The Isolation and Characterisation of an *Escherichia coli* Mutant Resistant to the Voltage Operated Calcium Channel Inhibitor, Verapamil.

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Doctor of Philosophy
at the University of Leicester

by

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To my wife, Ana,

and to my family.

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Abstract

The Isolation and Characterisation of an *Escherichia coli* Mutant Resistant to the Eukaryotic Voltage Operated Calcium Channel Inhibitor, Verapamil.

by Martin D. Goldberg

In eukaryotes, sophisticated mechanisms have been evolved to ensure that key events in the cell cycle take place at the correct time. The triggering of these events is largely controlled by transient increases in the concentration of intracellular free calcium ions $[Ca^{2+}]_i$. If these transient increases in $[Ca^{2+}]_i$ are prevented by the use of drugs that either block the voltage operated calcium channels (VOCCs), inhibit the release of Ca^{2+} from the cells' internal reserves, or block the receptor proteins that bind intracellular Ca^{2+} ions, the cell cycle will stop at specific points. The cell cycle will only recommence following removal of the Ca^{2+} blockade.

In bacteria, many genes have now been identified that are essential for specific cell cycle events. However, no regulator has been identified that actually controls the timing of these events. Many theoretical models have been developed to explain bacterial cell cycle control, but there is little solid evidence to support them.

A hypothesis has been developed that states that Ca^{2+} ions regulate the bacterial cell cycle in a manner analogous to eukaryotes. To test this hypothesis, temperature sensitive mutants of the *E. coli* strain, N43, resistant to the eukaryotic VOCC inhibitors verapamil and diltiazem were isolated, although detailed characterisation was confined to a verapamil resistant mutant, N43*verA1*. All the mutants are affected at a specific locus on the *E. coli* chromosome consisting either of deletions or major genetic rearrangements of three genes, *hns*, *galU* and *hnrG* (renamed *rrx* in this work). These genes do not constitute the expected receptor, but nevertheless, detailed examination of N43*verA1* demonstrated that it appears to be unable to regulate its $[Ca^{2+}]_i$, since it is hypersensitive both to the concentration of Ca^{2+} in the medium and the Ca^{2+}-chelator, EGTA. Moreover, when N43*verA1* was transferred from the permissive to non-permissive temperature, it formed filaments, minicells and "chains of sausages", demonstrating some defect in cell division. An examination of the wild-type and mutant's responses to treatment with verapamil, EGTA or Ca^{2+} by labelling cell proteins with [^{35}S-] methionine, indicated an extremely complex response. N43*verA1* constitutively expresses a number of heat-stable proteins, which are further induced following treatment with verapamil or EGTA. Preliminary data suggests that some of the EGTA inducible proteins possess calmodulin-like properties.

The three genes, *hns*, *galU* and *rrx* appear to interact, either at the genetic or protein level. Measurements of *hns* expression indicated that it is induced by verapamil, but not by EGTA. Overexpression of *galU* results in hyper-resistance to verapamil and Ca^{2+}, which is modulated by *hns* and *rrx*, respectively. It is proposed that these genes in some way regulate the $[Ca^{2+}]_i$, in addition to, or in relation to their role in nucleoid organisation and control of the stationary phase sigma factor, RpoS.

Contents

Abbreviations

A	Absorbance
Amp	Ampicillin
APS	Ammonium persulphate
bp	Base pairs
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bq	Becqerel
BSA	Bovine serum albumin
°C	Degrees Celsius
Ca^{2+}	Calcium ion(s)
$[Ca^{2+}]_i$	Intracellular free calcium concentration
$[Ca^{2+}]_{ext}$	Extracellular free calcium concentration
CaM	Calmodulin
Ca.CaM	Calmodulin bound to Ca^{2+}
cfu	Colony forming units
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra-acetic acid, disodium salt (Disodium diaminoethane tetra acetate)
EGTA	Ethylene glycol-bis(β-amino-ethyl ether)N,N,N',N'-tetra acetic acid
EtBr	Ethidium bromide
g	Angular velocity of a centrifuge as multiples of the acceleration due to gravity (g =9.81m s^{-2}).
h	Hour(s)
HGT	High gelling temperature agarose
H_2O	Water
IPTG	Isopropyl-β-D-thiogalactoside
k	Kilo (10^3)
kb	Kilobases
kDa	KiloDaltons
Km	Kanamycin
l	Litre(s)
LUA	Luria-Bertani agar
LUB	Luria-Bertani broth
M	Mole(s)
m	Milli (10^{-3})
μ	Micro (10^{-6})

MIC	Minimal inhibitory concentration
min	Minute(s)
mJ/cm^2	Milli joule per square centimetre
MOPS	3-[N-morpholino] propane sulphonic acid
M_r	Molecular weight
mRNA	Messenger ribonucleic acid
n	Nano (10^{-9})
NA	Nutrient agar
NB	Nutrient broth
NBT	Nitroblue tetrazolium. 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy 4,4'diphenylene] -ditetrazolium chloride
OD	Optical density
ONPG	o-Nitrophenyl-β-D-galactopyranoside
p	Pico (10^{-12})
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PEG	Polyethylene glycol
pH	Measurement of acidity
PKA	Protein kinase A
PKC	Protein kinase C
psi	Pounds force per square inch
s	Second(s)
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ssDNA	Single stranded deoxyribonucleic acid
Tet	Tetracycline
TEMED	N, N, N',N',-tetra methyl ethylene diamine
Tn	Transposon
Tris	Tris-(hydroxymethyl)-aminomethane
tRNA	Transfer ribonucleic acid
ts	Temperature sensitive
ts^+	Non-temperature sensitive
Tween 20	Polyoxyethylene-sorbitan-monolaurate
UV	Ultraviolet
V	Volts
VOCC	Voltage operated calcium channel

vol	Volume(s)
$^{v}/_{v}$	Volume per volume
$^{w}/_{v}$	Weight per volume
wt	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1
Introduction

"*In each of our bodies there are molecular choreographers programming a minuet in which chromosomes appear from obscurity, line up with their partners, separate, rejoin, and then disperse.*" Daniel E. Koshland, Jr., Editor, Science (Koshland, 1989).

1.1 General introduction

The use of music by Koshland to describe one of the more tangible aspects of the cell cycle is indeed profound and can be extended much further. Music consists of a programme in the form of musical notes which instruct the musician what pitch to play, and for how long. In addition, a plethora of terms tell the musician how to play the notes, whether they be *staccato*, *legato*, *sostenuto* and how fast: *allegro*, *lento*, *adagio*. This programme is equivalent to the DNA of the cell, containing all the instructions for reproducing the music. The DNA of the cell contains all the instructions needed to make identical copies of the cell. However the cell, like an orchestra needs a conductor to regulate the sequence of events, to ensure that certain events occur at the correct time and with the correct intensity. As in an orchestra, where for example, a trumpet interrupts a violin solo, resulting in chaos, the conductor must be able to stop the music if necessary, and to recommence when ready. Precisely the same is true in the cell, one of the most extreme examples of failure by the conductor being cancer, where the cell continues to grow and divide rapidly in a totally uncontrolled manner, ultimately resulting in the death of the organism.

The cell cycle is the term used to describe all the processes that take place during the growth and division of the cell, to ensure that each daughter cell inherits a complete set of chromosomes and all the biochemical machinery necessary for continued survival and multiplication. In eukaryotes, the cell cycle has been divided into four functional and temporal phases: M (Mitosis / Meiosis), G_1 (Gap 1), S (Synthesis) and G_2 (Gap 2) (Howard and Pelc, 1953) (see **Fig. 1**). M phase is the stage during which the chromosomes condense, align along the mitotic spindles of the cell and segregate to the opposite poles, followed by division of the cell into two identical daughter cells. During G_1, a decision is made whether to re-enter the cycle or to exit the cell cycle and enter the quiescent G_0 phase (Whitfield *et al.*, 1986). Such cells either become terminally differentiated or await stimulation by an appropriate mitogenic factor to re-enter G_1 in preparation for another round of cell division. It is during G_1 that many of the proto-oncogenes that are so important in cancer act to regulate cell proliferation. In addition, much of the cell's protein synthesis takes place

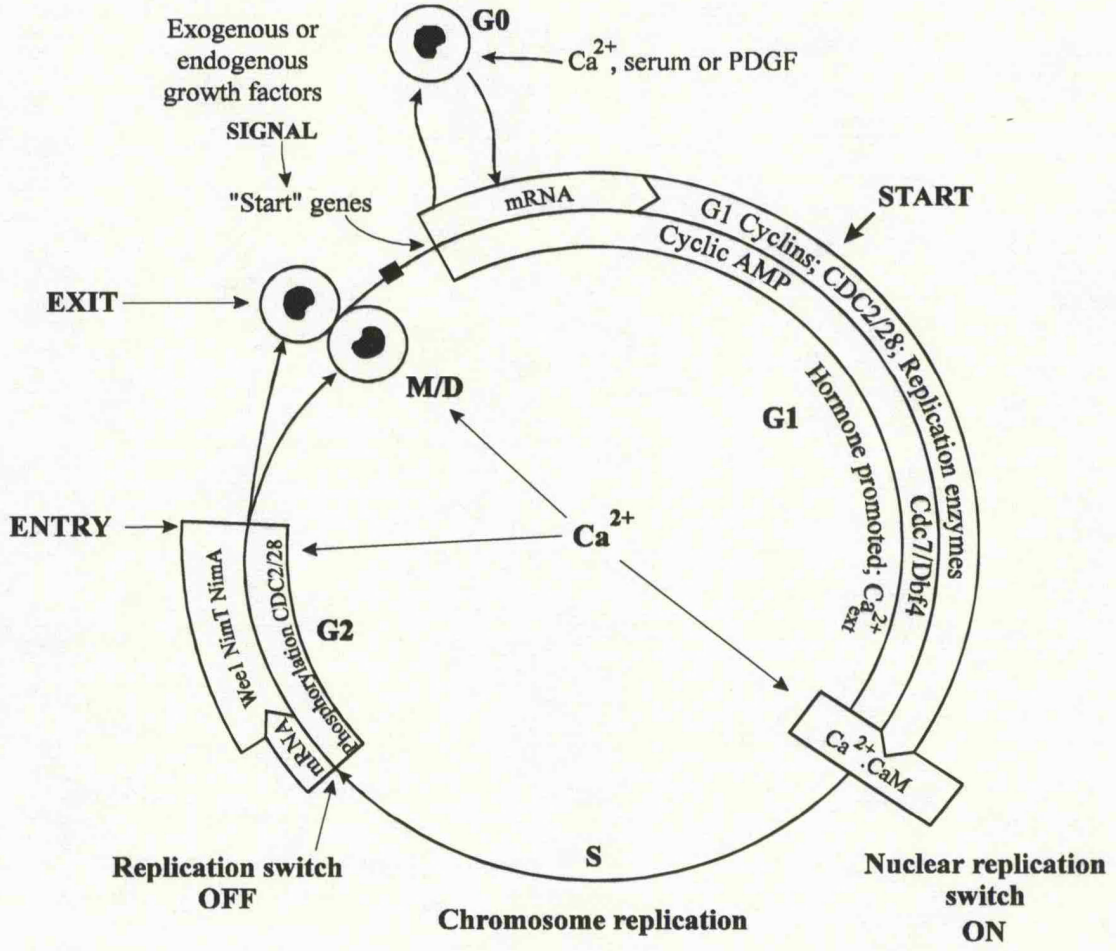

Fig 1. Schematic representation of the eukaryotic cell cycle.

during this period. The S phase is the period during which DNA synthesis occurs. During G_2, the cell makes preparations for the next meiosis / mitosis.

In bacteria, historically, the cell cycle has been traditionally regarded as much simpler, with only two main phases, C (chromosome replication) and D (preparation for division), terms first defined by Cooper and Helmstetter (Cooper and Helmstetter, 1968; Helmstetter and Cooper, 1968). However, more recent studies have shown that the bacterial cell cycle is more complex, and indeed similarities have been shown to exist between the cell cycles of prokaryotes and eukaryotes (Holland, 1987).

1.2 The eukaryotic cell cycle

Since the eukaryotic cell cycle is profoundly complex, and a vast (and growing) number of genes and proteins shown to be involved with its regulation, only the most important key aspects of eukaryotic cell cycle regulation will be discussed in this brief overview. Detailed reviews covering aspects of eukaryotic cell cycle regulation can be found in (Whitaker and Patel, 1990; Murray and Hunt, 1993a; Sherlock and Rosamond, 1993). Much of the experimental work on unravelling the eukaryotic cell cycle has been performed upon the single celled yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, the fungus *Aspergillus nidulans*, and the eggs and oocytes of aquatic animals such as the sea urchin, star fish, frog and clam. Yeasts and fungi have been used because of the comparative ease of manipulation, whereas the eggs and oocytes of higher eukaryotes possess rapid and relatively straightforward cell cycles. Cell cycle control points have been identified in oocytes and eggs as a result of the natural cell cycle pauses that they have evolved (Kanatani, 1973; Masui and Clarke, 1979; Whitaker, 1989). These pauses are relieved either by hormonal triggers or upon fertilisation by a sperm. Three major checkpoints have been identified: START, ENTRY and EXIT, which take place shortly before the G_1/S transition, entry into mitosis / meiosis and at the metaphase / anaphase transition respectively, and have since been identified in all normal cells so far examined. START, ENTRY and EXIT form commitment or restriction points which commit the cell to advance to the next stage of the cell cycle. Upon passing START, the cell becomes committed to entering the cell cycle whereupon it begins to synthesise DNA. Certain biochemical events must be completed before the cell can pass START. Similarly, the cell cannot ENTER mitosis / meiosis until certain requirements have been fulfilled, such as duplication of the chromosomes and the nucleolar organisers. Once the mitosis or meiosis phase has been entered, and the condensed chromosomes are aligned upon the microtubule spindle, the third restriction point, EXIT must be passed. Again, certain events must be completed in order for this to take place. Only then can the duplicated chromosomes migrate to the daughter cells which re-enter G_1 and a decision is then made whether to

become quiescent G_0 cells or to prepare for another round of the cell cycle. Some of the key proteins and transducers involved in the co-ordination of these events are outlined below.

1.2.1 Maturation promoting factor

The first regulatory component to be identified in the eukaryotic cell cycle was identified from frog oocytes. Frog oocytes need to be activated by exposure to the hormone progesterone, whereupon they undergo meiosis I and arrest at meiosis II. Like the gametes of many animals, they remain in this state until fertilisation, when metaphase arrest of meiosis II is overcome and the early embryonic cell cycles commence. Following activation of the oocytes with progesterone, it was observed that cytoplasm could be removed and injected into untreated oocytes, which then became activated (Masui and Markert, 1971). Similarly, cytoplasm from these activated oocytes could be used to induce further oocytes to undergo meiosis I. This sequence could be continued indefinitely. The factor that was found to be activating the immature oocyte was named maturation promoting factor, also known as mitosis promoting factor or M-phase promoting factor (MPF). Following fertilisation, the levels of MPF were shown to oscillate during the mitotic cell cycles, and were independent of DNA replication (Gerhard *et al.*, 1984). Protein synthetic capability was shown to be required however (Newport and Kirschner, 1984), since injection of inhibitors of protein synthesis such as emetine into the zygote blocked further rises in MPF production and the cell cycle arrested. Other studies demonstrated that the information for synthesising MPF during the first few cell divisions was encoded by maternally derived mRNA in the oocyte (Davidson *et al.*, 1982).

1.2.2 Cyclins

The first demonstration of the cyclical appearance and spectacular destruction of a protein during the sea urchin cell cycle, was reported by Evans *et al.* (1983). This "cyclin" protein was shown to commence accumulation shortly after the completion of mitosis, and gradually accumulated throughout the cell cycle. Just prior to anaphase, the protein was rapidly destroyed, and its synthesis only recommenced following completion of mitosis. Minshull *et al.* (1989) demonstrated that when sperm nuclei were added to activated frog egg extracts, the nuclei swelled, the chromosomes duplicated, the chromosomes condensed, the nuclear envelope broke down and, following cyclin degradation, the nuclear envelope reformed and the chromosomes decondensed. By adding ribonuclease to the extract during interphase, Minshull *et al* also showed that the cyclical changes in the nuclei were inhibited. However, if ribonuclease inhibitor was added to the ribonuclease-treated extract, followed by the

introduction of cyclin B mRNA, the nuclei regained the ability to perform cyclical events, thus illustrating the key role of cyclin synthesis in cell cycle progression (Murray and Kirschner, 1989). An interesting observation from these experiments was the fact that centriole duplication and microtubule formation were not essential prerequisites for completion of these early cell cycles.

Although the cell cycle can be arrested during G_2 by removing cyclin mRNA, overexpression of cyclin mRNA does not result in an accelerated cell cycle. Injection of frog eggs with compounds known to increase the levels of cyclin, such as 2mM ammonium chloride or 12-tetradecanoyl phorbol-13-acetate (TPA, an activator of protein kinase-C), failed to accelerate the cell cycle (Patel *et al.*, 1989). This provided evidence for an additional limiting factor for cell cycle progression and/or post transcriptional modification of the cyclin.

As mentioned earlier, cyclin is rapidly destroyed prior to completion of mitosis. Murray *et al.* (1989) demonstrated that the N-terminus of cyclin B is critical for this function, and showed that by deleting the 90 amino-terminal amino acid residues from the protein, that sea urchin eggs entered mitosis normally, but failed to exit mitosis, becoming arrested at metaphase. It has since been shown that the cyclins contain a "destruction box", a short amino acid sequence in the N-terminus with the consensus sequence RTXLGXIGX, which targets the ubiquitination of lysine residues downstream of the box for subsequent proteolysis by cytoplasmic protease complexes (Glotzer *et al.*, 1991); for a review of the ubiquitination mechanism, see Murray (1995).

A number of other cyclins have now been identified, which interact with CDC2/28 kinase (or its homologues; see below), and act at other restriction points in the cell cycle. For example, G_1 cyclins have now been identified from several different organisms (Lew and Reed, 1992), and have been shown to play a key role in passing START (Richardson *et al.*, 1989). Six G_1 cyclins have now been identified in *S. cerevisiae*, three of which have been well characterised, called CLN1, CLN2 and CLN3 and appear to be functionally redundant. The *S. cerevisiae* cell cycle is more complex than most unicellular organisms in that the progeny budding from the mother cell are considerably smaller than the mother. Therefore, they need to undergo a longer G_1 phase to reach the correct (critical) size in order to undergo mitosis than the parent cell, which is already large enough to re-enter the cell cycle. Indeed a characteristic feature of *S. cerevisiae* is the fact that under optimal conditions, the G_1 phase of the parent cell is almost non-existent whereas the G_1 phase of the daughter cell is by necessity longer, to attain the critical size (Hartwell and Unger, 1977). In *S. cerevisiae*, different cyclins have been shown to regulate START in the daughter cells and in the parent cell, CLN1 and CLN 2 are thought to be important in the daughter cells and

CLN3 in the parent cells, although deletion of any two of the three cylin genes is not lethal (Lew and Reed, 1992). Additional cyclins have been identified that appear to be important after START at the G_1/S transition, such as the mammalian cyclin E (Lew *et al.*, 1991). Thus, cyclins do not possess intrinsic enzymatic activity, but confer specificity of action upon the CDC2/28 kinase, presumably enabling the kinase to phosphorylate specific proteins at the correct points in the cell cycle.

A kinase, NIMA, first identified in *Aspergillus nidulans* has also been shown to behave like cyclins, and its pattern of synthesis mirrors that of the mitotic cyclin B. Like cyclin B, NIMA contains a regulatory domain, although in the case of NIMA, this domain is located in the C-terminus. Pu and Osmani showed that the C-terminus contains sequences required for nuclear localisation and two PEST sequences required for its destruction (Pu and Osmani, 1995). As for cyclin B, removal of the destruction box prevented degradation at mitosis and resulted in mitotic block. Interestingly, in the *Aspergillus* strains lacking the NIMA destruction box, at the point of the mitotic block, cyclin B levels were shown to degrade normally thus indicating that a) destruction of NIMA follows destruction of cyclin B, b) degradation of NIMA is probably mediated by the same mechanism as cyclin B, c) cyclin B is not the only protein that must be very rapidly broken down prior to the onset of anaphase and d) there are additional restriction points after cyclin B destruction in mitosis which must be passed for advancement of the cell cycle through mitosis. It should be emphasised that although both cyclin B and NIMA levels oscillate during the cell cycle and are probably degraded by the same mechanism, NIMA is not a cyclin since a) cyclins unlike NIMA, are not kinases *per se*, and b) NIMA does not interact with CDC2/28 kinase to activate the latter , unlike cyclins (see next section). However, the targets of NIMA kinase have not yet been identified.

1.2.3 CDC2/28 kinase

In *S. cerevisiae*, a temperature sensitive mutant was isolated which arrests cells in G_1. At the non-permissive temperature, the cells continued to grow in size, but were unable to initiate DNA replication, bud, form spindle pole bodies or ultimately divide (Hartwell *et al.*, 1974). The gene that was found to be affected encodes a kinase, and one of its points of action in the cell cycle is at START. It is regarded as an essential component, required for the process of committing the cell to a new round of DNA replication, followed by cell division. The activity of this 34kDa protein, known variously as $p34^{cdc2}$ in higher eukaryotes and Cdc2 and CDC28 in *S. pombe* and *S. cerevisiae* respectively (Murray and Hunt, 1993a), is regulated by phosphorylation of two amino acid residues, tyrosine-15 and threonine-161 in frogs or threonine-167 in *S. pombe*. Because of the different names for this kinase, depending upon the organism

being considered, the term CDC2/28 will be used here. Cyclin B has been shown to bind to CDC2/28 to form a complex that is now understood to be MPF (Labbe *et al.*, 1989), and is required for transit through mitosis. The target(s) of the CDC2/28 kinase have not yet been identified. Other cyclins (see above) also bind to CDC2/28 or their analogues and enable passage through the other restriction points in the cell cycle during G_1. Unlike most cyclins, CDC2/28 levels do not oscillate during the cell cycle.

1.2.4 Regulating activity of MPF and its homologues

As described above, MPF is the name of the mitotic cyclin-Cdc2/28 complex first identified in frogs eggs and this name for the complex is normally used only when discussing higher eukaryotes. The tyrosine-15 residue of Cdc2/28 has been shown to be important for inhibiting its kinase activity, and mutating this residue to a non-phosphorylatable amino acid results in cells dividing prematurely (Gould and Nurse, 1989). Moreover, the normal controls which require that DNA synthesis be completed prior to entry into mitosis are lost. Similarly, mutation of the kinase which phosphorylates this residue, Wee1, results in premature mitosis (Russell and Nurse, 1987). Prior to mitosis, the tyrosine-15 residue must be dephosphorylated, since mutants lacking the phosphatase, Cdc25 (or NimT from *Aspergillus*) that dephosphorylates tyrosine-15, arrest at metaphase (Moreno *et al.*, 1989). The threonine-14 residue adjacent to tyrosine-15 has also been shown to be phosphorylated during G_2 and dephosphorylated at mitosis in vertebrates (Krek and Nigg, 1991; Norbury *et al.*, 1991). Modification of this residue and tyrosine-15 to non-phosphorylatable amino acids, results in a more severe premature mitosis phenotype than by only mutating tyrosine-15. It is suspected that the Wee1 kinase and Cdc25 phosphatase are also responsible for phosphorylating and dephosphorylating the threonine-14 residue (Millar and Russell, 1992).

The threonine-161 (or 167, see section 1.2.3) residue becomes phosphorylated subsequent to phosphorylation of tyrosine-15, by the cyclin activating kinase (CAK) $p40^{MO15}$ (Fesquet *et al.*, 1993; Poon *et al.*, 1993), and this is an essential prerequisite for activation of CDC2/28 (Gould *et al.*, 1991). Activation of MPF only occurs upon dephosphorylation of tyrosine-15 by Cdc25. Phosphorylation of tyrosine-15 is therefore performed as a "fail-safe" mechanism in order to avoid premature activation of MPF before the cell is ready to enter mitosis, analogous to the safety-switch on a loaded gun.

1.2.5 Cyclic AMP and protein kinase A

Cyclic AMP (cAMP) is formed from ATP by the action of adenylate cyclase, encoded by the Cdc35 gene in of *S. cerevisiae*. cAMP was first recognised as being

important in regulating the cell cycle because temperature sensitive mutants defective in adenyl cyclase, when incubated at the non-permissive temperature, were unable to pass START or increase in mass (Moriya *et al.,* 1990). Moreover, cAMP has been shown to play an important role in signalling the nutritional status of cells, a high concentration of extracellular nutrients results in high levels of cAMP. cAMP acts by binding to the two regulatory subunits of cAMP-dependent protein kinase (PKA), and through a conformational change resulting in dissociation of the regulatory subunits, activates the kinase (Thevelein, 1991). In the absence of cAMP, the yeast cells respond by activating genes important for starvation survival *ie* glycogen hydrolysis, sporulation and meiosis. Temperature sensitive mutations in Cdc35 resulting in enhanced levels of cAMP are refractory to the START restriction point when incubated at the restrictive temperature, and starvation of these mutants fails to arrest the cell cycle at START (Gibbs and Marshall, 1989).

cAMP levels are regulated by the action of membrane bound GTP-binding proteins or G_s-proteins. In most instances, G_s-proteins are trimeric, and are associated with membrane-bound receptors such as hormone receptors. When a hormone such as insulin or platelet derived growth factor (PDGF) binds to the receptor, the receptors dimerise and auto-phosphorylate (see also section 1.4.2.2). One of the many consequences of this event is that the α-subunit of the G_s-protein exchanges its bound GDP for GTP and becomes activated. Upon activation of $G_{s\alpha}$, the α-subunit dissociates from the β and γ subunits, and activates adenylate cyclase, resulting in the formation of cAMP from ATP, which in turn activates PKA. In addition to activation of adenylate cyclase by $G_{s\alpha}$, some isoforms of adenylate cyclase are also activated by Ca^{2+}-calmodulin (Minocherhomjee *et al.,* 1988). PKA is inactivated by cAMP phosphodiesterases, some of which are also regulated by Ca^{2+}-calmodulin (Klee and Cohen, 1988). Ca^{2+}-calmodulin protein phosphatases (CaM-PP) have also been shown to be important in dephosphorylating several of the proteins phosphorylated by PKA (Klee and Cohen, 1988). In conclusion, changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) can regulate the activity of the cAMP pathway. This aspect of regulation of the cell cycle is extremely important, as will be discussed later.

1.2.6 Cdc7 and Dbf4

The two proteins Cdc7 and Dbf4 from *S. cerevisiae* have been shown to be crucial for entry into S phase, (for review see Sclafani and Jackson, 1994). In *cdc7*^{ts} or *dbf4*^{ts} mutants, the cell cycle becomes blocked at the G_1/S interface when incubated at the non-permissive temperature. Dbf4 behaves much like a classical cyclin, reaching maximal levels just prior to the G_1/S transition, and is destroyed during S phase. Levels of Cdc7 on the other hand remain constant throughout the cell cycle. It is

thought that Cdc7 becomes phosphorylated following interaction with Dbf4 by the same CAK that phosphorylates CDC2/28 at Threonine-161. Recent data has demonstrated the presence of Dbf4 associated with the origins of replication in *S. cerevisiae* (Dowell *et al.*, 1994). However, further work needs to be performed to show that this protein and Cdc7 are actually physically involved in the initiation of chromosome replication.

Having briefly described some of the more important proteins that are required for regulating eukaryotic cell cycle events (the musicians in the orchestra), the conductor, required for coordinating these events must still be considered (see later).

1.3 The bacterial cell cycle

The bacterial cell differs considerably from the eukaryotic cell. For example, bacteria possess a single circular chromosome which is not bounded by a nuclear membrane, unlike eukaryotes that contain multiple, linear chromosomes enclosed by a membrane. The bacterial envelope is complex, consisting of a cytoplasmic membrane, enclosed by a sacculus composed of peptidogycan and sometimes teichoic acids or in some instances complex mycolic acids. In the case of Gram-negative bacteria, the sacculus is surrounded by an outer membrane. The eukaryotic cell is bounded by a cytoplasmic membrane, and in some cases has an additional specialised external cell wall (cellulose in plants, chitin in fungi and yeasts). Eukaryotic cell functions are compartmentalised into organelles - energy is generated by mitochondria, proteins are synthesised within the endoplasmic reticulum, the DNA is contained within the nucleus and a special secretory apparatus (Golgi apparatus), has been evolved for exporting substances. Bacteria do not generally compartmentalise cellular functions, the DNA is surrounded by cytoplasm, energy is generated through the transfer of electrons and protons across the cytoplasmic membrane (proton motive force) and protein synthesis takes place in the cytoplasm. Eukaryotic cells possess a complex cytoskeleton which determines the shape and structure of the cell, and facilitates cytokinesis and cellular translocation. The cytoskeleton is composed of numerous structural proteins including actin and myosin. There is very little solid evidence for a bacterial cytoskeleton, although some encouraging data is now beginning to emerge to support this possibility (see later).

As mentioned earlier, Cooper and Helmstetter were the first to begin looking systematically at cell cycle parameters and regulation of the bacterial cell cycle, using *E. coli* B/r (Cooper and Helmstetter, 1968; Helmstetter and Cooper, 1968). They developed a model to relate chromosome replication with cell division. They established that under non-limiting growth conditions, the chromosome of *E. coli* B/r

takes 40 minutes to replicate and a further 20 minutes is required before cell division occurs, called the C and D periods, respectively. If the mean generation time (τ) is longer than 60 minutes, there is a "silent" period after cell division before a new round of chromosome replication initiates, called the B period. The C, D and B periods have been likened to the S, G_2 and G_1 phases of eukaryotes (Cooper, 1979). The length of the S and G_2 periods of eukaryotes is fairly constant, even with large changes in growth rate (Prescott, 1976), as is the case for the C and D periods of *E. coli* (Cooper and Helmstetter, 1968). On the other hand, the G_1 period of eukaryotes is highly variable, for example, in *S. cerevisiae* (Hartwell and Unger, 1977), where the parental cell re-enters the cell cycle almost immediately after completing mitosis, but the much smaller progeny cell must grow to a critical size during G_1 before reaching START and entering the cell cycle. Since *E. coli* can grow with τ values of as little as 20 minutes and the C and D values remain relatively constant at 40 and 20 minutes respectively, *E. coli* has evolved a system which allows for initiating new rounds of chromosomal replication before completion of the previous one. The consequence of this strategy is that during rapid growth, cells can contain multiple replication forks. A problem therefore emerges on how the nucleoids can be efficiently and accurately partitioned into individual cells under such conditions. Nevertheless, wild-type strains of *E. coli* can give rise to anucleate cells at a frequency of less than 0.03% (Hiraga *et al.,* 1989). Since eukaryotic cells must divide following chromosome replication, and before a new round of DNA synthesis can take place, it seems that the mechanisms determining the allocation of chromosomes to daughter cells may need to be different from those of eukaryotes.

In eukaryotes, all the chromosomes are bounded by a single nuclear envelope whose presence is required to initiate simultaneous replication of all the chromosomes, (Leno and Laskey, 1991). Chicken erythrocytes are nucleated, and Leno and Laskey showed that by adding the erythrocyte nuclei to a cell free *Xenopus laevis* oocyte extract, some of the nuclei formed multinuclear aggregates that shared a common nuclear membrane. All the nuclei within a single aggregate entered M phase simultaneously, whereas the nuclei within different aggregates entered M phase at different times. In rapidly growing *E. coli*, it appears that initiation of chromosome replication from multiple origins of replication occurs simultaneously (von Meyenburg and Hansen, 1987) (see later). This therefore poses the question of whether the bacterial cytoplasmic membrane can perform the task of the eukaryotic nuclear membrane.

1.3.1 Theoretical considerations on the timing of initiation of chromosome replication

A number of models have been developed to try to determine what triggers the initiation of chromosome replication. The accumulation of an initiator protein, something akin to cyclins has been postulated by several authors (Donachie, 1968; Sompayrac and Maaloe, 1973; Lobner-Olesen *et al.,* 1989). In contrast, (Pritchard *et al.,* 1969) postulated the inhibitor dilution model, where a fixed amount of an inhibitor is synthesised, which becomes diluted during growth until the cell reaches a critical volume.

The model of Sompayrac and Maaloe (1973), relies upon the constitutive synthesis of an initiator. This would be subject to gene dosage effects following replication of the gene during the cell cycle. Therefore, the model incorporates a repressor whose gene is closely linked to the gene encoding the initiator protein, and would regulate the levels of initiator, since its expression would also be subject to dosage effects. According to Bremer and Churchward (1991), such a mechanism could not work in practice since the expression of a given gene is dependent upon the amount of its mRNA relative to the total mRNA. The mRNA levels must vary according to the varying rate of bulk mRNA synthesis, thus gene expression could not be maintained at a constant level after changes in growth medium.

Pritchard's inhibitor dilution model (1969) relies upon a burst of synthesis of an inhibitor immediately after initiation of chromosome replication, which prevents immediate re-initiation. The model then suggests that when the repressor concentration becomes diluted by a factor of 2 as a result of cell growth, a further round of initiation can commence. On one hand, this mechanism would take into account differing growth rates, however, a fatal flaw in the model is the fact that bacteria growing at different rates produce cells of differing lengths - slow growing cells tend to be smaller than rapidly growing cells. If a fixed amount of repressor is synthesised, some form of compensatory mechanism would be needed to take into account differences in cell size. Secondly, a doubling in cell size of a bacterium results in a very small literal change in volume. The mechanism measuring the repressor concentration would need to be exquisitely sensitive to sense precisely when the concentration of repressor is sufficiently low to re-initiate replication.

On the basis of size measurements made by Schaechter *et al* (1958) and using the model for DNA replication in *E. coli* B/r (Cooper and Helmstetter, 1968), Donachie concluded that cell mass alone is critical for the initiation of replication (Donachie, 1968) and established the concept of initiation mass, as the cell mass at the time of initiation of a round of chromosome replication, divided by the number of chromosomal *oriC* sites present at the time. He determined that the average initiation

mass is constant, and does not vary with growth rate (τ). This concept has become a central dogma of cell cycle regulation, although tested using few experimental approaches. Churchward *et al.* (1981) measured the initiation mass, by measuring the relative increase in DNA following the inhibition of initiation with rifampicin added to to exponentially growing cultures. The initiation mass per chromosomal origin was determined for cultures growing at different rates and was found to increase with increasing growth rate in slow growing bacteria, until a plateau was reached when τ was less than 60 minutes. In contrast to the findings of Churchward *et al* (1981), a recent study (Wold *et al.,* 1994), which also showed that initiation mass is affected by the growth rate, found that the initiation mass increased with decreasing growth rate such that the initiation mass increased by a factor of 1.6 and 2.1 when light scattering and protein content were measured respectively, over a growth rate range of 0.3 to 2.5 generations per hour. Considering the differences in growth rate, the actual significance of the calculated initiation mass values over the range is debatable, since a certain amount of error will be introduced by the measurement techniques, and is even hinted at in the article by Wold *et al* (1994). On the other hand, Hiraga *et al.* (1989) provide evidence in support of Donachie's initiation mass model using a mutant *mukA,* that is partially defective in chromosome partitioning. Thus, in cells where segregation fails, resulting in daughter cells where one cell contains two chromosomes and the other none, the cell containing two chromosomes did not initiate a new round of chromosome replication before the next division cycle, in order to keep the ratio of DNA to cell mass constant. In the eukaryotic yeast *S. cerevisiae*, Donachie's model for a "constant" initiation mass is again supported by the observation that the daughter cell needs a longer period in G_1-phase before passing START, than the mother cell, which has already attained the critical initiation mass, and passes START almost immediately after completion of M-phase (Hartwell and Unger, 1977).

Clearly, if Donachie is correct and initiation only occurs when a critical mass is attained, the model by itself gives no clue to the mechanism involved, for example, a negative control, following initiation, must prevent further re-initiations from taking place before the cell has reached the critical initiation mass again. Consequently, it is neccessary to consider more explicit models for the regulation of the initiation of DNA replication, through the achievement of a relatively specific and constant cell mass.

During the *E. coli* cell cycle, there is only a brief period just prior to the initiation of chromosomal replication, where RNA and protein synthesis is required (Maaløe and Hanawalt, 1961; Schaechter, 1961; Lark, 1972; Messer, 1972). Skarstad *et al* (1985) showed that when protein synthesis was inhibited by the addition of chloramphenicol to rapidly growing cultures of *E. coli*, the cell cycle stopped at the point of initiation of DNA replication, with the majority of cells (95%) containing

either 2, 4 or 8 chromosome equivalents, thus indicating that each of the origins must have initiated simultaneously prior to the addition of chloramphenicol. If initiation synchrony had not been taking place, any number of chromosome equivalents would have been seen within a given cell, ranging from 2 to 8, rather than 2^1, 2^2 or 2^3. From these observations, it becomes clear that any model to explain the initiation of chromosome replication must be able to accommodate the fact that multiple origins trigger virtually simultaneously.

The precise models for the initiation of chromosome replication described by Pritchard, Donachie, Sompayrac and Maaløe have been seriously questioned as a result of minichromosome experiments (see next section), which have shown that large increases in the number of chromosomal origins in the cell or massive increases in the amount of DnaA protein, a key protein involved in the initiation of DNA replication (see below), fail to demonstrate the dramatic effects on the timing of initiation of DNA replication that would be expected, simply if critical levels of an initiator or inhibitor were in control. Indeed, the *dnaA* gene and its gene-product, DnaA, proposed as replication initiator by Hansen *et al* (1991), has been demonstrated to be autoregulated (Atlung *et al.,* 1985) (see also Skarstad and Boye, 1994). Thus, overexpression of DnaA from a high copy-number plasmid results in transcriptional repression of the *dnaA* gene, and conversely, elevation of a *dnaA*^{ts} strain to the non-permissive temperature results in transcriptional derepression, as determined by measuring β-galactosidase levels from p*dnaA-lacZ* fusions. Moreover, a number of GATC sequences can be found in the promoter region of *dnaA*, which are the methylation targets of Dam-methyltransferase (see the next section). It is believed that immediately following replication of the region containing *oriC* and *dnaA*, this hemi-methylated region becomes sequestered by the membrane, rendering it inaccessible to Dam-methyltransferase and RNA polymerase, thus greatly reducing transcription of *dnaA* for a period of approximately a third of the cell cycle (Campbell and Kleckner, 1990). Since *dnaA* expression is determined to a large extent by the accessibility of its promoter to RNA polymerase, and therefore to other regulatory mechanisms, it cannot logically be *the* trigger of DNA replication initiation as proposed by (Hansen *et al.,* 1991).

Both the inhibitor dilution model (Pritchard *et al.,* 1969) and the initiator accumulation model proposed by Sompayrac and Maaloe (1973) would require additional elements in order to accommodate the precision of chromosomal initiation observed by flow cytometry (Skarstad *et al.,* 1985), and cannot support the synchronous initiation of replication of minichromosomes (see below), which would titrate-out repressor or initiator factors.

Models have been proposed (Norris *et al.*, 1988; Norris, 1989) whereby the key cell cycle events including initiation of DNA replication are triggered by a flux of Ca^{2+} ions into the cell as observed in some eukaryotic systems. Further modifications were subsequently incorporated into the model, introducing the possible roles of membrane phospholipids and how they might play a part in the control of the cell cycle (Norris, 1992). The models proposed by Norris *et al* (1988) and Norris (1989) in which Ca^{2+} ions are proposed to regulate the cell cycle appear to be accumulating supportive evidence for example, Masaaki *et al.* (1995) recently presented initial results that Ca^{2+} ions were required to suppress the mutant phenotype in a temperature sensitive strain of *E. coli* that is defective in nucleoid segregation.

Support for the role of phospholipids in the control of the initiation of chromosome replication has emerged from studies by Crooke *et al.* (1992) who showed that acidic phospholipids play a role in activating DnaA protein. Apparently, acidic phospholipids assist in the reactivation of DnaA in the replacement of ADP with ATP, but only when DnaA is bound to *oriC*. In the unbound form, the phospholipids appear to inhibit DnaA, preventing it from binding to *oriC* and initiating chromosome replication. However, no mutants have been reported that are defective in phospholipid synthesis at the non-permissive temperature, and have altered timing of initiation of chromosome replication.

1.3.2 The initiation of chromosome replication

A useful tool for identifying genes / proteins involved in the initiation of replication has been the isolation of temperature sensitive mutants that are unable to initiate replication at the non-permissive temperature. Many of these mutants were found to be affected in the gene *dnaA* (Hansen and von Meyenburg, 1979). A demonstration of the role of DnaA in the initiation of chromosome replication was shown by the fact that temperature sensitive *dnaA* mutants cannot initiate replication at the non-permissive temperature. However, once initiation has commenced, incubation at the non-permissive temperature has no further effect upon the cell cycle until the cell is ready to initiate a new round of chromosome replication. Additional evidence was obtained by showing that addition of rifampicin to inhibit transcription permitted DNA synthesis to continue where initiation had already taken place, but no new rounds of initiation could occur because protein and/or RNA factors required for a new round could not be synthesised (Maaloe and Hanawalt, 1961; Schaechter, 1961; Lark, 1972; Messer, 1972; Zyskind *et al.*, 1977). This provided elegant early evidence for the requirement of *de novo* protein and RNA synthesis at the beginning of each cell cycle. Studies on *B. subtilis* showed that the same features appear to exist for this organism too (Séror-Laurent, 1973, 1974; Moriya *et al.*, 1994). In several other unrelated

species of bacteria, the origins of chromosome replication have been examined and in all cases appear to have the same common features (Moriya *et al.,* 1994).

Two of the components that have been intensively investigated in the control of replication initiation are the origin of replication, *oriC* (see **Fig. 2**), located at 84' on the *E. coli* chromosome, from which replication takes place bidirectionally (Bird *et al.,* 1972; Marsh and Worcel, 1977), and the protein DnaA, encoded by the gene *dnaA*, which is located at 83' on the linkage map (for a review of DnaA, see Skarstad and Boye (1994)). Løbner-Olesen *et al* (1989) postulated that DnaA monomers accumulate during the cell cycle until initiation occurs. Following initiation of chromosome replication, the presence of two origins would titrate-out the DnaA below the threshold concentration required to initiate a further round of chromosome replication. If this hypothesis were true, over-expression of DnaA should cause runaway chromosome replication, which does not happen. Only a very modest 20% transient increase in replication initiation occured when DnaA was overexpressed approximately 20-fold (Lobner-Olesen *et al.,* 1989; Pierucci *et al.,* 1989). However, it appears that many of the extra replication forks pause during replication or become stalled (Lobner-Olesen *et al.,* 1989).

Plasmids containing the chromosomal *oriC* apparently replicate synchronously with the chromosome (Leonard and Helmstetter, 1986) and establish copy numbers of 8-10 plasmids per chromosomal origin (Lobner-Olesen *et al.,* 1987). Thus, in very rapidly growing cultures where there are potentially 8 chromosomal replication forks in a cell (based upon the data of Cooper and Helmstetter (1968)), there can be upto 80 *oriC* plasmids in the cell. This should therefore lead to a 10-fold increase in initiation mass if DnaA, required for initiation, is the rate limiting factor. According to von Meyenburg and Hansen (1987), this does not happen, again suggesting that another initiating factor(s) which perhaps regulates DnaA or independently acts upon *oriC*, is important for controlling the cell cycle oscillator (see for example, Hughes *et al* (1988); Norris *et al* (1988)). Moreover, if DnaA were the clock regulator, it would need to be capable of very tight autoregulation, always restoring constant levels of DnaA per *oriC*, which it does (Atlung *et al.,* 1985). Equally however, if DnaA is tightly controlled at all times during the normal cell cycle through feedback regulation, this argues against a critical concentration of DnaA protein being the trigger for chromosomal replication initiation. This would contradict F. Hansen's model, which specifically argues in favour of an increase in concentration, reaching a critical threshold which triggers initiation (Hansen *et al.,* 1991). Despite the controversy over the role of DnaA in controlling the timing of replication, there can be no doubt concerning the vital, mechanistic role of DnaA in driving initiation, once the control signal has been given.

The 52kDa DnaA protein is normally present in the cell as approximately 800-2100 molecules (Sekimizu *et al.*, 1988), and possesses two effector binding sites for ATP/ADP (Sekimizu *et al.*, 1987) and cAMP (Hughes *et al.*, 1988). When bound to ATP, DnaA binds to four 9bp target sequences TTAT(A/C)CA(A/C)A in the *oriC* region (Fuller *et al.*, 1984; Samitt *et al.*, 1989; Messer *et al.*, 1991) and is required for opening of the 13-mer AT-rich sequences in *oriC* (Sekimizu *et al.*, 1988). A complex of 10-20 DnaA monomers interacts with the four DnaA boxes to form the 'initial complex' and is believed to be an important prerequisite for the assembly of the primosome and initiation of replication by mediating the local unwinding of the AT rich part of *oriC* (Bramhill and Kornberg, 1988; Gille and Messer, 1991). Following formation of the initial complex, the proteins DnaB helicase, DnaC, primase (DnaG), and DNA polymerase III holoenzyme are acquired for the formation of the primosome (Kornberg, 1988). Hughes *et al.* (1988) demonstrated that cAMP is important in regulating the activity of DnaA. DnaA requires bound ATP for activity, and has ATPase activity which gradually converts the ATP to ADP. DnaA protein bound to ADP is inactive and cAMP is required for the exchange of ADP with ATP.

In addition to phospholipids, membrane fluidity also plays an important role in DnaA regulation. The *in vivo* activity of DnaA has been shown to be dependent upon the presence of acidic phospholipids, of which, oleic acid is an important constituent. Inhibition of oleic acid synthesis was specifically shown by Fralick and Lark (cited in Skarstad and Boye, 1994) to inhibit initiation of DNA replication. Upon addition of exogenous oleic acid, DNA synthesis re-commenced. In an equivalent *in vitro* experiment performed by Yung and Kornberg (cited in Skarstad and Boye, 1994), the ability of purified DnaA to exchange ADP for ATP was absent when bacterial phospholipid extracts lacking oleic acid were added to an *in vitro* replication assay, but did occur in the presence of oleic acid.

The *oriC* region (see **Fig. 2**) is flanked by two genes *mioC* (modulation of initiation at *oriC*) and *gidA* (glucose inhibition of division). Using *oriC* plasmids (minichromosomes), studies have shown that the promoters of *gidA* and *mioC* are essential for efficient replication of the minichromosomes. The promoter of *mioC* contains a 9bp DnaA binding sequence (DnaA box) and DnaA acts to repress expression of this gene by binding to the DnaA box (Lobner-Olesen *et al.*, 1987). Deletion of the *mioC* gene from the chromosome is not lethal, however deletion of the *mioC* promoter from the *oriC* region of minichromosomes results in a marked reduction in copy number and stability (Stuitje *et al.*, 1986), indicating that the *mioC* promoter is required for efficient minichromosome replication. Evidence that *gidA* and *mioC* are important in the stringent response (see later) emerged from experiments showing that guanosine 3',5'-bispyrophosphate (ppGpp), an alarmone important in the

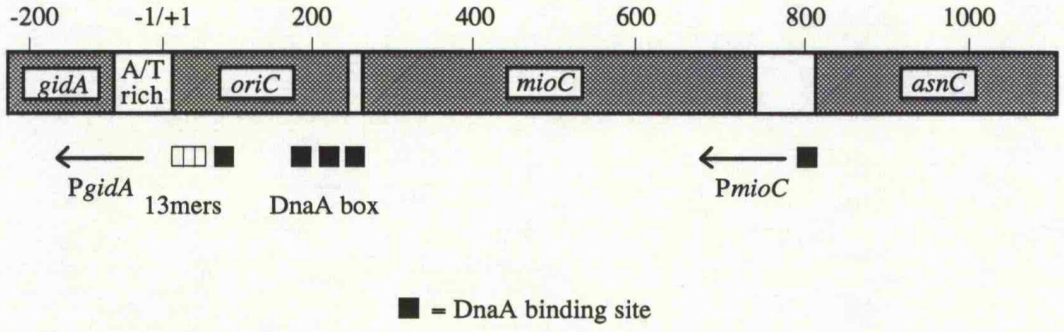

Fig 2. Structure of the *E. coli* origin of the replication, *oriC* and flanking DNA.

Taken from Ogawa and Okazaki (1994).

inhibition of chromosome replication (Levine *et al.,* 1991) as a result of nutrient deprivation, inhibits transcription of *gidA* and *mioC* (Rokeach *et al.,* 1987; Ogawa and Okazaki, 1991). Since the concentration of ppGpp is inversely proportional to the growth rate (Baracchini and Bremer, 1988), Weinberger and Helmstetter (1989) proposed that ppGpp could at least be partly involved in coupling the rate of initiation of chromosome replication with changes in growth rate. Indeed, in mutants devoid of ppGpp, the initiation mass does decrease (Hernandez and Bremer, 1993). A possible explanation for the requirement of RNA synthesis in replication initiation has come from measurements of *gidA* and *mioC* expression, in synchronised cultures of *E. coli*, which indicate that *mioC* is repressed just prior to initiation of chromosome replication by DnaA, whereas *gidA* transcription is repressed shortly after initiation of replication, probably as a result of the DNA becoming hemimethylated and the sequestration of *oriC* in the membrane (Theisen *et al.,* 1993; Ogawa and Okazaki, 1994) (see below). In minichromosomes, transcription of *mioC* appears to be involved in the stability and maintenance of copy number (Stuitje *et al.,* 1986; Lobner-Olesen *et al.,* 1987). Ogawa and Okazaki (1994) postulated that *mioC* transcription might be important in opening the 13-mer region through increases in negative supercoiling, as a preliminary step in the formation of the primosome complex under suboptimal conditions *eg* in DnaA-limiting conditions. The *mioC* RNA transcript could also be important for priming DNA synthesis. The transcription of *gidA* has also been postulated as a mechanism for facilitating unwinding of the DNA duplex, transcriptional activation, in readiness for replication initiation (Asai *et al.,* 1990).

An examination of the proteins that interact with *oriC* prior to the initiation of replication, has established that a number of histone-like proteins play an important role in the initiation process (Messer *et al.,* 1991; Roth *et al.,* 1994). It appears that the main role of these proteins is in modifying the topology of *oriC*, by introducing bends in the DNA, which are thought to assist DnaA in the initial unwinding of the AT-rich region of *oriC*. The key histone-like proteins that have so-far been identified in binding to *oriC* are integration host factor (IHF), a factor for inversion stimulation (FIS) or HU. By specifically mutating the binding sites for FIS of IHF in the *oriC* region of *oriC* plasmids, replication of the plasmids becomes severely impared or blocked (Roth *et al.,* 1994). Chromosomal replication is also affected in strains deficient in IHF or FIS. As described earlier, under normal conditions, multiple origins initiate simultaneously. However, deficiencies in any of the histone-like proteins results in asynchronous initiations, although these strains are still viable (Roth *et al.,* 1994). Using antibodies, some evidence has been obtained to indicate that another histone-like protein, H-NS is also associated with *oriC* (Kaidow *et al.,* 1995). No data is currently

available regarding the role of H-NS and *oriC*, although *hns* mutants apparently show reduced ploidy (Kaidow *et al.*, 1995).

Methylation of the DNA is believed to be important in regulating chromosome replication re-initiation. Like all *E. coli* DNA, the DNA around the origin is methylated *ie* the adenine nucleotide in the sequence GATC is methylated. Normally, following DNA replication, the newly synthesised strands are methylated by *dam* methyltransferase. However, the DNA around *oriC* remains "hemimethylated" for considerably longer than DNA elsewhere on the chromosome (Campbell and Kleckner, 1990). This hemimethylation has been shown to be important in sequestering *oriC* in the membrane, whereas unmethylated or fully methylated DNA do not bind to membrane *in vitro* (Ogden *et al.*, 1988). In addition, hemimethylation of the origin of minichromosomes has been shown to prevent plasmid replication in *dam*$^-$ strains (Russell and Zinder, 1987). Sequestration of the origin is thought to be important for creating a refractory period during which replication re-initiation is blocked, by preventing access of the initiator protein DnaA to *oriC*. This idea is strengthened by the fact that the 11 hemimethylated GATC sequences in *oriC* and the *dnaA* promoter region remain hemimethylated for approximately one third of the cell cycle in rapidly growing cells, whereas the same sequences in other parts of the chromosome become fully methylated within 2-3 minutes (Campbell and Kleckner, 1990). In *dam*$^-$ mutants and Dam overproducing strains, synchrony of initiation of multiple chromosome origins is lost and over replication is observed (Bakker and Smith, 1989; Boye and Lobner-Olesen, 1990). If these conclusions are true for *E. coli*, how do Gram-positive bacteria regulate re-initiation of chromosome replication, since they do not possess GATC methylation sites?

Recent studies have identified two different *E. coli* proteins, SeqA (Lu *et al.*, 1994; von Freiesleben *et al.*, 1994) and HobH (Herrick *et al.*, 1994) that appear to negatively regulate re-initiation of replication. SeqA appears to interact directly with DnaA, and to restrict its activity. Mutants of *seqA* re-initiate chromosomal replication at a much higher frequency than normal (von Freiesleben *et al.*, 1994). It has been postulated that SeqA might be involved in the ADP / ATP exchange reaction on the DnaA protein (Lu *et al.*, 1994 ;von Freiesleben *et al.*, 1994), and that removal of SeqA results in a much more rapid exchange. Alternatively, von Freiesleben *et al* (1994) suggest that in the presence of SeqA, only the ATP-bound form of DnaA is functional, whereas either form will function in its absence. The 24kDa HobH protein is reported to localise in the outer membrane (Herrick *et al.*, 1994) and raises the interesting possibility (although hotly debated, especially since HobH does not appear to contain a signal sequence required for its translocation through the cytoplasmic membrane), that *oriC* does not just associate with the inner membrane, but in fact traverses the

periplasm, and associates with the outer membrane, through interaction with outer membrane proteins. In *hobH* mutants, a partial loss of synchrony of replication initiation occurs, resulting in cells containing irregular numbers of chromosomal origins (*ie* not 2^n, see previous section). HobH apparently has a very strong affinity for the parent-strand (*ie* methylated strand) of hemimethylated DNA and may also be involved in controlling or inhibiting immediate re-initiations by DnaA.

Another negative regulator of chromosome replication initiation in *E. coli* has been identified, IciA (Crooke *et al.*, 1991; Hwang and Kornberg, 1992; Hwang *et al.*, 1992; Yoo *et al.*, 1993). The 33kDa IciA protein binds to the 13-mer regions of *oriC*, preventing the interaction with DnaA and therefore prevents opening of the AT rich sequences with concomitant entry of the DNA replication proteins. Overexpression of *iciA* results in a pronounced growth lag following transfer of culture to fresh medium. Moreover, the cellular concentration of IciA increases four-fold during entry into stationary phase. Apparently, the serine protease, HtrA, which has been reported to be located in the periplasm (Lipinska *et al.*, 1989), although this is disputed by Yoo *et al* (1993), who claim that the protease is cytoplasmic or membrane associated, is capable of cleaving IciA and preventing it from binding to *oriC in vitro*, thereby permitting initiation of chromosome replication (Yoo *et al.*, 1993). Disruption of the *htrA* gene results in a decrease in the overall rate of intracellular proteolysis, and a loss of viability at elevated temperatures. No information is currently available, regarding the *in-vivo* effects of *htrA* inactivation on replication initiation. Disappointingly, strains that either lack or overexpress IciA, do not have any obvious phenotype (Thöny *et al.*, 1991). Reports of another protein of 150kDa, that apparently inactivates DnaA have appeared recently, which is different to IciA, SeqA and HobA (Katayama and Crooke, 1995). However, no information is currently available regarding the nature of the protein, its gene or mode of action.

Bacteria clearly need multiple mechanisms for regulating the initiation of chromosome replication, since, hemimethylated DNA only remains sequestered within the membrane for approximately 10 minutes, and therefore, if no other inhibitory mechanisms existed, new rounds of initiation would occur much more frequently than is observed (Campbell and Kleckner, 1990). Some of these regulators are now being investigated, with a view to unravelling this key restriction point in the bacterial cell cycle.

1.3.3 Termination of chromosome replication

The termination region for chromosomal replication, *ter* is centred at approximately 32' on the the *E. coli* chromosome, diametrically opposite to *oriC* (for a review, see Baker, 1995). In the *ter* region there are six 22bp termination sites, *terA*-

terF to which the 36kDa protein Tus (termination utilisation substance) binds (see **Fig. 3**). The Tus-*ter* complexes form a ratchet mechanism such that replication forks can enter the *ter* region, but are prevented from exiting and continuing around the chromosome, back towards the origin. The component of the replisome that is believed to be sensitive to the Tus-*ter* complexes is the DnaB protein (helicase). Several other *E. coli* and bacteriophage helicases and the mouse helicase B are also impeded by the Tus-*ter* complexes (Hidaka *et al.*, 1992). Using plasmid models, it has been established that the Tus-*ter* region is used to prevent runaway replication and consequent multimerisation (Lee *et al.*, 1989; Hiasa and Marians, 1994). A high level of recombination activity has been observed in the Tus-*ter* region (Louarn *et al.*, 1994; Horiuchi *et al.*, 1994). It has been suggested that the pausing of the replication forks at the Tus-*ter* region provides a target site for the homologous recombination machinery, RecBCD (Horiuchi *et al.*, 1994).

In most instances, decatenation of the duplicated chromosomes is mediated by the DNA topoisomerases (see below). However, even under optimal conditions, sister chromatid exchange can result in the formation of circular dimers that cannot be resolved by the topoisomerases, and occurs at a frequency of approximately 3.5% (Lobner-Olesen and Kuempel, 1992). Resolution of these dimers is achieved through recombination at *dif* and is mediated by the two recombinases, XerC and XerD (Blakely *et al.*, 1993; Arciszewska and Sherratt, 1995). In addition, XerC and XerD function in combination with additional proteins ArgR and PepA in the resolution of a number of plasmids (Sherratt *et al.*, 1995). The use of the two recombinases ensures inter-molecular recombination (Blakely *et al.*, 1993). The *dif* site on the chromosome and the equivalent site, *cer* on ColE1 based plasmids are asymmetric, and it is suggested that this ensures correct alignment of the DNA strands prior to recombination (Blakely *et al.*, 1993).

1.3.4 Chromosome segregation

In eukaryotes, contractile force-generating proteins (kinesins and dyneins in conjunction with microtubules) are used to segregate the duplicated chromosomes, aligned along the centre of the cell at metaphase, to the cell poles prior to cell division. Bacteria, as mentioned earlier, can contain several replicating chromosomes at different stages of completion. Ultimately, each chromosome needs to be segregated to separate daughter cells. There has been considerable speculation as to the mechanism that leads to segregation. The earliest model proposed that the *oriC* of the chromosome is anchored to the cell membrane at the mid-point of the cell and following duplication of the origins, cell wall growth occurring from the mid-point outwards drags the origins towards opposing cell poles (Jacob *et al.*, 1963). This model is now widely discounted

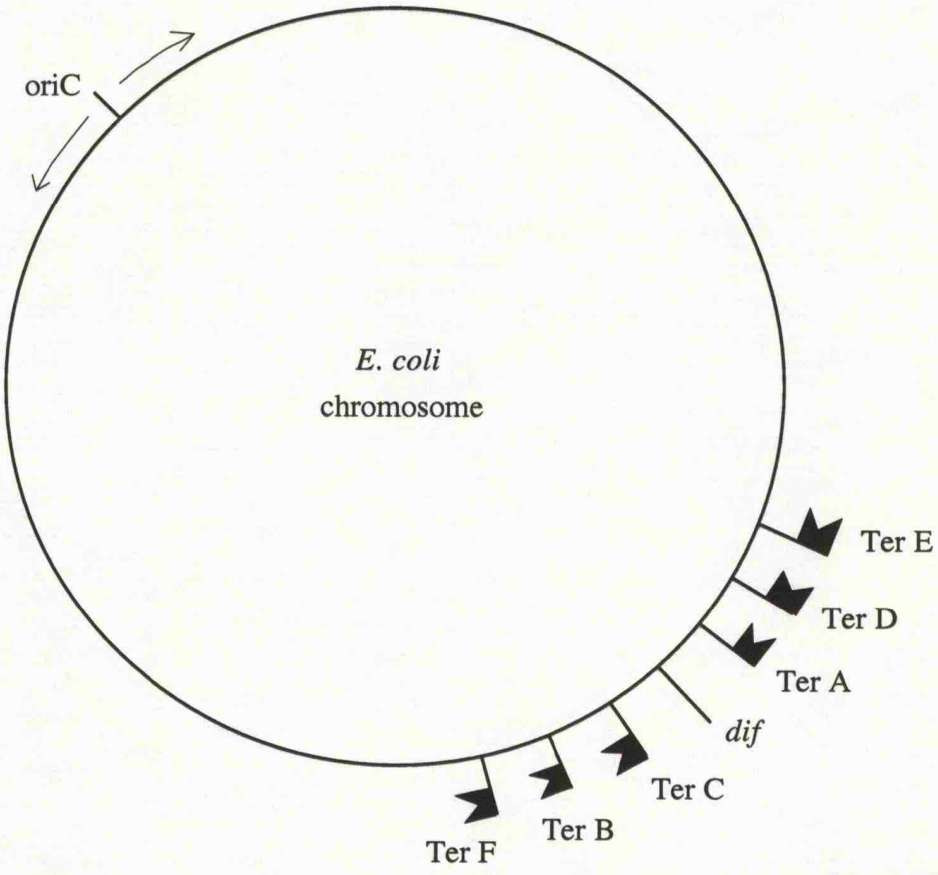

Fig. 3 Organisation of the *oriC* and *ter* regions of the *E. coli* chromosome.

since the nucleoids detach from the membrane periodically (see above) and cell wall material is inserted throughout the cell surface and during the entire cell cycle (Woldringh *et al.*, 1987). Moreover, the nucleoids of *B. subtilis* (Sargent, 1974) and *E. coli* (Donachie and Begg, 1989; Hiraga *et al.*, 1990), when examined by phase contrast microscopy, appear to "jump" to the quarter positions of the cell prior to division (*ie* not a smooth or gradual movement as would be expected from the model of Jacob *et al*). In addition, post-replication protein synthesis is required for chromosome segregation (Donachie and Begg, 1989; Hiraga *et al.*, 1990).

A typical feature of mutants affected in chromosome partitioning/segregation is the accumulation of DNA at the centre of the cell followed by division which results either in one cell inheriting two chromosomes and the other becoming anucleate or the septum acting as a guillotine, cutting across the nucleoids. Many of the mutants displaying these phenotypes (*par*$^-$), are defective in either DNA synthesis, DNA gyrase, or in the topoisomerases (Norris *et al.*, 1986; Hussain *et al.*, 1987; Kato *et al.*, 1988, 1989; Adams *et al.*, 1992). Both DNA gyrase and topoisomerase IV are required for decatenation of duplicated plasmids and chromosomes. In addition, gyrase introduces negative supercoiling and reduces the linking number of the duplicated chromosomes, whereas topoisomerases III and IV decatenate the chromosomes and plasmids (topoisomerase III appears to be dispensible) (reviewed in Luttinger, 1995). Two further *E. coli* mutants which display an inability to segegate their nucleoids efficiently (*muk*) were isolated. The first mutant, *mukA* (Japanese *mukaku* = anucleate) is affected in the *tolC* gene (Hiraga *et al.*, 1989). TolC mutants are hyper-sensitive to SDS and have altered levels of porin synthesis. In addition, TolC, which is an outer membrane protein (Morona and Reeves, 1982), is required for secretion of haemolysin (Wandersman and Delepelaire, 1990). The role of TolC in chromosome segregation is not understood although it has been suggested that the cell membrane or specific membrane sites associated with TolC might be required for active positioning of the daughter chromosomes (Kunitoshi *et al.*, 1992). The second gene, *mukB* (Niki *et al.*, 1991) encodes a 177kDa protein with many properties in common with force-generating proteins such as myosin heavy chain or kinesin heavy chain. The N-terminus has homology with the microtubule-associated mechanochemical enzyme dynamin (D100) (Niki *et al.*, 1991). The predicted tertiary structure of MukB suggests that it has a globular N-terminus, and two paired rod-shaped internal domains connected by a hinge region. The primary sequence indicates the presence of an ATP binding site and three zinc-fingers (common amongst eukaryotic DNA binding proteins). The purified protein forms homo-dimers. A consequence of deleting the *mukB* gene is that 5% of cells become anucleate compared with less than 0.03% in the wild-type but are normal sized at permissive temperatures below 22°C. At higher temperatures, a mixture of

normal sized and filamentous cells are formed, often with replicated but unsegregated nucleoids.

A very recent and exciting discovery (for reasons that will become clear later) emerged from the observation that mutants affected in the histone-like DNA protein, H-NS give rise at high frequency, to anucleate cells (Kaidow *et al.*, 1995). The protein H-NS is rapidly becoming one of the most intensively studied proteins in *E. coli*, due to its role in the regulation of a plethora of often unrelated genes (for a review of its properties, see Higgins *et al.*, 1990; Hulton *et al.*, 1990). No data is currently available to explain how H-NS might be involved in chromosome partitioning, since it does not demonstrate any properties that in themselves would appear to be useful in the partitioning process (*cf* MukB). The most likely hypothesis is that H-NS probably regulates a gene whose product *is* required for the partitioning process. Alternatively, the fact that H-NS is involved in nucleoid compaction could be important in segregation, as is the case in eukaryotes.

1.3.5 Cell division

The first major attempts to study the mechanism of cell division in *E. coli* were performed by Hirota *et al* (1968), who isolated a number of temperature sensitive mutants that were unable to septate correctly at the non-permissive temperature. Although these mutants were unable to septate, they were nevertheless able to continue growing. Such cells formed filaments with nucleoids evenly distributed along them at the non-permissive temperature. Because of this phenotype, these mutants became known as *fts* (filamentation temperature sensitive). A second set of mutants, *par* were also isolated, based on their inability to *par*tition their nucleoids at the non-permissive temperature (see above). The isolation of conditional mutants in many of the genes now understood to be involved in cell division, has been useful but has permitted the temporal sequence of events catalysed by their corresponding proteins to be determined to only a limited extent so far.

1.3.5.1 Models for regulating the cell division cycle

Numerous questions regarding the sequence of events leading to division of the cell need to be addressed, and the answers to many of them have yet to be completely resolved. For example, what determines when the cell commences septum formation, switching from lateral cell wall growth to inward cell wall growth? What determines the positioning of the septum? What checkpoints exist to inhibit the septation process

if a deleterious event occurs such as inhibition of DNA replication, DNA damage or heat shock?

It is widely recognised that the sacculus (the peptidoglycan 'bag' that surrounds the cytoplasmic membrane and provides the support and rigidity required for survival in a hostile environment), plays a major role both in determining the shape of the cell and the division process. Since the sacculus is "seamless", certain logistical problems exist in incorporating new cell wall material into the sacculus to permit growth to take place, and yet constrain the enormous pressures exerted from within. In addition, at a certain stage during the growth cycle of the bacterial cell, cell wall synthesis must undergo an abrupt change from lateral growth (elongation) to invagination (septum formation) (Nanninga, 1991). Numerous models have been developed to try and understand how these processes are regulated and coordinated.

Cooper has developed models based upon the premise that different biosynthetic mechanisms synthesising DNA, cytoplasmic components and the cell envelope function essentially independently and are regulated by local passive mechanisms (Cooper, 1991). The main driving force is considered to be the synthesis of cytoplasmic components which is a direct consequence of the interaction of nutrients, precursors, energy and environmental factors. He argues that synthesis of lateral cell wall is continuous and that formation of the septum has preference over lateral wall, forcing interruptions in the synthesis of lateral cell wall material. He also argues against a specific cell cycle event triggering the initiation of septum formation and that the cell surface grows or stretches to accommodate increased cell mass. All the experimental data used to formulate this model was performed using the membrane elution method of Helmstetter and Cooper to generate cells at an identical stage in the cycle, by minimising the introduction of errors through invasive experimental techniques. Using this system, an exponential increase in cellular RNA and protein was demonstrated (Boyd and Holland, 1979; Cooper, 1988).

An alternative model of cell division control was developed by Kubitschek (1990) whereby cells grow at a constant, linear rate until they reach a critical mass or volume or length upon which, the growth rate doubles. Evidence in support of this hypothesis is the apparent doubling in the rate of synthesis of outer membrane proteins at a specific time during the cell cycle (Churchward and Holland, 1976; Boyd and Holland, 1979). Kubitschek extended these latter findings in proposing that transport systems may double abruptly and he found that RNA and protein precursor pools doubled at mid-cycle, followed by a return to normal levels at the end of the cell cycle. Several studies also showed a linear (with doubling) rate of phospholipid synthesis during the cell cycle, which coincided with the initiation of chromosome replication (Pierucci, 1979; Pierucci et al., 1981).

The Two Competing Site model (Satta *et al.*, 1994) attempts to define the regulation of bacterial cell shape and growth. The reference to Site does not specifically imply physical positions (or sites), but can also apply to chemical reactions that create a dichotomy. For example, in rod-shaped bacteria such as *E. coli*, there are two competing reactions resulting in cell elongation and septum formation, mediated by different penicillin binding proteins (PBPs) (Spratt, 1975). By using sublethal concentrations of antibiotics which selectively inhibit the formation of the septum (penicillin G) or elongation of the cell wall (mecillinam), filaments and cocci are formed, respectively. The model goes on to suggest that bacteria that evolved early only possess a single site for peptidoglycan assembly and therefore naturally only form cocci. Thus, blockage of peptidoglycan synthesis from this site results in cessation of growth, whereas more recently evolved bacteria with two competing sites for peptidoglycan synthesis, such as *E. coli* either form cocci or filaments depending upon which site has been blocked (for elongation or septation respectively). Superimposed upon the basic motor driving the two competing sites, there are a multitude of additional competing mechanisms modulating and fine tuning the basic system. These are neccessarily required to protect the cell when for example, DNA damage takes place, or the cell is subjected to thermal stress. Systems such as the *minB* operon (see later) which controls the positioning of the septum, or the *sfiA* SOS system which is capable of inhibiting septation following DNA-damage or interference of DNA replication are competing and/or interacting with activators of septation such as SdiA (see later). The protein FtsZ functions to integrate all of these signals, the consequence of this being the commencement of formation of a correctly positioned septum at the correct time in the cell cycle (as described below).

Although the above models attempt to explain how the bacterial cell regulates the morphology of the cell and relates it to cell division, they still do not provide an adequate model for the switch mechanism that triggers the transition from lateral cell growth to septation. Nor do these models take into account that bacteria have evolved biochemical controls to regulate proliferation, and control the periods during which particular processes take place, *ie* many processes are compartmentalised in time to particular stages in the cell cycle. In other words, what cellular parameters does the cell measure and use to determine when septation should commence? What is the actual signal which initiates the changes?

1.3.5.2 The role of the sacculus in cell division

As indicated above, the *E. coli* cell envelope is comprised of 3 layers, the cytoplasmic membrane, the periplasm containing the peptidoglycan sacculus, and the outer membrane. The sacculus is a dynamic structure of great strength due to its 3-

dimensional structure, consisting of sugar backbones made from alternating N-acetyl muramic acid and N-acetyl glucosmine residues, and crosslinked to other sugar backbones by short peptides, which are in turn crosslinked to other peptides (see Nanninga, 1991, for a review). It is the peptidoglycan that ultimately determines the shape of the bacterium, and mutations affecting enzymes involved in the synthesis of the sacculus often result in morphological changes (Spratt, 1975; Wientjes and Nanninga, 1989). During cell elongation, components of the sacculus are being constantly incorporated into the sacculus. This involves making specific breaks in the peptidoglycan by autolysins, followed by incorporation of new material, mediated by the PBPs.

Evidence for a generalised incorporation of peptidoglycan components throughout the entire cell envelope during cell elongation, followed by a switch to a localised ingrowth at the septum, has accumulated in recent years, for example, the data from Wientjes and Nanninga (1989), who developed the concept of the Leading Edge to explain the penicillin-binding protein 3 (PBP3)-mediated ingrowth of peptidoglycan during septation. PBP3 is probably a peptidoglycan transglycolase (involved in the elongation of new glycan strands in the peptidoglycan layer) and transpeptidase (crosslinks the individual strands of peptidoglycan) (Ishino *et al.*, 1986). During growth of the cell, there is an apparent topological shift from generalised incorporation of peptidoglycan throughout the cell envelope to the centre of the cell, where septation occurs (reviewed in Nanninga, 1991).

Attempts have been made to determine whether PBP3 levels fluctuate during the cell cycle, but the apparently low levels of this protein (at most, 50 molecules per cell (Spratt, 1975)) have prevented definitive conclusions so far. The gene *ftsI* that encodes PBP3 is negatively regulated by the *mreB* gene product (Wachi and Matsuhashi, 1989). By inactivating the *mreB* gene, cells become coccoid in shape, a feature also seen following the inactivation of PBP2, which is involved in cell elongation (Donachie,1993). Thus, there appears to be a balance between the levels of PBP2 and PBP3, which determines the shape of the cell.

1.3.5.3 Formation of the septum

One of the earliest events which has been postulated to be required for the determination of the future division site is the formation of two periseptal annuli - concentric rings of modified cell envelope (MacAlister *et al.*, 1983; Cook *et al.*, 1986). The periseptal annuli appear to form a continuous zone of adhesion linking the cytoplasmic membrane with the murein sacculus and outer membrane, and are thought to form a domain of the cell envelope which is distinct from elsewhere in the cell. The presence of periseptal annuli can be readily visualised microscopically, by subjecting *E.*

coli to hyper-osmotic treatment. This results in the formation of plasmolysis bays - zones where the cytoplasmic membrane separates from the murein layer, and are bounded by annular adhesion zones (Cook *et al.,* 1986). These observations suggested that the annuli might originate at the centre of the cell and be moved apart towards the 1/4 and 3/4 positions of the elongating cell. However, the mechanism promoting such movement is obscure and cannot be due to surface growth since, as described above, cell wall growth involves incorporation of cell wall material in a diffuse pattern for most of the cell cycle, rather than localised to the centre of the cell (Burman *et al.,* 1983; Wientjes and Nanninga, 1989). In addition, Mulder and Woldringh (1993) studied the displacement of plasmolysis bays, and failed to confirm that the plasmolysis bays are influenced by gradual movement of periseptal annuli from the centre of young cells towards the 1/4 and 3/4 positions of older pre-divisional cells.

1.3.5.3.1 FtsZ and the prokaryotic cytokinetic ring

The first observable change in the cell which leads to the initiation of septation is the appearance of a protein ring composed of polymerised FtsZ (Bi and Lutkenhaus, 1991). The discovery of this ring initiated a series of exciting observations about the nature of FtsZ and provided the first strong evidence of a tubulin-like protein which could be responsible, through relative rates of polymerisation and depolymerisation, for drawing the mid-point of the cell closed like a camera shutter. Bi and Lutkenhaus (1991) used gold-labelled anti-FtsZ antibody to study the cellular distribution of FtsZ during the *E. coli* cell cycle. During the majority of the cell cycle, FtsZ exists as a monomer, distributed evenly throughout the cytoplasm. However, just prior to the initiation of septation, FtsZ assembles into a ring on the cytoplasmic surface of the inner membrane. The FtsZ ring remains at the leading edge of the invaginating septum and only dissociates upon septum completion.

Studies on the levels of FtsZ in the cell during the cell cycle indicate that mRNA expression from at least one promoter is of a cyclical nature (Garrido *et al.,* 1993; Zhou and Helmstetter, 1994). Garrido *et al* claimed that maximal *ftsZ* expression is seen at the time of initiation of chromosome replication. However, Zhou and Helmstetter subsequently disputed the observations of Garrido *et al.,* finding that a pause or refactory period in *ftsZ* expression occurs at the time of replication of the 2min cluster of genes (see **Fig.** 4), giving in their view, the illusion of controlled periodic synthesis. Nevertheless, strikingly in the Garrido *et al* (1993) experiments, the level of FtsZ required for normal length control was greater when expressed from the constitutive (with respect to the cell cycle) *lac* promoter, rather than from its own promoters.

Further evidence about the possible similarity between FtsZ and eukaryotic α, β and γ-tubulins emerged from the finding that FtsZ possesses an Mg^{2+} dependent GTPase activity which is essential for its function (de Boer *et al.,* 1992; RayChaudhuri and Park, 1992). The GTP binding / GTPase activity in FtsZ was found to be associated with a 7 amino-acid motif GGGTGTG which is virtually identical to the GTP/GDP binding domain in the tubulins. A single amino-acid substitution within this motif is sufficient to eliminate GTPase activity (de Boer *et al.,* 1992; RayChaudhuri and Park, 1992) and prevent septum formation at elevated temperatures (de Boer *et al.,* 1992). Activation of FtsZ by GTP is dependent upon protein concentration, and shows the sort of kinetics that are normally seen in self-associating reactions, for example, the nucleotide-dependent assembly of microtubules and actin filaments. By far the most convincing evidence relating to the ability of FtsZ to form microtubule-like structures came from work performed by Mukherjee and Lutkenhaus (1994), and by Bramhill and Thompson (1994). This purified FtsZ was shown to polymerize to form filaments and tubules that have similar dimensions to those of microtubules, as visualised by electron microscopy (Mukherjee and Lutkenhaus, 1994). Reversible *in vitro* polymerization and depolymerization of FtsZ was demonstrated in the respective presence and absence of GTP+Mg^{2+}, followed by a second round of polymerization upon a further addition of GTP and Mg^{2+} (Bramhill and Thompson, 1994). However, using a number of *ftsZ* mutants defective in GTP binding and/or GTPase activity *in vitro*, it was found that the GTPase activity of FtsZ may not be essential for its function (Dai *et al.,* 1994), contrary to earlier published observations, since certain mutants that lack GTPase activity still support growth.

Coupled to its role in the initiation of septation, the concentration of FtsZ is also critical, since excess FtsZ results in division at the cell poles, resulting in the formation of chromosomeless minicells in addition to the normal divisions at the cell centre (Ward and Lutkenhaus, 1985). The concentration of FtsZ also appears to be important in coupling nucleoid separation with the initiation of septation, since reducing the levels of FtsZ results in a delay in septation, but does not affect the actual septation process (Tétart *et al.,* 1992). Tétart *et al* therefore suggested that the concentration of FtsZ actually determines the timing of septation. In addition, they observed that in strains with sub-optimal levels of FtsZ, nucleoid separation is delayed by the same period as septation. Moreover, a *ts ftsZ* mutant containing suboptimal levels of FtsZ protein, when transferred to the nonpermissive temperature showed a severe defect in nucleoid segregation (normally, nucleoid segregation in *ftsZ* mutants containing correct levels of the FtsZ protein is not affected).

Since FtsZ plays a pivotal role in the initiation of the septation process, it provides the ideal element in the cell cycle to act as a restriction point. As will be

described later, FtsZ is the receptor for numerous signals relating to the cells metabolic and environmental status, and to DNA or cellular damage. FtsZ activity can be blocked by a number of cellular inhibitors or its expression enhanced to induce cell division.

1.3.5.3.2 The formation of the septalsome (divisisome)

As mentioned above, the formation of the FtsZ ring is only the first stage in the septation process. PBP3, a transmembrane protein, encoded by the *ftsI* gene (see section 1.3.5.2 and **Fig. 4**) is believed to interact with a number of cell division specific proteins such as FtsZ (Walker *et al.,* 1975), FtsA (Tormo *et al.,* 1986) and FtsQ (Mukherjee *et al.,* 1993). In addition, other proteins FtsW and RodA, which act together with PBP2 in lateral wall synthesisis, are also thought to interact with PBP3 (Matsuhashi *et al.,* 1990). Such a complex of proteins is suggested to form a structure called the septalsome (Holland and Jones, 1985) or divisisome (Nanninga, 1991) which links cytoplasmic, cytoplasmic membrane and periplasmic components which comprise the biochemical machinery that forms the septum. It is further proposed that the septalsome couples cytokinetic or contractile proteins with the division mechanism. Nanninga proposes a model for the interaction of the different septation proteins (Nanninga, 1991) and brings in the idea first postulated by Norris *et al.* (1988); Norris, (1989) that calcium ions, like magnesium ions (Holland and Jones, 1985) might play a role in activating the cytoplasmic form of FtsZ, and enabling it to polymerise and interact with the membrane thus initiating the sequence of events leading to cell division.

Completion of the septation process requires the action of the EnvA protein to split the double layer of peptidoglycan formed during invagination (Wolf-Watz and Normark, 1976). Inactivation of *envA* results in the the formation of chains of cells resembling "strings of sausages" (Donachie and Robinson, 1987).

1.3.6 Regulating bacterial cell division

As indicated previously, there are numerous mechanisms potentially involved in the regulation of the bacterial cell division cycle. For example, the positioning of the septum is critical, since it is both wasteful and potentially deleterious to divide in such a way as to generate chromosomeless and polyploid cells, or to 'guillotine' the nucleoid. In addition, the cell needs to be able to respond to DNA damage, and to repair the damage before dividing, or to respond to heat shock which might damage critical proteins, and finally to respond to starvation.

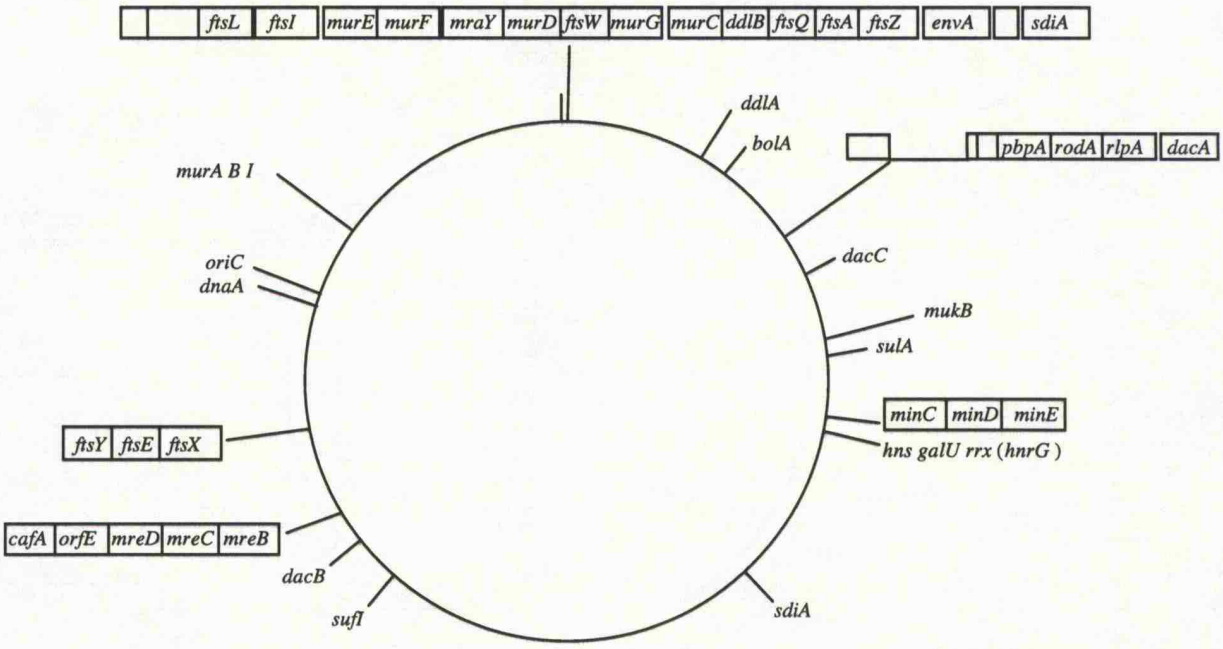

Fig. 4 Chromosomal location of some of the *E. coli* cell cycle genes, taken from (Donachie, 1993). The genes *hns*, *galU* and *rrx* (*hnrG*) have been indicated for later reference.

1.3.6.1 Ensuring correct positioning of the septum

Adler *et al* (1967) first isolated a mutant *E. coli* strain that produced a mixture of miniature, chromosomeless, normal-sized and elongated cells. The *minB* operon, which when mutated, leads to minicell production, was found to encode three genes, *minC*, *minD* and *minE* (de Boer *et al.,* 1989). These mutants were found to divide either centrally (symmetrically) or at one of the cell poles (asymmetrically). It was then established that the central parental division site, that subsequently forms one pole of each daughter cell, needs to be "blocked" or inactivated such that it ceases to be accessible or usable as a septation site during subsequent cell division events. In addition, the mid-point of the cell needs to remain accessible to the the septation machinery.

The function of the MinE protein has yet to be fully established. However, it has been shown to be required for topological specificity of the the cell division inhibition system. By over-expressing this protein, specificity is lost, and septation frequently occurs at the cell poles. Conversely, deletion of the *minE* gene results in a filamentous phenotype (de Boer *et al.,* 1989). The MinC protein appears to be the effector of this cell division inhibition system since over-expression of the *minC* gene results in cell division inhibition in the absence of the *minD* and *minE* genes, whereas deletion of this gene causes the classical minicell phenotype. Deletion of the *minD* gene also results in the minicell phenotype, however, overexpression does not lead to a general inhibition of septation in the presence of MinC. Evidence regarding the separate roles of MinC and MinD emerged through studies on another cell division inhibitor, DicB (Béjar and Bouché, 1985; Béjar *et al.,* 1988). Normally, expression of DicB is tightly repressed by the action of the *dicA* and *dicC* gene products (Béjar *et al.,* 1986). Derepression of DicB by mutations in either *dicA* or *dicC* or by coupling *dicB* to a different promoter leads to a block in cell division, resulting in filamentation. The relationship between DicB and the *minB* operon was established when *ftsZ* mutants were isolated that failed to filament upon induction of *dicB* expression (Labie *et al.,* 1989). Later experiments showed that DicB interacts with MinC to inhibit division, but is independent of MinD. Further evidence regarding the independence of the two division inhibition reactions was shown by experiments that demonstrated that whereas MinE is required for correct positioning of the septum in conjunction with MinC and MinD, over-expression of MinE has no effect upon DicB/MinC division inhibition (de Boer *et al.,* 1990b). From these experiments, it was concluded that MinD and DicF are activators of MinC which inhibits cell division through interaction with the central component of the the cell division machine, FtsZ. Indeed, recent studies have now confirmed this to be true (see below). MinD in addition to activating MinC, also couples the topological specificity factor MinE with MinC. More recent studies

have shown that MinD is membrane-bound, capable of binding ATP, and has ATPase activity. Moreover, mutagenesis of the putative ATP binding site in MinD was shown to inhibit its function in activating MinC (de Boer *et al.*, 1991). Nevertheless, attempts to detect the location of the Min proteins at specific sites in the membrane by electron microscopy have so far been unsuccessful.

Evidence for the interaction of the MinCDE system with FtsZ began with the observations that over-expression of FtsZ resulted in the minicell phenotype (Ward and Lutkenhaus, 1985). Overexpression of FtsZ also suppresses the MinC/MinD and MinC/DicB cell division blocks (de Boer *et al.*, 1990b). Moreover, in strains containing certain *ftsZ* alleles, over-expression of MinC failed to cause filamentation. An increase in the cell length distribution occurs in *min* mutants (Teather *et al.*, 1974), which is restored by a slight increase in the levels of FtsZ, thus suggesting that FtsZ is rate limiting for cell division (Bi and Lutkenhaus, 1990), and elevated levels of FtsZ leads to more division events per mass doubling.

1.3.6.2 The SOS response

Bacteria have evolved a number of complex interlinking regulatory networks for responding to environmental stresses and cellular perturbations. One of the best understood regulatory networks, and the prototype cell cycle checkpoint is responsible for protecting the cell if DNA damage is sustained or when DNA replication is blocked. Damage can be sustained, for example, through exposure to ionising or ultraviolet radiation, chemical mutagens such as mitomycin C, or drugs which inhibit DNA gyrase, for example, nalidixic acid. The role of the SOS system is two-fold a) to activate DNA repair systems and b) stop the cell cycle, usually by inhibiting division until the damage has been repaired. An excellent review of the SOS system can be found in (Walker, 1987), where a description of many of the SOS-regulated genes is given.

The two key proteins regulating the SOS response are RecA and LexA, encoded by the *recA* and *lexA* genes respectively. RecA is a 37.8kDa protein, and is essential for homologous recombination, catalysing synapsis and homologous strand exchange. Its role in the SOS response is as the major regulator, since inactivation of the *recA* gene prevents induction of the SOS response (Clark, 1973). The 22.7kDa LexA protein is a repressor, which binds to the promoters of a number of different genes, including *recA* and *lexA*, containing the consensus sequence TACTGATATA-A-ACAGTA (Walker, 1984). Inactivation of the *lexA* gene was shown to result in constitutive expression of the SOS regulon, thus providing early evidence for the role of LexA as a repressor (Mount *et al.*, 1972).

RecA regulates LexA promoter-binding by inducing cleavage of the LexA repressor and thus removing the transcriptional block imposed by LexA (Little, 1991). RecA does not possess proteolytic activity *per se*, but, following activation, induces LexA to undergo an autolytic event (for a review, see Little, 1993). This is further demonstrated by the fact that LexA undergoes self-cleavage at elevated pH *in vitro*, in the absence of RecA (Little, 1984). Activation of RecA is believed to result from binding to single stranded DNA and is nucleoside triphosphate dependent (Higashitani, N. *et al.,* 1995). Moreover, the SOS response is modulated by two additional proteins, RecF and the single stranded-DNA-binding protein, Ssb. Ssb is understood to bind and to protect single stranded DNA resulting from DNA damage. Proteins RecO and RecR act to remove Ssb, enabling RecA to bind, and initiate the SOS response (Umezu *et al.,* 1993).

At least 21 genes have now been shown to be regulated by LexA (Hegde *et al.,* 1995), and are primarily concerned with repairing damage caused by DNA-damaging agents. In addition to the cellular SOS-responsive genes, the repressors required for the maintenance of lysogeny by a number of prophages are also cleaved by the action of activated RecA, for example bacteriophage λ (Walker, 1987). This is clearly vital for the lysogenic phage, since it needs to "evacuate the burning ship" if its host is subjected to potentially harmful DNA damage. Of particular interest here is how the SOS response affects the cell cycle.

As described earlier, the protein FtsZ acts as a cell division initiator, being a target for the cell division inhibitor MinCD (Bi and Lutkenhaus, 1993). It has been shown that the SOS-regulon also acts upon FtsZ to reversibly block septation through the 18kDa protein SulA (SfiA) (Jones and Holland, 1985; Jaffé *et al.,* 1986). In addition to MinCD, SulA also blocks the formation of the FtsZ ring (see section 1.3.5.3.1) which highlights the importance of the ring as a critical precursor to septation (Bi and Lutkenhaus, 1993). A recent report indicates that SulA forms a stable complex with FtsZ which is dependent upon FtsZ-mediated GTP hydrolysis in the presence of Mg^{2+} ions (Higashitani, *et al.,* 1995). Apparently, Ca^{2+} ions cannot substitute for Mg^{2+} in this reaction (Higashitani, *et al.,* 1995). However, a study by (Dai *et al.,* 1994) showed that some *ftsZ* mutants that have reduced GTPase activity did not become resistant to the SulA division inhibitor. One possible explanation for this result is that binding GTP changes the conformation of FtsZ to activate it, and that some of the mutations that lead to reduced GTPase activity, result in a change in the FtsZ conformation so that it mimics the wild type GTP-bound protein.

A second SOS-dependent division inhibitor, SfiC is found in some *E. coli* strains, and is encoded by an excisable genetic element, e14, that possesses properties suggestive of a defective prophage (de Boer *et al.,* 1990a). SfiC differs in one major

aspect from SulA in that although it is RecA dependent for activation, it is LexA independent for regulation (de Boer *et al.*, 1990a).

SulA is completely dispensible for normal growth of *E. coli* (Donachie and Robinson, 1987). Nevertheless, an excess of SulA results in lethal filamentation (Gottesman *et al.*, 1981). *E. coli* utilises the protease, Lon to degrade SulA (Mizusawa and Gottesman, 1983). Indeed the half-life of SulA in *lon*$^{+}$ cells is only 3 min, whereas the half-life in *lon*$^{-}$ strains is 30 mins (Mizusawa and Gottesman, 1983; Jones and Holland, 1985; Canceill *et al.*, 1990). Therefore, the instability of SulA and the presence of the Lon protease provides an exquisitely sharp and precise division control, ensuring that cell division can recommence as soon as the synthesis of SulA is blocked by the re-establishment of LexA repressor, when the source of the SOS-response induction has been removed.

As indicated above, FtsZ is the target for a number of cell division inhibitors. However, FtsZ is also the target of a cell division promoter, SdiA (Wang *et al.*, 1991). SdiA has been shown to enhance expression of the *ftsZ* gene by specifically binding to the P2 promoter upstream of the *ftsQAZ* gene cluster. A protein sequence comparison indicated that SdiA is related to the response regulator components of a number of two-component response regulators. Overexpression of SdiA results in minicell formation (as does FtsZ) and renders cells resistant to the division inhibitors MinC/MinD, DicF/MinC and SulA. However, deletion of SdiA has no discernible effect upon cell division (Wang *et al.*, 1991). Thus SdiA appears to be a non-essential enhancer of cell division.

1.3.6.3 The heat-shock response

A complex regulatory network, the heat-shock regulon involves component proteins which have been highly conserved during evolution from bacteria to Man. Excellent reviews of this subject can be found in Georgopoulos *et al.* (1990) and Yura *et al.* (1993). The heat-shock response fulfills three functions: 1) prevention of inactivation of cellular proteins, 2) reactivation of heat-inactivated proteins and 3) degradation of irreparably denatured proteins that occur either during normal growth or due to heat. Like the SOS-regulon, the heat-shock regulon has a specialised means of regulating the expression of heat-shock proteins (HSPs), in this case through the use of a specific RNA polymerase (E), containing the σ^{32} subunit, encoded by the *rpoH* gene. The σ^{32} subunit recognises the following promoter consensus sequence, found upstream of all HSP genes:

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TctCxcCcTTGAA  13~17  CCCCATtTA    σ32
      TTGACa   16~18     TAtAaT    σ70
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(Georgopoulos *et al.*, 1990; Yura *et al.*, 1993)

In *E. coli*, an increase in temperature from 30 to 42°C results in a rapid induction of HSPs, reaching maximum levels after 5 min, which then rapidly attenuate to a level only slightly higher than the basal levels. The extreme rapidity of the heat-shock response, is largely due to rapid, transient changes in the stability of $E\sigma^{32}$, which normally has a half life of <1 min, but following induction of the heat-shock response, the half-life is increased 10 to 30-fold (Yura *et al.*, 1993). Although the mechanism responsible for the increased stability of $E\sigma^{32}$ is unknown, it is thought that some of the HSPs such as DnaK, DnaJ and GrpE, although lacking any inherrent protease activity, normally act to promote its degradation through binding and presenting the E σ^{32} to the HflB-governed protease (also known as FtsH) (Herman *et al.*, 1995). Thus, reducing the levels of these HSPs markedly increases the half-life of $E\sigma^{32}$ and therefore the levels of this transcription factor. DnaK, DnaJ and GrpE are also thought to affect the ability of $E\sigma^{32}$ to bind to heat-shock promoters through complexing with it and effectively sequestering it. The initial induction of $E\sigma^{32}$ expression is believed to be mediated through translational control. An analysis of the predicted mRNA secondary structure suggests that the 5' end of the transcript anneals to the 3' region through complementary base-pairing, and this is thought to inhibit translation under normal conditions. However, upon raising the temperature, the secondary structure is predicted to collapse, permitting the ribosomes to bind to the Shine-Dalgarno box. This model is supported by the observation that point-mutations interfering with the complementary base-pairing result in constitutive expression of $E\sigma^{32}$ (Yura *et al.*, 1993).

As mentioned above, $E\sigma^{32}$ is required for the transcription of genes involved in the heat-shock response. The proteins encoded by such genes are required for protein degradation, protein folding, protein synthesis, oligomerisation and DNA replication. Of the heat-shock proteins currently identified, DnaK, DnaJ and GrpE have been shown to be essential for replication of bacteriophage λ (Friedman *et al.*, 1984; Georgopoulos *et al.*, 1990). Mutations in these genes also affect DNA synthesis (Friedman *et al.*, 1984) and cell division (Neidhardt and VanBogelen, 1987). Deletion of the *dnaK* gene results in a block in cell division (*ie* mutants filament), although its actual role in the cell cycle process is unclear (McCarty and Walker, 1994). Moreover, *dnaK* null mutants are both cold and heat sensitive, with a vary narrow temperature range for growth (McCarty and Walker, 1994). DnaK is a prokaryotic equivalent of the eukaryotic hsp70 protein, having 48% identity with the Drosophila hsp70 protein (Bardwell and Craig, 1987). The DnaK protein is capable of autophosphorylation in the presence of ATP at a threonine residue, and *in vitro*, this is apparently enhanced by a factor of 10 in the presence of calcium ions (Cegielska and Georgopoulos, 1989a, b).

Recently, another protein CbpA was identified (Ueguchi *et al.,* 1994), which has homology with DnaJ. Whereas deletion of *dnaK* has gross effects upon cell division and growth in *E. coli*, as described above, deletion of *dnaJ* was found to be far less severe (Sell *et al.,* 1990). Ueguchi *et al* (1995) postulated that this might be due to the compensating action of CbpA. By constructing *dnaJ* / *cbpA* double mutants, it was shown that a phenotype more or less identical to that of *dnaK* null mutants could be obtained (Ueguchi *et al.,* 1995). Of particular interest was the observation that whereas *dnaJ* has a classical $E\sigma^{32}$ heat-shock promoter, *cbpA* has a $E\sigma^{s}$ promoter suggestive of a role in adaptations to stationary phase survival (see below) (Ueguchi *et al* 1995), although expression of *cbpA* was not found to increase upon entry into stationary phase in a wild-type strain (Yamashino *et al.,* 1995).

The Lon protease, required for the destruction of SulA in the SOS response, is also required for degradation of heat denatured proteins, although inactivation of its gene is not lethal.

A comparatively new area to be explored with respect to the heat shock response and its regulation, is the role of nucleoid topology. Numerous genes are regulated by the levels of negative supercoiling of the DNA in response to environmental stimuli (Higgins *et al.,* 1988; Hulton *et al.,* 1990; Dorman, 1991). Recent experiments monitoring the changes in plasmid topoisomers following heat shock showed a relaxation in the degree of supercoiling, which failed to be restored to normal in a *gyrA* mutant (Mizushima *et al.,* 1993; 1994). Even more recent experiments showed the opposite response to heat-shock, with an increase in the levels of supercoiling (Camacho-Carranza *et al.,* 1995), which again were attributed to changes in gyrase activity. In both sets of experiments however, expression of *dnaK* and *groE* were shown to be induced as a result of changes in the levels of supercoiling. Upon removal of the heat-shock stimulus in the strains in which gyrase activity was either blocked or absent, expression of *dnaK* and *groE* continued (Mizushima *et al.,* 1993). Expression of *cbpA* has been shown to be influenced by the histone-like DNA binding protein, H-NS through the action of the stationary phase regulator, RpoS (Yamashino *et al.,* 1995). RpoS directly regulates the expression of *cbpA*, and since RpoS levels in *hns* mutants are elevated, the levels of *cbpA* expression are therefore also elevated (Yamasino *et al.,* 1995). These experiments elegantly demonstrate an additional layer of complexity regulating the heat-shock response.

1.3.6.7 The stringent response

Upon depletion of nutrients, bacteria have evolved a system for down-regulating their metabolism in order to conserve their resources. In addition to shutting down many of their metabolic processes, the cell cycle is blocked (for a general review,

see Cashel and Rudd, 1987). The stringent response, triggered by amino acid starvation, is mediated by the alarmones, ppGpp and pppGpp which are derived from GDP and GTP respectively. The 77kDa enzyme (p)ppGpp synthetase I is encoded by the *relA* gene which maps at 60 min on the *E. coli* chromosome. This enzyme is responsible for (p)ppGpp synthesis in response to a failure to maintain levels of aminoacylated tRNA required to fulfil the demands of the cell. Facillitating its role in responding to changes in the availability of charged tRNA residues, (p)ppGpp synthetase I has been shown to be associated with the ribosomes (Cashel and Rudd, 1987). Termination of the ppGpp signal is mediated by the 80 kDa *spoT* gene product, which removes the 3' pyrophosphate residue from ppGpp (Cashel and Rudd, 1987).

Although (p)ppGpp is responsible for the regulation of numerous metabolic systems within the cell, in particular the control of macromolecular synthesis, only those concerned with the cell cycle will be considered here. In *E. coli*, ppGpp has been shown to be responsible for blocking the initiation of chromosome replication at *oriC*, by inhibiting transcription of the *mioC* and *gidA* genes, as already discussed in section 1.3.2 (Rokeach *et al.*, 1987; Ogawa and Okazaki, 1991). Moreover, *dnaA* promoters are also apparently subjected to stringent control (Chiaramello and Zyskind, 1990). Séror and co-workers, (Séror *et al.*, 1986; Levine *et al.*, 1991) showed that the stringent response is required for controlling the rate of initiation of chromosomal replication in *E. coli* and *B. subtilis*. The site and means of regulation differ in these two species, in *E. coli*, regulation takes place solely at the origin, with high levels of ppGpp blocking any movement of the replisome from the origin. In *B. subtilis*, over-initiation of chromosome replication is controlled downstream of the origin by pausing of the replication forks at specific points approximately 180kb either side of *oriC* (post-initiation control) (Henckes *et al.*, 1989). The mechanism behind this pausing has been recently attributed to participation of the replication terminator protein (RTP), which normally binds to the terminus region of the *B. subtilis* chromosome, but can also apparently bind to the Stringent Terminus (STer) sites flanking *oriC* (Levine *et al.*, 1995). It is hypothesised that ppGpp is responsible for interaction of RTP with the STer sites. Following removal of the Stringent Response stimulus, chromosomal replication resumes from the blocked forks, unlike in *E. coli* (Levine *et al.*, 1991).

A recent report describes experiments that identify a target for ppGpp as the β-subunit of RNA polymerase (Reddy *et al.*, 1995). This evidence fits well with the postulated roles of *mioC* and *gidA* transcription being prerequisites for initiation of chromosome replication. If RNA polymerase activity is blocked by the binding of ppGpp, transcription of the *mioC* and *gidA* promoters cannot take place thus blocking initiation of replication.

1.3.6.8 Cyclic AMP in bacteria

As in eukaryotes, cAMP is produced from ATP by the enzyme adenylate cyclase, encoded by the *cya* gene (for a review, see Botsford and Harman, 1992). The target of cAMP is the 47kDa cAMP receptor protein (CRP), encoded by the *crp* gene, which in the absence of cAMP, is remarkably protease resistant and exhibits sequence independent affinity for DNA. However, upon binding cAMP, CRP changes conformation, becoming protease sensitive and acquires sequence specific DNA binding characteristics. The consensus CRP-binding site is a palindromic sequence AAATGTGATCT*AGATCACATTT, and is often found for example, upstream of genes concerned with metabolism, such as the *lac*, *ara*, *gal*, *mal* and *mel* operons (Botsford and Harman, 1992). Upon binding to promoters containing CRP consensus sequences, the DNA undergoes a conformational change, resulting in a bend of between 90 and 130°. Promoter curvature has been shown to be important for the binding of other regulatory proteins such as H-NS (Yamada *et al.*, 1991). The interaction of cAMP-CRP can both positively and negatively regulate gene expression as highlighted in the review by Botsford and Harman (1992).

Overexpression of the *cya* gene, resulting in an overproduction of cAMP causes cell filamentation (Kumar *et al.*, 1979), however, deletion of the *cya* or *crp* is not lethal. Amongst the many pleiotropic effects of these mutations, cells become spherical (Kumar, 1976), and become resistant to the antibiotic mecillinam, that normally targets the septum-specific penicillin binding protein, PBP2 (D'Ari *et al.*, 1988). Moreover, in *cya* or *crp* mutants lacking the SOS-response division inhibitor SfiA, a higher incidence of anucleate cells is produced when DNA synthesis is blocked (Jaffé *et al.*, 1986), thus indicating a role for cAMP-CRP complex, as yet unclear, in the division process.

In eukaryotes, some forms of adenyl cyclase can be activated by Ca^{2+}.CaM (see section 1.2.5), and protein kinase-A (PKA) and its targets can also be dephosphorylated by Ca^{2+} regulated phosphodiesterases and phosphatases. Some evidence for Ca^{2+}-CaM regulated adenyl cyclase has emerged from studies with a cyanobacterium *Anabeana spp* (Botsford and Harman, 1992), where the protein adenyl cyclase is apparently activated by Ca^{2+}.CaM from bovine brain, and from a CaM-like protein from the bacterium.

1.3.6.9 Regulation of entry into stationary phase

Outside the highly artificial environment of the laboratory, bacteria are subjected to considerable constraints on their ability to multiply. Frequently, nutrients are in short supply, or water becomes scarce. Some bacteria such as *Bacillus sp* and *Clostridium sp* have evolved specialised structures called spores that permit them to

survive for very considerable periods (several decades at least, but a recent report (Cano and Borucki, 1995) has described the isolation of viable bacterial spores from the gut of bees that were trapped in amber 25 to 40 million years ago!). These spores can withstand extremes of heat and desiccation, as well as ultraviolet radiation and toxic chemicals. Upon the return of favourable conditions, the spores germinate and bacterial growth resumes. There are however, many species of bacteria that do not produce spores, including *E. coli*. Like the spore-forming bacteria, they have evolved mechanisms to assist their survival during lean periods. This physiological adaptation to growth conditions can be likened to the G_0 phase of eukaryotes.

The term stationary phase normally follows the so-called exponential phase of growth seen in bacterial cultures grown *in vitro* under ideal conditions, where the bacteria are constantly doubling in numbers at a rate determined by the conditions and genetic constitution of the strain. When nutrients become limiting, the bacteria stop dividing (see Stringent Response, section **1.3.6.7**) and enter the stationary phase - a state in which the metabolic levels are reduced to the minimum neccessary to maintain survival. A number of changes take place in the cell during entry into stationary phase (for a review, see Loewen and Hengge-Aronis, 1994). The changes seen upon entry into stationary phase include increased resistance to oxidative stress, osmotic stress, thermal stress and anaerobiosis.

Genes that constitute the stationary phase regulon contain promoters that are recognised by a specific RNA polymerase sigma factor, $E\sigma^s$, encoded by the *rpoS* (formerly *katF*) gene (Hengge-Aronis, 1993). During exponential phase, the levels of σ^s remain low. However, upon entry into stationary phase, there is a sudden increase in transcription of *rpoS*, the efficiency of translation of its mRNA and stability of the RpoS protein itself (Lange and Hengge-Aronis, 1994). As a direct consequence, elevated levels of RpoS lead to the expression of genes required for survival during stationary phase. Considerable amounts of work are currently being done to identify what factors regulate expression of *rpoS*. Interestingly, studies on the expression of *hns*, the gene that encodes the histone-like DNA binding protein H-NS (see section **1.3.6.3**) demonstrated that the level of this protein also increases dramatically upon entry into stationary phase (Spassky *et al.*, 1984; Dersch *et al.*, 1993; Ueguchi *et al.*, 1993), and is accompanied by changes in the levels of chromosomal supercoiling. Another protein, whose gene, *galU*, is coincidentally located adjacent to *hns*, has been shown to regulate levels of σ^s through the synthesis of UDP-glucose. The gene *galU* encodes the enzyme α-D-glucose-1-phosphate uridylyltransferase (UDPGP) which catalyses the reaction: UTP + glucose--> UDP-glucose (Weissborn *et al.*, 1994).

Clearly, as the molecular mechanisms controlling the bacterial cell cycle become unravelled, patterns are emerging, regarding the nature of the controlling

proteins and their actions. It remains to be seen whether proteins concerned with DNA topology, which are known to play a vital role in the regulation of many genes might also play pivotal roles in the regulation of the cell cycle. It is certainly true that some of these proteins are required for the correct initiation of chromosome replication *viz* IHF, HU and Fis (Crooke *et al.*, 1991; Gille *et al.*, 1991; Messer *et al.*, 1991; Kaidow *et al.*, 1995). Recent experiments, involving an *hns* mutant showed that nucleoid segregation is aberrant (Kaidow *et al.*, 1995). Moreover, H-NS was reported to be detected in the *oriC* complex and has been suggested to play a role in the initiation of chromosome replication (Crooke *et al.*, 1991). However, as already indicated above, no single central regulator for the bacterial cell cycle has yet been identified which can coordinate the timing of the various cell cycle events such as the initiation of chromosome replication and initiation of cell division.

1.4 The role of Ca^{2+} in the regulation of the eukaryotic cell cycle

At high concentrations, calcium is a potent intracellular toxin, and both eukaryotic and prokaryotic cells expend large quantities of energy maintaining low intracellular concentrations (Kretsinger, 1990; Rasmussen *et al.*, 1990). One of the primary reasons for this toxicity is that calcium readily forms insoluble precipitates in the presence of phosphates, which are in abundance inside the cell (Rasmussen *et al.*, 1990; Clapham, 1995). Indeed, Rasmussen *et al* liken the use of calcium for signalling to Prometheus' theft of fire from the gods - an immensely useful and powerful tool but also extremely hazardous if it is out of control. This is highlighted by the fact that certain pathogenic bacteria cause disease through disrupting the normal regulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$), resulting in catastrophic derangement of host cellular metabolism and the cytoskeleton (Baldwin *et al.*, 1991, 1993). There are also inherited diseases that affect the cells ability to regulate its $[Ca^{2+}]_i$, such as malignant hypothermia, which is potentially fatal (Rasmussen *et al.*, 1990). The problems of expelling calcium from the cell are exemplified by the fact that there is typically a 10-20,000-fold difference between the extracellular and intracellular concentrations of the cation (Rasmussen *et al.*, 1990; Clapham, 1995).

1.4.1 Evidence for Ca^{2+} regulating the cell cycle

A considerable volume of evidence has now accumulated to demonstrate the role of calcium in the regulation of the eukaryotic cell cycle (for reviews, see Whitaker and Patel, 1990; Lu and Means, 1993). As indicated in section 1.2, much of the early research investigating the roles of calcium and its main target protein calmodulin (CaM), centered around the early cell cycles of the fertilised eggs and oocytes of sea urchins, star fish, frog and clams. More recently, work has also included the yeasts *S.*

cerevisiae and *S. pombe*, the fungus *A. nidulans* and mammalian cell lines. Measurements of intracellular Ca^{2+} have been made possible by the development of dyes that change their fluorescence characteristics upon binding Ca^{2+} (Borle, 1990). More recently, a chemiluminescent protein aequorin has been purified from the jelly fish *Aequorea victoria* and its gene cloned (Knight *et al.,* 1991). By transforming a plasmid containing the aequorin gene into the cell-type to be tested, constant measurements of calcium can be made. This method is much less invasive, since aequorin does not chelate the Ca^{2+}, but spontaneously emits light upon transient binding Ca^{2+} which can be directly measured (Watkins *et al.,* 1995). The fluorescent probes such as Quin-2, Fura-2 and Indo-1 are technically easier to use however (Borle, 1990), and are frequently the method of choice when making measurements of $[Ca^{2+}]_i$.

Measurements of the $[Ca^{2+}]_i$ in both eukaryotes (see Rasmussen *et al.,* 1990) and bacterial cells established a basal concentration of 0.1μM of free, unbound Ca^{2+} (Gangola and Rosen, 1987; Watkins *et al.,* 1995), compared with an extracellular concentration of typically 2-10 mM. In cultured untransformed mammalian cells, there is a positive requirement for an extracellular calcium concentration ($[Ca^{2+}]_{ext}$) of 1-1.2 mM for proliferation to take place (Swierenga *et al.,* 1980). Thus, Ca^{2+}-deprivation of such cell lines causes a block in the cell cycle, which is removed upon the addition of physiological concentrations of Ca^{2+}. However, in transformed cell lines, this Ca^{2+} requirement is lost (Swierenga *et al.,* 1980; Whitfield *et al.,* 1980). Moreover, the $[Ca^{2+}]_i$ in transformed cell lines has been shown to be considerably higher than in primary cell lines (Veigl *et al.,* 1984), which is believed to be extremely important for their autonomous growth. When the $[Ca^{2+}]_i$ of synchronised primary cell lines loaded with Fura-2 was measured during the cell cycle, increases in $[Ca^{2+}]_i$ were observed during the completion of mitosis and again at the G_1-S interface (Lu and Means, 1993). By chelating the intracellular Ca^{2+}, through microinjection of the calcium chelator 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (Quin-2), DNA synthesis was shown to be blocked in C127 cells (a non-transformed cell line derived from a mouse mammary tumour). Similar experiments demonstrated the requirement for an increase in the $[Ca^{2+}]_i$ upon entry into mitosis in Swiss albino 3T3 mouse fibroblasts (Kao *et al.,* 1990).

Using sea urchin eggs, measurements of the $[Ca^{2+}]_i$ were made during fertilisation (cited in Whitaker and Patel, 1990). It was noted that the $[Ca^{2+}]_i$ increased from a resting level of 0.1 μM to 1-5 μM following fertilisation of the sea urchin eggs, and returned to the basal level after 5-10 min. Further subsequent transient increases were observed during progression of the cell cycle at the G_1/S boundary, at the point of nuclear envelope breakdown, anaphase and during cleavage of the nuclei. Microinjection of calcium into fertilised sea urchin eggs has been shown to induce

mitotic events (Steinhardt and Alderton, 1988), and induces precocious mitosis in cells that have not yet reached M-phase (Twigg *et al.,* 1988). As with the mammalian cell lines, microinjection of Ca^{2+} chelators blocked the cell cycle (Steinhardt and Alderton, 1988). More remarkable was the demonstration that treatment of unfertilised eggs with the calcium ionophore A23187 resulted in parthenogenetic activation of the cell cycle, leading to the normal (albeit haploid) development of the embryo (cited in Whitaker and Patel, 1990).

1.4.2 The eukaryotic calcium cascade

The calcium cascade refers to the intracellular signalling pathways that become activated as a consequence of an increase in the $[Ca^{2+}]_i$ (see **Fig. 5** for a simplified overview), and is reviewed in Clapham (1995). The cascade serves to amplify the initial signal and to route the stimulus to the appropriate effector molecules that in turn modulate gene expression. The initial signal that triggers the increase in $[Ca^{2+}]_i$ can originate either extracellularly, for example through the interaction of hormones with membrane-bound receptors, or from an intracellular source (see below). Equally, the source of calcium can be either the extracellular space or from intracellular stores. The usual store of intracellular Ca^{2+} is the endoplasmic reticulum (ER) which, for example, stores the Ca^{2+} bound to the protein calsequestrin (Henson *et al.,* 1989). It appears that the major source of Ca^{2+} required for cell cycle regulation is intracellular, since inhibition of the Ca^{2+} pumps that normally pump Ca^{2+} from the cytoplasm back into the ER, with the drugs thapsigargin (TG) or 2,5-di-*tert*-butyl-hydroquinone (DBHQ), causes a G_0 arrest in the cell cycle of DDT1MF-2 smooth muscle cells, which is only relieved upon removal of the blockade, when the intracellular stores have refilled (Short *et al.,* 1993). Further evidence for the role of the intracellular Ca^{2+} store in providing the Ca^{2+} required for regulating the cell cycle was shown by Ciapa *et al.,* (1994), who demonstrated that by blocking the $InsP_3$ receptor (see below) with heparin, and thus preventing the release of Ca^{2+} from the intracellular stores, the cell cycle became blocked at mitosis. Release of intracellular calcium is mediated by the intracellular second messenger, inositol (1,4,5)-trisphosphate ($InsP_3$), which binds to a receptor on the endoplasmic reticulum that is inhibited by the addition of heparin (Ciapa *et al.,* 1994). Elegant experiments showed that the levels of $InsP_3$ in the sea urchin embryo oscillate independently of protein synthesis and therefore of cyclins. When protein synthesis was blocked, $InsP_3$ levels peaked at the point of cell cycle blockage, where nuclear envelope breakdown should have occurred, accompanied by futile transient increases in the $[Ca^{2+}]_i$ (Ciapa *et al.,* 1994). Thus, the authors conclude

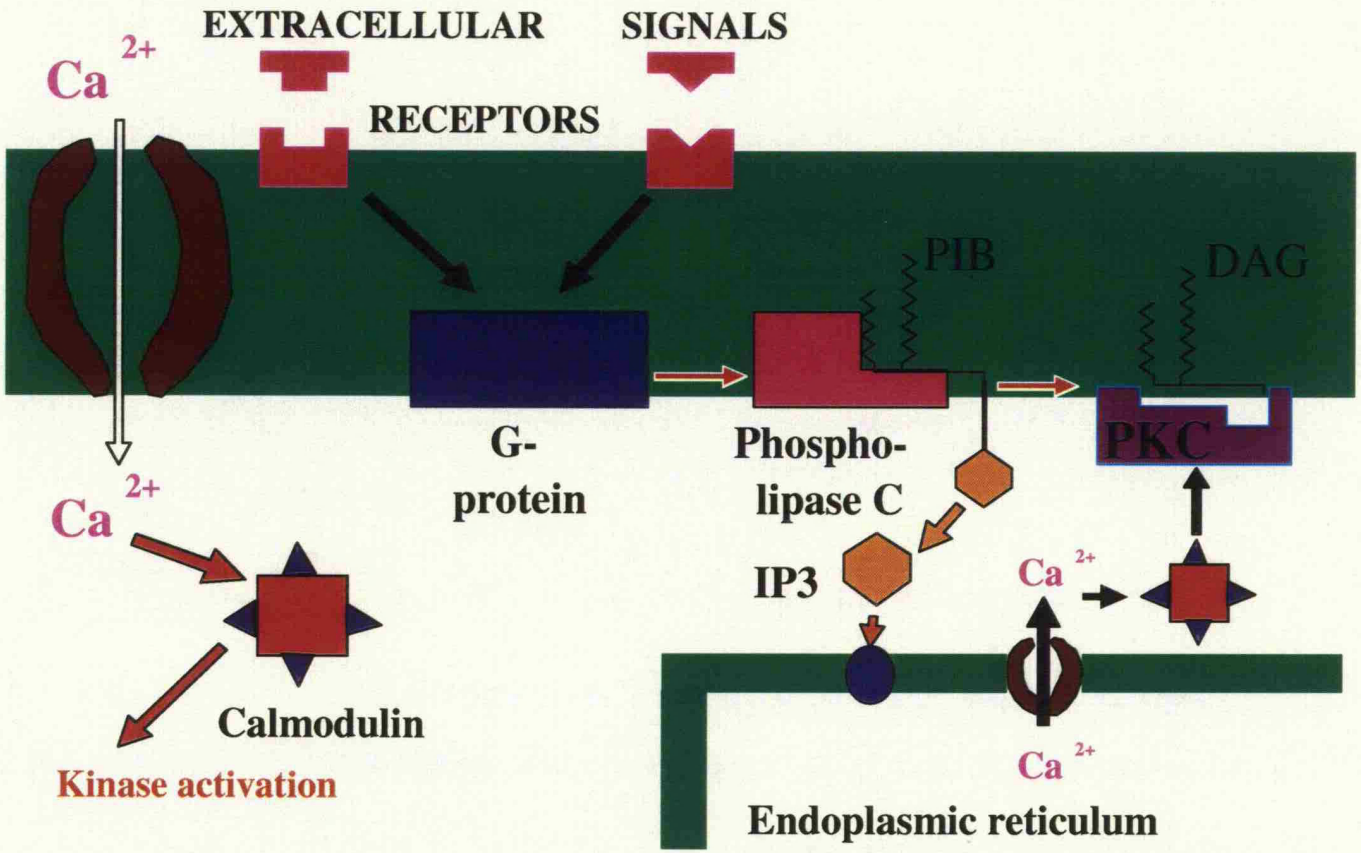


Fig 5. Overview of key components of the calcium cascade in eukaryotes.

Key: PIB, phosphatidyl (4,5)-bisphosphate; DAG, diacyl glycerol; IP3, inositol (1,4,5)-trisphosphate ($InsP_3$); PKC, protein C-kinase;

that in sea urchin embryos, there is an endogenous oscillator producing $InsP_3$, which controls the timing of mitosis onset, through triggering the release of Ca^{2+} from the intracellular stores. During the cell cycle, another factor which has been shown to specifically induce a release of stored intracellular Ca^{2+} is the cyclin dependent kinase, CDC2/28 (see section 1.3.2). More specifically, the presence of a highly conserved 16 amino acid sequence designated PSTAIR, found within CDC2/28 and its homologues in higher eukaryotes ($p34^{cdc2}$), has been shown to induce an elevation of $[Ca^{2+}]_i$ (Picard *et al.,* 1990). Moreover, microinjection of PSTAIR is capable of inducing meiosis in starfish oocytes and accelerating the action of added maturation promoting factor (MPF) in *Xenopus laevis*. This therefore provides a classical "chicken and egg" situation, which clearly needs to be resolved.

1.4.2.1 Calmodulin is an essential component of the calcium cascade

Calmodulin (CaM) has been shown to be the main receptor for intracellular Ca^{2+} (for excellent reviews, see Whitaker and Patel, 1990; Lu and Means, 1993). Moreover, CaM is essential for the normal regulation of the cell cycle. By adjusting the levels of CaM, the length of the cell cycle has been shown to be greatly affected (Rasmussen and Means, 1987; Means and Rasmussen, 1988). This was done by cloning the CaM gene into bovine papilloma virus (BPV) based vectors designed either to express CaM from its own promoter (CM); or to allow controlled overexpression from the metallothionein promoter cloned upstream of the CaM gene, (MCM). Levels of CaM were also manipulated by expression of antisense CaM mRNA (AS) and intracellular free Ca^{2+} levels were altered by expression of parvalbumin (PV) (another calcium binding protein, but lacking the regulatory functions of CaM). The C127 mouse cell-line containing the CM construct constitutively produced a 4-fold increase in CaM, compared to the BPV vector control. This resulted in a shortening of the cell cycle which was attributed to a decrease in the length of the G_1 phase. Induction of CaM expression in the MCM cell line led to a transient 50% increase in CaM levels which again resulted in a shortening of the G_1 phase during the period of induction. Inducing expression of the antisense CaM construct resulted in a complete cessation of growth during the entire period of expression. An analysis of the cells showed that their cell cycles had stopped either during G_1 phase or at mitotic metaphase. Overexpression of parvalbumin resulted in a dramatic slowing down of the cell cycle which was found to be due to increases in the lengths of G_1 and mitosis, and was attributed to the effective chelation of Ca^{2+} by the parvalbumin. In a normal C127 mouse cell line, measurement of CaM levels during the cell cycle showed a 2-fold increase at the G_1/S interface.

Inhibition of CaM activity by the addition of CaM antagonists such as the naphthalene sulphonamide compound W13, has been shown to result in a cessation of cell growth at specific points in the cell cycle *ie* at G_1/S and mitosis, which is only alleviated by the removal of the antagonist (Chafouleas *et al.*, 1982). In contrast, inactive analogues of the CaM inhibitors had no effect on cell cycle progression.

A more elegant, and unequivocal demonstration of the role of CaM in cell proliferation was performed using transgenic mice, containing a CaM gene linked to an inducible promoter (Gruver *et al.*, 1993). An increase in cardiomyocyte CaM levels resulted in an increase in cardiac mass of between 31-72%, and was accompanied by elevated levels of DNA, RNA and protein synthesis, compared with the non-transgenic controls. This experiment was apparently the first to directly link CaM levels with tissue hyperplasia.

As described above, in mouse C127 cells, a transient increase in the $[Ca^{2+}]_i$ is correlated with an advancement of the cell cycle, and depletion of the extracellular calcium with primary cell lines results in a block in the cell cycle, often during late G_1 phase (Whitfield *et al.*, 1986). During the cell cycle block, the cells remain competent for several hours, to continue cell cycle advancement, once the $[Ca^{2+}]_{ext}$ is restored. However, if this competence period is exceeded, the cells enter the quiescent G_0 phase. Cell lines that have become transformed, for example as a result of infection by avian sarcoma virus, are often able to grow in medium depleted of calcium (Swierenga *et al.*, 1980), and this has been shown to be coincident with greatly elevated levels of CaM, compared with their untransformed counterparts. However, the cell cycle can still be blocked by treatment with CaM inhibitors (Durkin *et al.*, 1983). Apparently, depletion of extracellular calcium or treatment of cells with CaM inhibitors during G_1, results in cell cycle blockages at different points during G_1, indicating that there are at least two kinds of Ca^{2+} dependent control mechanism acting during G_1, one acting at the cell surface, which is sensitive to $[Ca^{2+}]_{ext}$, and the second which is sensitive to $[Ca^{2+}]_i$ transients (Whitfield *et al.*, 1986). The cell surface Ca^{2+} receptor is now widely believed to be protein kinase-C (Fig. 5; see later). There are a number of potential cytoplasmic targets for the intracellular Ca^{2+}, required for passage through G_1, including some isoforms of adenylate cyclase, which synthesises cAMP from ATP (see section 1.2.5).

Ca^{2+}.CaM has been shown to be important for DNA synthesis during S-phase (López-Girona *et al.*, 1992). Apparently, DNA polymerase α is activated by phosphorylation upon entry into S-phase. Treatment of mammalian cell lines with the CaM inhibitors W13 or trifluoperazine significantly inhibited DNA synthesis. It is believed that the kinase required for activating DNA polymerase α is activated by Ca^{2+}.CaM, and postulated to be a CaM kinase.

Much work has been performed concerning the identication of the target(s) of Ca^{2+}.CaM at the end of G_2 and mitosis, which are then required for completion of the cell cycle. As described in section 1.2.4, cell cycle progression is dependent upon the phosphorylation of the amino acid residue threonine-167 in *S. pombe* (or 161 in *Xenopus laevis*) and dephosphorylation of threonine-14 and tyrosine-15 of CDC2/28, and then upon degradation of the mitotic kinase, NIMA, which occurs following cyclin B degradation. Experiments with *A. nidulans* have shown that Ca^{2+}.CaM is vital for activation of the phosphatase, NIMT, that is required to dephosphorylate threonine-14 and tyrosine-15 on CDC2/28, thus activating the cyclin B - CDC2/28 complex (MPF in higher eukaryotes); and for activation of the NIMA kinase, which also has cyclin-like properties (see section 1.2.2) (Lu *et al.*, 1993). By artificially regulating the levels of CaM expression, Lu *et al.* (1993) demonstrated that entry into mitosis could be blocked, and showed that the blockage was due to a failure to activate (phosphorylate) NIMT and NIMA. The kinase responsible for activating NIMA and NIMT in *Aspergillus* is suspected to be a CaM kinase, which has been shown to phosphorylate *in vitro*, both NIMT from *A. nidulans* and Cyclin B from *S. pombe* in a Ca^{2+} / CaM dependent manner (Lu and Means, 1993). As mentioned earlier (see section 1.4.1), at fertilisation, there is a transient increase in the $[Ca^{2+}]_i$ of sea urchin oocytes. Almost immediately after this transient increase in $[Ca^{2+}]_i$, cyclin B is degraded and meiosis II is completed. Lorca *et al.* (1991) demonstrated that the degradation of cyclin is Ca^{2+}.CaM dependent, since blocking the increase in $[Ca^{2+}]_i$ prevented cyclin degradation and thus mitosis arrest. More recent experiments have in fact identified the kinase responsible for Cyclin B degradation as the type II calmodulin-dependent protein kinase (CaM kinase II) (Lorca *et al.*, 1994).

1.4.2.2 Properties of calmodulin

The calmodulin family of proteins is diverse in both structure and function (Wylie and Vanaman, 1988), their common feature being the presence of the so-called EF-hands. Calmodulin (CaM) is a small, heat-stable, acidic protein with an approximate molecular weight of 17kDa, and in most cases has four Ca^{2+} binding sites (EF-hands), each consisting of a helix-loop-helix motif. The structure of CaM has been highly conserved throughout evolution, and has been identified in organisms tested, from the yeasts *S. cerevisiae* and *S. pombe*, up to Man, and consists of a dumbell structure, with two EF-hands in each half, separated by a long central α-helix.

There are 3 classes of metal binding proteins, 1) those such as haemoglobin, cytochromes and ferredoxin, that bind their metal ion avidly, without a significant change in structure; 2) highly flexible, extracellular proteins such as prothrombin and osteocalcin, that do not possess specialised metal ion binding sites; and 3) the

calmodulin family, that are semi-rigid in structure, and require the interaction of Ca^{2+} ions to modulate their structure (Wylie and Vanaman, 1988). Amongst this latter class of proteins are those with regulatory functions, cytoskeletal/contractile activity, calcium buffering properties, proteolytic activity and bioluminescence characteristics. A Ca^{2+} binding protein apparently only found in tumour tissue has also been reported, called oncomodulin (Veigl *et al.*, 1984).

Upon binding Ca^{2+} ions to the EF-hands of CaM, a conformational change takes place. Ca^{2+}.CaM is capable of binding to, and regulating the activity of a diverse range of enzymes, and it has been shown using X-ray crystallography, that Ca^{2+}.CaM interacts, not with specific amino acid sequences, but with basic amphiphilic α-helices in the target proteins (for a review, see O'Neil and DeGrado, 1990). Amongst the many proteins with which Ca^{2+}.CaM interacts, of particular importance in this context are the enzymes protein kinase-C (PKC) (Nishizuka, 1988)), diacyl glycerol kinase (DGK) (Sakane *et al.*, 1990) and the CaM dependent protein kinases (Lu and Means, 1993; Lorca *et al.*, 1994).

1.4.2.2 Phosphorylation and the signal transduction pathway

As indicated in **Fig. 5**, there are two main branches to the calcium cascade. The first involves entry of Ca^{2+} via the voltage operated calcium channels (VOCCs), and binding of the Ca^{2+} to CaM, followed by the activation of various response elements. The second branch requires the interaction of an external effector (signalling or hormone) molecule with specific cell surface receptors. Upon interaction of the receptors with the signalling molecule, a conformational change occurs, resulting in dimerisation and autophosphorylation of the receptors on tyrosine residues (Williams *et al.*, 1991; Heldin, 1995). The process of receptor phosphorylation has two consequences, 1) other enzymes can attach to the phosphorylated residues through specialised SH2 (src homology 2) domains and b) the receptor-kinase is activated. Interaction of the enzymes with the receptors, promotes their activation by phosphorylation from the receptor's tyrosine kinase. The enzymes binding to the receptor play pivotal roles in activating the signal transduction cascades (see **Fig. 5**). Two of the key enzymes are phospholipase C-γ (PLC-γ) and phosphatidyl inositol-3-phosphate kinase (PIP_3K), which catalyse a) the cleavage of membrane phospholipids (phosphatidyl inositol (4,5) bisphosphate; PIB), to yield diacyl glycerol (DAG) and inositol trisphosphate (IP_3); and b) phosphorylates the 3 position of phosphatidyl inositol (4,5) bisphosphate to form PIP_3, respectively. Another membrane-bound signalling protein family that interacts with the dimerised receptors are the GTP-binding G-proteins described earlier in section 1.2.5 (see also **Fig. 5**). Activation of G-proteins results in the activation of adenyl cyclase, which in turn catalyses the

conversion of ATP to cAMP. This series of events is required for passage through the G_1-phase of the cell cycle (see section 1.2.5) and also results in phosphorylation of L-type voltage operated Ca^{2+}-channels (VOCC), permitting uptake of Ca^{2+} (Rasmussen *et al.*, 1990). Activated G-proteins can also activate PLC which, as described above, cleaves PIB to yield DAG and IP_3. Both DAG and IP_3 are also potent intracellular second messengers, since DAG binds and activates protein kinase-C (PKC) in conjunction with Ca^{2+}. IP_3 triggers the release of Ca^{2+} from the intracellular stores (endoplasmic reticulum), which binds to CaM and activates Ca^{2+}.CaM dependent enzymes. PKC is an important regulator of a plethora of signal transduction pathways, including those affecting the cell cycle (Nishizuka, 1984, 1988).

PKC was first identified in 1977, and has become the focus for a considerable amount of attention due to its implication in tumourigenesis (Nishizuka, 1984, 1988), which largely stems from the discovery that the tumour-promoting phorbol esters (see below) are capable of activating PKC (reviewed in Nishizuka, 1984). PKC is a polypeptide of approximately 80-85kDa, that occurs either in the cytoplasm, in its inactive state, or in the membrane in its activated form. There are a large number of PKC isozymes, which have different properties (Nishizuka, 1988). Classically however, PKC binds acidic phospholipids or biological membranes in a Ca^{2+}-dependent manner, is activated by diacyl glycerol (DAG), and by tumour promoting compounds such as the phorbol ester, 12-tetradecanoylphorbol 13-acetate (TPA), which resembles DAG, and binds to PKC. Upon activation of PKC, this kinase is capable of phosphorylating the serine and threonine residues of a wide range of basic polypeptide substrates (Farago and Nishizuka, 1990). The different isozymes are distinguished by their activation requirements *ie* whether they require DAG, Ca^{2+}, phospholipids or arachadonic acid (Nishizuka, 1988; Divecha and Irvine, 1995). Although little is currently known about the targets of PKC phosphorylation (Murray and Hunt, 1993b; Divecha and Irvine, 1995) due to the promiscuous nature of this kinase *in vitro* (*ie* its ability to phosphorylate a large number of proteins not normally phosphorylated by this protein *in vivo*), evidence for its role in regulating the cell cycle is exemplified by the fact that 1) PKC activity rises during the Ca^{2+} dependent phase of G_1 prior to entry into S-phase; 2) pharmacological inhibition of PKC blocks the cell cycle during G_1; 3) treatment of primary cell lines with TPA causes the cells to behave like neoplastic cell lines, initiating DNA synthesis and proliferating in a Ca^{2+}-deficient medium; and 4) only external treatment (but not microinjection) of immature *Xenopus* oocytes with TPA will induce the replication of λ bacteriophage DNA due to stimulation of the active, membrane-bound form of the enzyme (cited in Whitfield *et al.*, 1986).

1.4.3 Voltage operated calcium channels

As indicated above, the $[Ca^{2+}]_{ext}$ is extremely high, compared with the $[Ca^{2+}]_i$. This therefore presents the cell with major problems in maintaining the low intracellular concentrations required for a) survival, and b) regulation of the Ca^{2+} controlled processes. To achieve this, the cell has evolved a cell membrane which is impermeable to Ca^{2+} ions. However, the cell needs to be able to allow Ca^{2+} to enter the cell in a controlled manner, in order to replenish intracellular stores and to permit the transient intracellular rises in $[Ca^{2+}]_i$ required for activation of cellular processes. This controlled uptake is mediated through specialised channels in the membrane. A wide variety of ion channels exist in the cell membrane, allowing the entry of metal ions such as K^+, Na^+, Mg^{2+} and Ca^{2+}, as well as anions such as Cl^-. Moreover, there are numerous mechanisms controlling the opening and closure of the different types of channels: changes in the voltage across the membrane (voltage operated), ligand gated (sensitive to *eg* neurotransmitters), second messenger operated (SMOC) and mechanosensitive (MS) channels (*ie* respond to changes in the differential pressures in the inner and outer leaflets of the membrane) (Tsien and Tsien, 1990; Stephenson, 1991).

The voltage operated channels (VOCs) are the most heavily studied of the ion channels (Tsien and Tsien, 1990). There are a number of methods for performing physiological experiments upon either native or reconstituted channels, the two commonest being the patch clamp method (Jan and Jan, 1989), where small regions (patches) of cell membrane are studied, using a modified pipette; and the voltage clamp method (A. Williams, Brompton Hospital, London, personal communication; see **Fig. 6**), where ion channels or membrane vesicles are incorporated into a synthetic lipid bi-layer separating two ionic solutions. The principle behind both methods is to create a potential difference across the membrane / channel. Ion channels are highly selective towards a given ion (voltage operated Ca^{2+} channels have a selectivity of >1000-fold for Ca^{2+} over Na^+ or K^+) (Trautwein and Pelzer, 1986; Tsien and Tsien, 1990). By gradually altering the potential difference across the membrane, the activating potential is reached, and the channels open. Ion channels function by allowing ions to flow through the channel in single file, down an electrochemical gradient, thus, each ion traversing the membrane via the channel can be detected by a sensitive amplifier connected to an oscilloscope or chart recorder (see **Fig. 6**). The ionic current passing through the membrane is directly proportional to the number of ion channels in the patch pipette / voltage clamp apparatus (Trautwein and Pelzer, 1986). Using patch-clamp or a voltage-clamp apparatus, different types of calcium channels can be distinguished on the basis of their triggering potential, ionic

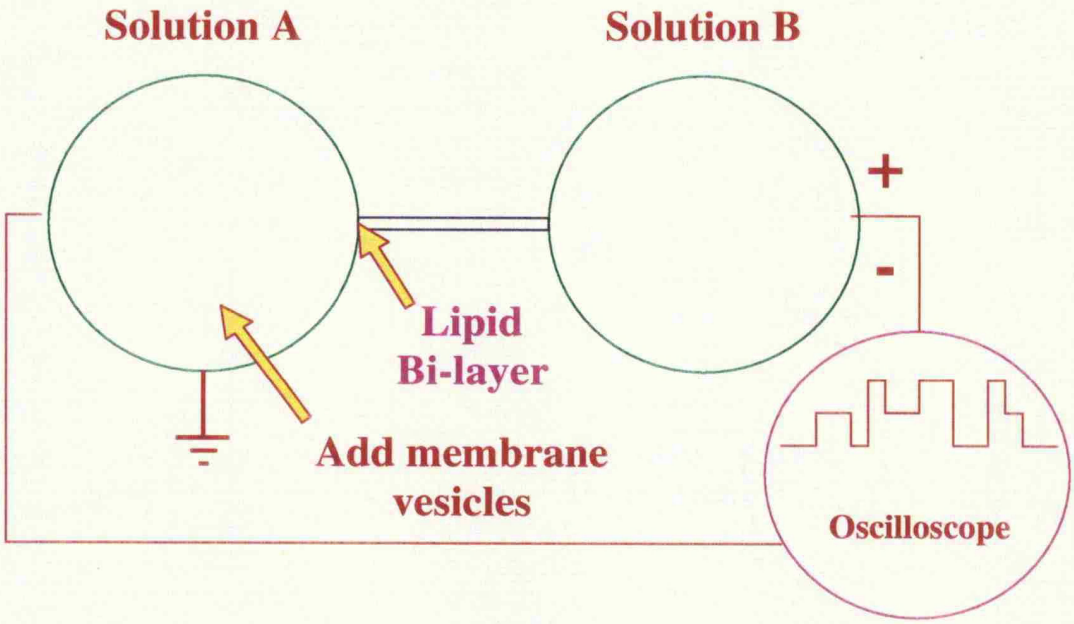
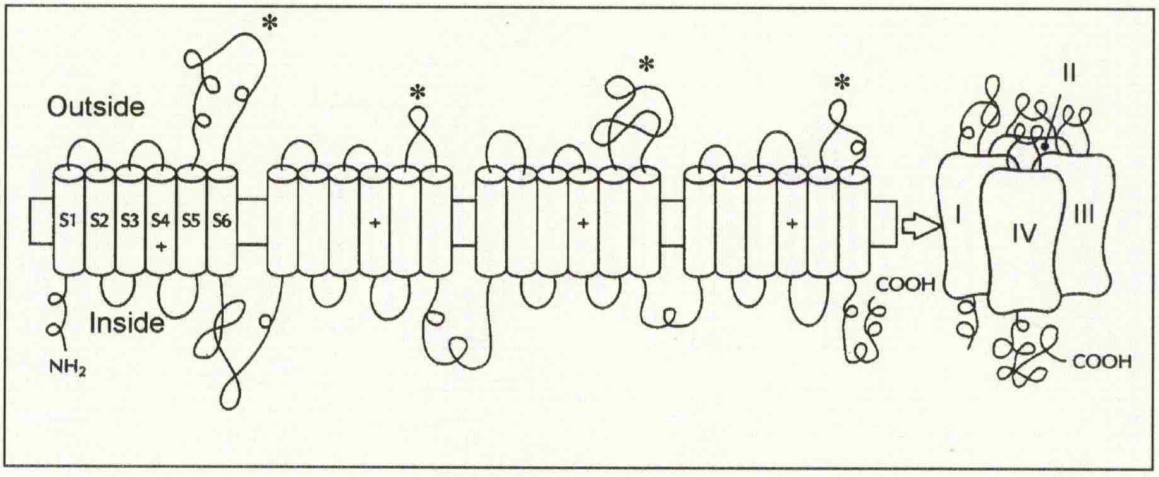


Fig 6. Diagram of the voltage clamp technique used to measure the conductance of ions through voltage operated ion channels in the membrane.



*=SS1-SS2 connecting loops (see text)

Fig 7. Structure of the voltage operated calcium channel.
(Taken from Stephenson, 1991)

conductance, opening duration, sensitivity to pharmacological agonists / antagonists and cellular distribution (Tsien and Tsien, 1990).

There are four types of voltage operated calcium channel (VOCC), L-, T-, N- (Nowycky *et al.,* 1985) and P-type (Llinas *et al.,* 1989). The commonest, and most widely distributed amongst cell types is the L-type channel (see **Fig. 7**), which was initially characterised by its requirement for a comparatively high activation voltage, and sensitivity to 1,4-dihydropyridines (DHPs), although DHP-insensitive L-type channels have subsequently been identified (Tsien and Tsien, 1990). L-type (L=long lasting (Nowycky *et al.,* 1985)) channels remain open for a longer period than other channels, thus permitting higher currents to flow, with a typical throughput of 10^6-10^8 ions per second (Yellen, 1993). L-type channels are abundant in heart and skeletal muscle, but differ in their activation kinetics, conductance and permeability to Mg^{2+} (cited in Tsien and Tsien, 1990). L-type channels are the major pathway for the uptake of Ca^{2+} and play a vital role in heart contraction, the control of transmitter release from endocrine cells and sensory neurones and in events requiring large influxes of Ca^{2+} into the cell. The opening/closing parameters of L-type channels can be modified as a result of phosphorylation by protein kinase-A (PKA), through the cAMP pathway described earlier (see section **1.2.5**) (Lazdunski *et al.,* 1988), interaction with the G_s subunit of the GTP-binding G-proteins (Brown *et al.,* 1989), or by Ca^{2+}.CaM dependent processes (Lazdunski *et al.,* 1988; Saimi and Kung, 1994). A number of drugs are available to regulate the activity of L-type channels, these inhibitors include phenylalkylamines, amongst which, verapamil was the first synthetic compound to be identified, and gallopamil; benzothiazepines, which include diltiazem and the DHPs mentioned earlier (Fleckenstein, 1988). L-type channels are in most instances, resistant to ω-conotoxin, which characteristically inhibits mammalian N-type Ca^{2+}-channels (see below, (Tsien and Tsien, 1990)), although according to Wagner *et al* (1988), ω-conotoxin is also a potent inhibitor of L-type channels. It must be strongly emphasised however, that none of the inhibitors are completely specific, and may block the activity of other proteins. In addition to the inhibitors mentioned above, compound BAY K 8644 has been shown to specifically activate L-type channels (Cognard *et al.,* 1986). An analysis of L-type channels from different cell types and organisms has indicated that the channels are composed of two covalently linked subunits of 30-32 and 140kDa (Tanabe *et al.,* 1987; Lazdunski *et al.,* 1988) (the large subunit is illustrated in **Fig. 7**). However, according to Tsien and Tsien (1990), there are a total of 5 subunits α_1, α_2, β, γ and δ, although the α_1 fragment was found to function as a channel in the absence of the other subunits, binds DHP antagonists and allows Ca^{2+} permeation (Perez-Reyes *et al.,* 1989). The other subunits are therefore postulated to confer tissue specific properties. Of the two subunits described by Lazdunski *et al* (1988) and

Tanabe *et al* (1987), the larger (α_1) has again been shown to be the DHP receptor (Vandaele *et al.,* 1987), and is phosphorylated by cAMP and Ca^{2+}.CaM dependent processes (Hosey *et al.,* 1986).

A number of recent studies have examined the basis of ion specificity in VOCCs (Mikala *et al.,* 1993; Yang *et al.,* 1993; Yellen, 1993). Apparently, the specificity is determined primarily by four glutamate residues located in the SS1-SS2 connecting loops between the S5 and S6 transmembrane helices of each of the four Ca^{2+} channel α-helical domains (see **Fig.** 7). By site-directed mutagenesis of the four glutamate residues, it has been shown that remarkable changes in ion permeability and specificity can be effected, allowing the permeation of other ions (Yellen, 1993; Yang *et al.,* 1993). Moreover, evidence has been presented to indicate that the SS1-SS2 connecting loop actually forms the lining of the channel pore (Stevens, 1991). The S4 transmembrane domains are found in Na^+, K^+ and Ca^{2+} VOCs, and are thought to contain the voltage sensing apparatus (Stühmer *et al.,* 1989; Liman and Hess, 1991).

The T-type channels (T = transient (Nowycky *et al.,* 1985)) are found primarily in excitable tissues such as the heart, and are activated at lower potentials than L-channels. In addition, the T-channel becomes inactivated much more rapidly, and are ideally suited for rapid responses. Unlike the L- channels, the T-type is insensitive to DHP, benzothiazepine and phenylalkylamine antagonists, and is also less sensitive to Cd^{2+} ions, (which competes with Ca^{2+} for the high affinity binding sites in the L-type Ca^{2+}-channel) (Yang *et al.,* 1993; Tsien and Tsien, 1990). One of the main functions of these channels is to support cardiac pacemaker activity (Tsien and Tsien, 1990).

The N-type Ca^{2+} channels (N = neither T nor L (Nowycky *et al.,* 1985)) are almost exclusively found in neurones and adrenal glomerulosa cells (Tsien and Tsien, 1990). N-type channels are resistant to the L- channel antagonists, but are sensitive to ω-conotoxin (ω-CgTx) produced by the fish-hunting cone snail *Conus geographus* (Olivera *et al.,* 1984). The conductivity of N- channels is intermediate to the L- and T-type, and their activation kinetics are clearly distinct (Nowycky *et al.,* 1985). The primary role of N-type channels appears to be in mediating the release of neurotransmitters, in response to the uptake of Ca^{2+}.

A more recently isolated class of VOCC is the P-type channel, which is also involved in neurotransmitter release (Llinas *et al.,* 1989). Both L- and P- channels have a relatively high activation voltage, but the latter are not affected by the L- channel inhibitors or ω-CgTx. However, a toxin, ω-agatoxin-1A from the funnel web spider *Agelenopsis aperta* is specific for these channels.

1.5 The control of the prokaryotic cell cycle: is there a role for calcium?

"*These prokaryotes, which directly couple cell growth to chromosome replication by hanging and replicating their chromosomes on their membranes, did not, and still do not use* Ca^{2+} *for anything important internally.*" Taken from Whitfield *et al* (1986).

As explained above, calcium and calmodulin play a crucial role in regulating the eukaryotic cell cycle. In prokaryotes, although many of the genes involved in the cell cycle and its control, have now been identified, no global regulator such as Ca^{2+} functioning in eukaryotes, has been identified. Many theoretical models have been proposed which attempt to apply mathematical principles to the cell cycle, but since the bacterial cell is ultimately a "bag of enzymes and chemical reactions", some form of physical regulator is needed to direct all of these processes, and to ensure that like the conductor of the orchestra, all the reactions progress at the correct rate, and at the correct time. Whitfield *et al* (1986) (see above), like many eukaryotic molecular biologists regard Ca^{2+} signalling as a uniquely eukaryotic domain. There is however, a rapidly accumulating body of evidence in contradiction to this belief.

Some of the earliest findings relating to Ca^{2+} in bacteria included the discovery that the concentration of intracellular free Ca^{2+} in *E. coli* is maintained at the same levels as for eukaryotes, at a concentration of approximately 10^{-7}M (Gangola and Rosen, 1987), and has been subsequently confirmed (Watkins *et al.,* 1995; L. Tisa, personal communication). This has been further supported by measurements in other bacteria, such as *Propionibacterium acnes* (Futsaether and Johnsson, 1994), where $[Ca^{2+}]_i$ values ranged from between $7x10^{-8}$-$2.5x10^{-7}$, depending upon the $[Ca^{2+}]_{ext}$ and the extracellular pH. Moreover, mutants in *calD*, defective in Ca^{2+} export, have intracellular Ca^{2+} levels 5 to 10 fold higher than wild-type strains (L. Tisa, personal communication). Such *calD* mutants are hypersensitive to extracellular Ca^{2+} and display defective cell division (M. Tempête, personal communication). In addition to the above findings, other evidence indicates that the $[Ca^{2+}]_i$ is maintained through the use of Ca^{2+} pumps (as in eukaryotes). Two types of secondary Ca^{2+} pump have been detected in *Escherichia coli*, a Ca^{2+} -HPO_4^{2-} /H^+ antiporter and a Ca^{2+}/H^+ antiporter (Rosen, 1987). A Ca^{2+}/H^+ antiport system has also been detected in *B. subtilis* (de Vrij *et al.,* 1985). Such pumping mechanisms can be driven by either proton motive force ($\Delta\psi$) or by a pH gradient (ΔpH). A Ca^{2+}-HPO_4^{2-} symport transporter, for Ca^{2+} uptake has also been cloned and characterised from *E. coli* (van Veen *et al.,* 1994). In addition to these "secondary pumps", primary (*ie* ATP-driven) Ca^{2+} pumps have been demonstrated in a number of bacteria such as *Streptococcus faecalis* (Kobayashi *et al.,* 1978), *Streptococcus pneumoniae* (Trombe *et al.,* 1992, 1994; Trombe, 1993) and *Flavobacterium odoratum* (Gambel *et al.,* 1992). On the subject of Ca^{2+} transport, a

number of researchers have examined bacterial membranes for the presence of ion channels, using the patch clamping technique (Berrier *et al.*, 1989; Delcour *et al.*, 1989; Saimi *et al.*, 1992; Martinac *et al.*, 1994; Sukharev *et al.*, 1994). In this way, at least two mechanosensitive channels have been identified in *E. coli*, MscL and MscS (Sukharev *et al.*, 1994), which have been postulated to play a role in osmoregulation. The large (MscL) channel does not appear to be particularly ion selective, whereas the small (MscS) channel apparently has a slight preference for anions. Although the mechanosensitive channels are permeable to Ca^{2+}, they are not specific for this ion. There has been a report (Preston *et al.*, 1992) of VOCC activity, using the patch-clamp method on *E. coli* in which the channels exclude the passage of K^+ ions but are slightly Na^+ permeable. When *E. coli* everted inner membrane vesicles were placed into a voltage-clamp apparatus, possible VOCC activity was observed, which was blocked by the addition of La^{3+} ions (M. Goldberg, unpublished data).

A further very encouraging demonstration of the possible existence of VOCCs in *E. coli* emerged from experiments measuring chemotactic behaviour following treatment of *E. coli* with the eukaryotic N-type calcium channel inhibitor, ω-conotoxin (Tisa *et al.*, 1993). These experiments showed that the concentration of toxin required to inhibit chemotaxis in *E. coli* is the same as the concentration required to inhibit eukaryotic channels. Moreover, Tisa *et al* (1993) demonstrated that the target for the toxin is located in the cytoplasmic membrane. Elegant experiments have been performed to show that chemotaxis *is* regulated by the $[Ca^{2+}]_i$, by electroporating caged Ca^{2+} compounds and caged Ca^{2+} chelators into *E. coli*, and showing that following exposure of the bacteria to light of the wavelengths required to activate the caged compounds and release / chelate Ca^{2+}, a remarkable change in the chemotactic behavior could be observed (Tisa and Adler, 1992). Using *E. coli* containing the photoprotein aequorin, which emits light upon binding Ca^{2+}, measurements of the $[Ca^{2+}]_i$ in response to changes resulting from exposing *E. coli* to attractants and repellents (Watkins *et al.*, 1995). An increase in the $[Ca^{2+}]_i$ was detected, associated with tumbling behaviour following exposure to repellents, and a decrease in $[Ca^{2+}]_i$ resulted in "smooth swimming" upon encountering attractants. These findings were in direct agreement with the findings of Tisa and Adler (1992). Most exciting in respect to the discovery that changes in the $[Ca^{2+}]_i$ regulate chemotactic behavior, is that chemotaxis is controlled by a complex signal transduction pathway involving a number of 2-component response regulators (Ninfa *et al.*, 1991). Another example of Ca^{2+}-enhanced phosphorylation of a signal transduction pathway has been described for the EnvZ-OmpR 2-component response regulator system (Rampersaud *et al.*, 1991). The membrane-bound sensor-kinase, EnvZ, phosphorylates the DNA-binding response regulator, OmpR, and activates the expression of a number of genes concerned with

osmoregulation and virulence. A hybrid sensor-kinase was constructed, Taz, which was shown to phosphorylate OmpR upon binding aspartic acid, and this phosphotransfer was found to be greatly enhanced specifically by Ca^{2+} *in vitro* (Rampersaud *et al.,* 1991). However, the concentrations of Ca^{2+} used in this experiment were very high (60μM), compared with the physiological concentrations of intracellular Ca^{2+} (~0.1μM).

Ca^{2+} is being increasingly shown to play an important role in bacterial virulence mechanisms. In *Yersinia sp*, if these pathogens are grown at 37°C, in the absence of Ca^{2+}, induction of a number of plasmid-encoded virulence determinants occurs. This effect is regulated by a group of genes forming the low calcium response stimulon (LCR) (for a review, see Straley *et al.,* 1993). At 26°C, in the absence of Ca^{2+}, or at 37°C in the presence of micromolar amounts of Ca^{2+}, induction of LCR does not occur. Most curiously however, when grown at 37°C in the absence of Ca^{2+}, growth of the bacteria actually stops (growth restriction) (cited in Straley *et al.,* 1993), and transcription of virulence genes commences. The mechanism responsible for growth cessation is not understood, but a tempting analogy can be drawn with cell cycle blockage in eukaryotes when deprived of Ca^{2+}. Finally, extracellular Ca^{2+} is now being recognised as a requirement for the correct folding of some virulence determinants such as haemolysin A from *E. coli* (M A Blight, personal communication).

Deletion of the *dnaK* gene results in both cold and heat-sensitivity, and in defective cell division, leading to filamentation (Bukau and Walker, 1989; McCarty and Walker, 1994). Apparently, DnaK can be autophosphorylated by both ATP and GTP *in vitro*, and this autophosphorylation is greatly enhanced by 2-10mM Ca^{2+}. Although the role of autophosphorylation is not fully understood, is has been postulated that it could function to modulate the ability of DnaK in the possible transduction of signals from the environment and communicate them to the cytoplasm (Cegielska and Georgopoulos, 1989a, b). The *in vitro* experiment demonstrating the enhancement of autophosphorylation by Ca^{2+} must be regarded with caution however, since the concentrations of Ca^{2+} used were rather high, compared with the known intracellular concentrations of ~0.1μM. Also, the *in vivo* significance of this data is not clear.

Calmodulin-like proteins have been identified from a number of bacterial genera such as myxobacteria (Inouye *et al.,* 1983), mycobacteria (Falah *et al.,* 1988), *Saccharopolyspora erythraea* (Swan *et al.,* 1989) and *Halobacterium salinarium* (Rothärmel and Wagner, 1995). A CaM-like protein was isolated from *E. coli* (cited in Botsford and Harman, 1992) that was shown to activate bovine brain cAMP diesterase, ATPase from human erythrocytes and myosin light chain, these being features of authentic CaM. However, in this article it was noted that the bacteria had

been grown in nutrient broth and that the protein could have been a contaminant from the culture medium. In another group of bacteria, a species of the cyanobacterium *Anabaena sp* apparently produces a form of adenyl cyclase that is activated by Ca^{2+}.CaM from bovine brain and also by a Ca^{2+}-binding protein from the *Anabeana sp* (cited in Botsford and Harman, 1992).

In a search for the bacterial analogues of eukaryotic protein kinases, biochemical and immunological evidence has been presented for the existence of a protein kinase-C-like activity in *E. coli* (Norris *et al.,* 1991), although to date, the kinase has yet to be identified. In the soil-living bacterium *Myxococcus xanthus*, in addition to having a calmodulin-like protein, protein S (Inouye *et al.,* 1983), a serine/threonine protein kinase which is required for developmental transition during the formation of the fruiting bodies, has been identified, with a strong resemblance to the eukaryotic Ca^{2+}.CaM dependent protein kinase II (Munoz-Dorado *et al.,* 1991).

In an attempt to identify and clone possible prokaryotic homologues of eukaryotic cell cycle genes, *E. coli* and *B. subtilis* have been challenged with a number of antagonists known to antagonise components of the eukaryotic Ca^{2+} cascade. For example, a temperature sensitive mutant *feeB1*, resistant to the compound 48/80 (an inhibitor of CaM), which is defective in cell division, was found to be affected in a leucine tRNA gene, $tRNA_3^{Leu}$, with the anticodon, CUA, one of the rarest used in *E. coli* (Chen *et al.,* 1991). Although at the time of isolation, no obvious link between this mutation and the *E. coli* cell cycle could be ascertained, information derived from this report provides a tantalising explanation for the observed phenotype, indicating that such a mutation could indeed affect the expression of genes concerned with the regulation of the $[Ca^{2+}]_i$, and the cell cycle (see **Chapter 10**). Another *E. coli* temperature sensitive mutant, resistant to the CaM inhibitor, trifluoperazine, was found to be affected in the gene *fabD*, involved in fatty acid biosynthesis (Bouquin *et al.,* 1995). A link between this gene and Ca^{2+} is less clear, but one possibility, which ties in with ideas by Norris (1992), is that phospholipid structures in the membrane formed by domains of particular types of phospholipids are important in the localisation of membrane proteins and can allow a transient flux of Ca^{2+} ions into the cell under certain conditions, which could trigger Ca^{2+} regulated reactions. A mutation affecting phospholipid biosynthesis could affect this mechanism. Interestingly, a gene adjacent to *fabD*, *acp*, which encodes the acyl carrier protein, Acp, one of the most abundant proteins in *E. coli* (>10^4 copies / cell) binds tightly, between 2 and 6 Ca^{2+} ions (D. Laoudj and I. B. Holland, personal communication). A tempting possibility is that this protein could function as a Ca^{2+} buffering or storage protein.

It was first proposed (Norris *et al.,* 1988; Norris, 1989) that as in eukaryotes, bacteria use fluxes in the $[Ca^{2+}]_i$ to trigger cell cycle events such as initiation of

chromosome replication, chromosome partitioning and cell division. It was further proposed that these Ca^{2+} fluxes enter the cell via VOCCs, and are transduced by Ca^{2+}-binding proteins such as CaM-like, PKC-like, and related proteins. Also following this hypothesis, a search for myosin-like contractile proteins was undertaken (Casaregola *et al.*, 1990, 1991; Holland *et al.*, 1990), on the premise that cytokinesis must be mediated by a contractile protein. The protein initially named Hmp1 was identified on the basis of antibody recognition, using antibodies raised against the yeast heavy-chain myosin protein, but was subsequently identified as the essential protein, Ams or RNAse E (Casaregola *et al.*, 1992). It has been pointed out that the N-terminal portion of Ams nevertheless has some sequence homology with heavy chain myosin (McDowell *et al.*, 1993; Okada *et al.*, 1994), and is also highly homologous to CafA (Okada *et al.*, 1994), whose gene is located downstream of the *mre* cell-shape determining operon in *E. coli*. Moreover, when overexpressed, the CafA protein results in the formation of axial protein filaments and spectacular cell division defects. From the C-terminal portion of the Ams protein, short regions of coiled coils have been predicted as in the MukB protein and myosin (Niki *et al.*, 1991) (I B Holland, personal communication). Nevertheless, no cell cycle role has been specifically identified for Ams.

Proteins with potential cytokinetic properties have now been identified as FtsZ (Bi and Lutkenhaus, 1991) and MukB (Niki *et al.*, 1991, 1992), which interestingly co-purifies with the protein Acp (see above; Niki *et al.*, 1992).

From the above observations, it therefore seems likely that some form of Ca^{2+} signalling system exists in bacteria, and that Ca^{2+} may be used for regulating cell cycle events.

1.6 L-type voltage operated Ca^{2+} channel antagonists

A historical overview of the cardiac Ca^{2+} channel and the antagonists that have been developed can be found in Fleckenstein (1988). The discovery that certain drugs had the same effect (*ie* depressed cardiac muscle contraction) as Ca^{2+} withdrawal, was the first clue that these drugs actually blocked the calcium channels of the cardiac myocytes. These early drugs were verapamil and the less potent prenylamine. The Na^{+}-dependent action potentials actually triggering heart contraction remained unaffected during treatment with verapamil or prenylamine, only the intensity of heart contraction was reduced by the drugs. This has subsequently formed the basis for the treatment of patients with high blood-pressure. Following the identification of verapamil as a potent and relatively specific VOCC antagonist, numerous other drugs were subsequently tested, both for potency and specificity (Fleckenstein, 1988). Four drugs were

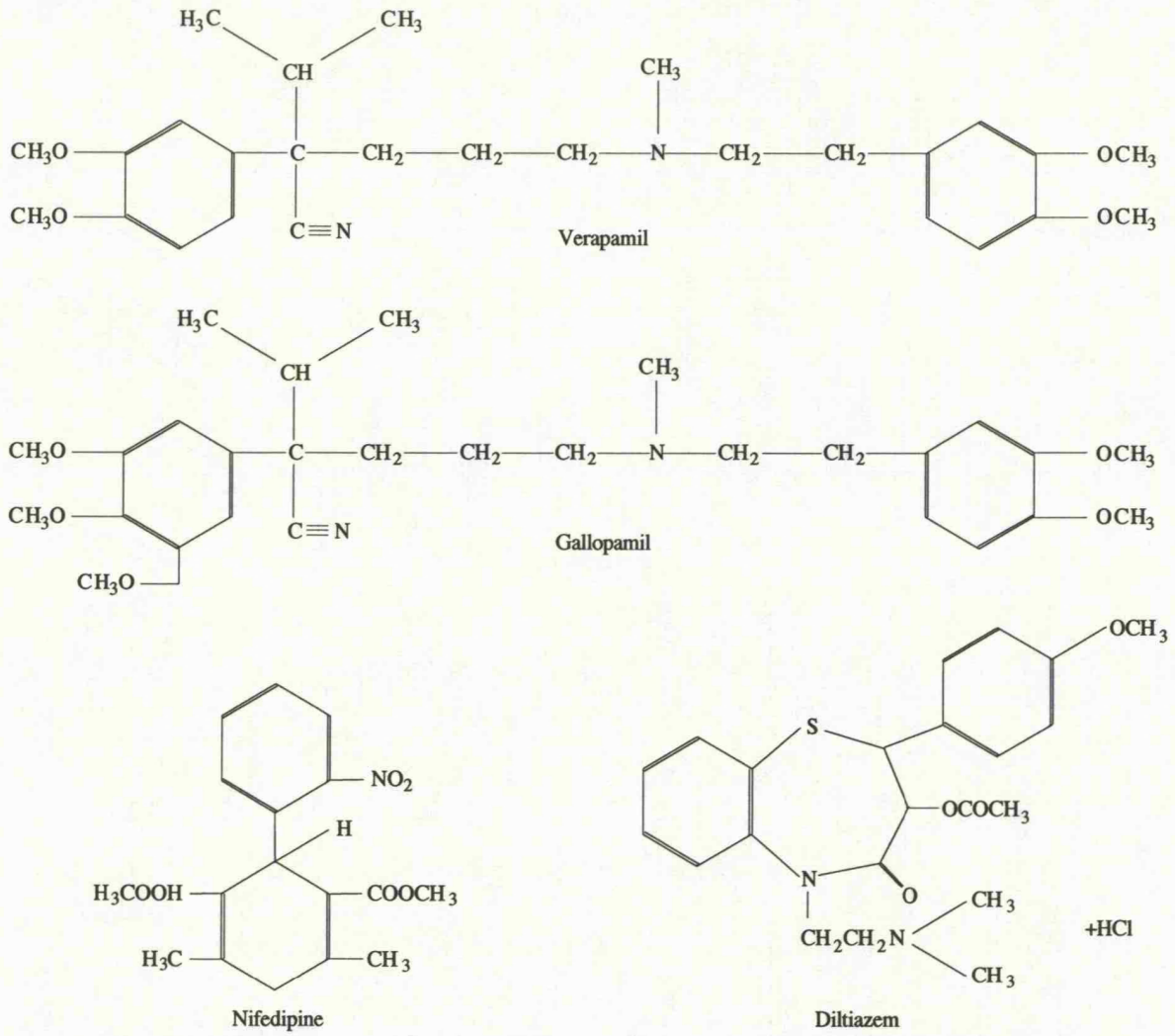


Fig 8. L-type Ca^{2+} antagonists in common usage, taken from Fleckenstein (1988).

apparently short-listed for their superior properties, verapamil, its methoxy-derivative gallopamil, nifedipine and its dihydropyridine (DHP) derivatives, and diltiazem (see **Fig. 8**). Other compounds were found to have a lower potency and specificity, affecting the transport of other ions in addition to Ca^{2+} (Fleckenstein, 1988).

The drugs mentioned above have been classified into three families: the verapamil-like compounds (including gallopamil) are members of the phenylalkylamine family (PAA); diltiazem is a member of the benzothiazepines (BT); and nifedipine, as mentioned above, is a member of the DHP family (Wagner *et al.*, 1988). Binding of DHPs to their receptors is voltage dependent, with maximal binding taking place when the membrane is depolarised. Moreover, binding of verapamil and diltiazem to the Ca^{2+} channel allosterically regulates the DHP binding site, such that BTs enhance the binding of DHPs, whereas PAAs reduce binding (Wagner *et al.*, 1988), thus providing evidence that the different classes of VOCC antagonist bind to different sites in the channel.

1.7 Aims of the project

Since the basic premise that my work has involved, states that like eukaryotes, bacteria utilise transient increases in $[Ca^{2+}]_i$ to initiate key cell cycle events, it was decided to try to isolate and to identify components of a possible Ca^{2+} cascade through the use of inhibitors of proteins constituting the eukaryotic Ca^{2+} cascade. The primary aim of this project was to isolate genes encoding voltage operated Ca^{2+} channels from *E. coli*. In order to isolate these genes, an attempt to raise mutants resistant to voltage operated Ca^{2+}-channel inhibitors was undertaken. Initially, the principal drugs chosen for this study were verapamil and ditiazem although subsequently, the main effort was concentrated on verapamil. Since, in eukaryotes, the levels of $[Ca^{2+}]_i$ are critical for normal cell functioning, it was considered likely that conditional lethal mutations in Ca^{2+}-channels might be isolated in *E. coli*, whereby drug resistant mutants would not be capable of growth at the non-permissive temperature. Such mutants would then be transformed with a low copy-number genomic library, and transformants isolated that were capable of growth at the non-permissive temperature. The cloned DNA fragments complementing the drug-resistant, temperature sensitive phenotype would then be sequenced and the encoded genes analysed for possible homology to Ca^{2+}-channels or other Ca^{2+} binding proteins. A phenotypic analysis of any mutants isolated would be performed and the nature of the mutation investigated. It was always anticipated that mutations conferring drug resistance might identify genes other than specific Ca^{2+} channels, either through the action of the drug on other targets in *E. coli* or through drug resistance being generated by other, non-target modifications. To maximise the possibility of identifying

physiologically important functions modified in drug resistant mutants, it was therefore an essential part of the initial screen that the mutants should be conditional lethal mutants and should display some cell cycle defect.

Chapter 2
Materials and Methods

N.B. All reagents used in media and solutions were of analytical grade, supplied by Fisons Scientific Equipment, Loughborough, Leicestershire, unless otherwise stated.

2.1 Bacterial strains

All strains were derivatives of *E. coli* K-12

N43 F$^-$, *acrA1, Δlac-85, ara-14, galK2, rpsL179, malA1*(λ^r), *xyl-5, mtl-1* (Nakamura and Suganuma, 1972)

N43*verA1* (This report); resistant to 0.8mM verapamil

N43*dilA1-4* (This report)

5K F$^-$, K-12, *thi-1, lacY1, tonA21,* r_k^- m_k^+, *mcrA*.

***E. coli* 12016** *zcg-3060*::Tn10. Derived from MG1655 (Bachmann, 1987). Contains a Tn10 transposon at 26.75' on the chromosome. Source C. Gross.

***E. coli* 12169** *zch-506*::Tn10. Derived from MG1655. Contains a Tn10 transposon at 27.25' on the chromosome. Source C. Gross.

MC4100 Δ(*argF-lac*)U169, *araD, rpsL, flbB, deoC, ptsF, rbsR* (Casadaban, 1976)

XL-1 Blue F'[*proA*$^+$ *B*$^+$, *lacI*^{q}ZΔM15, Tn10], *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^- m_k^+), *relA1,* Δ(*lac-pro*), *supE44* (Bullock *et al.*, 1987)

DH5α F$^-$, ϕ80d*lacZ*ΔM15, *recA1, endA1, gyrA96, thi-1, hsdR17*(r_k^- m_k^+), *supE44, relA1, deoR,* Δ(*lacIZYA-argF*)U169 (Hanahan, 1983)

W3110 Prototrophic strain

MC4100*hns*::Tn10 Source: F. Moreno, Unidad de Genética Molecular, Hospital Ramon y Cajal, Madrid, Spain.

GM37 MC4100 ϕ(*proU-lacZ*)hyb2, (λp*lac* Mu15) (May *et al.*, 1986)

GM230 MC4100 ϕ(*proU-lacZ*)hyb2, (λp*lac* Mu15), *hns205*::Tn10 (Higgins *et al.*, 1988)

SY327λ*pir* F$^-$, *araD*, Δ(*lac-pro*), $argE_{am}$, *recA56*, *rif*^{r}, *nalA* (Miller, V.L. and Mekalanos, 1988)

SM10λ*pir* F$^-$, *thi-1*, *thr-1*, *leuB6*, *supE44*, *tonA21*, *lacY1*, *recA*::RP4-2-Tc::Mu Km^r) (Simon *et al.*, 1983)

RB308 F$^+$

PD73 MC4100 ϕ(*hns-lacZ*) (Dersch *et al.*, 1993)

N43 ϕ(p*hns-lacZ*) (this report; derived from PD73 by P1 transduction)

N43*verA1* ϕ(p*hns-lacZ*) (this report; derived from PD73 by P1 transduction)

DS410 F$^-$, *minB*, *thi*, *ara*, *lacY*, *gal*, *malA*, *xyl*, *mtl*, *tonA*, *rpsL* (Dougan and Sherratt, 1977)

2.2 Bacteriophages

$P1_{vir}$ (Dr R Cooper, Dept. of Biochemistry, Univ. of Leicester, UK)

λ141 (6E2) from Kohara library (Kohara *et al.*, 1987)

λ250 (4D8) from Kohara library (Kohara *et al.*, 1987)

λ251 (3D5) from Kohara library (Kohara *et al.*, 1987)

2.3 Plasmids

pLG339. Low copy-number vector containing antibiotic resistance genes to tetracycline and kanamycin (see **Fig. 10**) (Stoker *et al.*, 1982).

pUC18 and 19. High copy-number vectors with versatile multiple cloning site within the region encoding the α-peptide of the *lacZ* (β-galactosidase) gene. When transformed into

suitable host strains containing *lacZ*ΔM15, will complement the deletion in the host strain to produce blue colonies on LUA plates containing X-gal (5-bromo-4-chloro-3-indolyl phosphate) and IPTG (isopropyl thio-β-D-galactoside). Insertion of DNA into the multiple cloning site interupts the α-peptide and prevents complementation, resulting in the formation of white colonies on X-gal / IPTG plates. These plasmids contain the β-lactamase gene for selection. (Yanisch-Perron *et al.*, 1985)

M13mp18RF and mp19RF. Replicative or double stranded form of the filamentous bacteriophage M13. These plasmids are similar to the pUC plasmids, but in addition, contain the functions for packaging into a single stranded infectious bacteriophage, capable of infecting F^+ cells, that can be harvested from the culture supernatant, and used for sequencing and site directed mutagenesis (Messing, 1981; Yanisch-Perron *et al.*, 1985).

pCVD442. Suicide vector based upon the λ*pir* system. This plasmid can only replicate in a pir^+ background and is therefore ideal for gene replacement or the introduction of mutations into the chromosome of λpir^- strains. Following integration of the plasmid into the chromosome of a pir^- strain, a second recombination event which removes the original version of the gene can be selected, by plating the strain onto Luria-Bertani agar (LUA; see below) containing 6% $^{w}/_{v}$ sucrose. pCVD442 contains the *sacB* gene, which confers sucrose sensitivity. Loss of the plasmid as a result of the second recombination event results in sucrose tolerance and consequent survival on these plates (Donnenberg and Kaper, 1991)

2.4 Media

Luria-Bertani broth (LUB) (Roth, 1970)

To 950ml of deionized water, add:
10g bacto-tryptone (Difco)
5g bacto-yeast extract (Difco)
10g NaCl

Adjust pH to 7.0 with 5N NaOH. Adjust volume to 1l. Autoclave at 121°C / 15psi for 15min.

Luria-Bertani Agar (LUA)

As above, but with 1.5%$^{w}/_{v}$ BBL grade A bacteriological agar added.

LUA + 6%$^{w}/_{v}$ sucrose

As for LUA but also containing 6%$^{w}/_{v}$ sucrose. This medium was autoclaved at 115 °C / 10psi for 20min to prevent caramelisation of the sucrose.

Nutrient Broth (NB)

13g Nutrient broth 'E' powder (Lab M)
1l Distilled water
Autoclave at 121°C for 15min.

Nutrient Agar (NA) As NB but with 1.5%$^{w}/_{v}$ BBL grade A bacteriological agar added.

Soft nutrient agar (SNA)

As NB, but containing 0.5%$^{w}/_{v}$ BBL grade A bacteriological agar.

M9 minimal medium

5x M9 salts:

0.24M $Na_2HPO_4.7H_2O$
0.11M KH_2PO_4
0.04M NaCl
0.1M NH_4Cl
Autoclave at 121°C for 15min.

To 750ml of autoclaved distilled H_2O, add:

200ml 5x M9 salts
10ml 40%$^{w}/_{v}$ sterile glucose solution (0.4%$^{w}/_{v}$ final concentration)
10ml 1M $MgCl_2$ (10mM final concentration)
1ml 10mg/ml thiamine (Sigma) (10µg/ml final concentration)

M9 agar as above, with the addition of 1.5%$^{w}/_{v}$ HGT agarose (Seakem).

BTL Agar

LUB
0.7%$^{w}/_{v}$ agar
10mM $MgSO_4$

TCG medium

Each component was autoclaved separately at 121°C for 15min prior to adding to the sterile distilled H_2O.

0.16mM	Na_2SO_4
10mM	$MgCl_2$
8.6mM	NaCl
3μM	$FeCl_3$
64mM	KH_2PO_4
100mM	Tris-HCl pH7.5
0.4%$^{w}/_{v}$	Glucose
10μg/ml	Thiamine (Sigma)
20mM	NH_4Cl

Sterile distilled H_2O.

SOC medium

2%$^{w}/_{v}$	Bactotryptone (Difco)
0.5%$^{w}/_{v}$	Yeast extract
10mM	NaCl
2.5mM	KCl
10mM	$MgCl_2$
10mM	$MgSO_4$
20mM	Glucose

Autoclave all the ingredients except for the glucose, at 121°C for 15min. Finally, add 40%$^{w}/_{v}$ glucose solution that has been sterilised at 110°C for 20min to obtain the desired final concentration of 20mM. Aliquot into 20ml Sterilin containers and freeze at -20°C until required.

Brain-heart infusion broth

37g of brain-heart infusion (Oxoid Unipath Ltd) was dissolved in 1l distilled water and autoclaved at 121°C for 15min.

2.5 Antibiotic supplements

Antibiotic	Solvent	Stock concentration	Working concentration
Kanamycin	Distilled H_2O	50mg/ml	50μg/ml
Ampicillin	Distilled H_2O	100mg/ml	100μg/ml
Tetracycline	50:50 Ethanol:H_2O	15mg/ml	10μg/ml
Streptomycin	Distilled H_2O	200mg/ml	200μg/ml

Table 1. Antibiotics used in this project, their preparation and working concentrations. NB. All antibiotics were filter sterilised prior to use.

2.6 General purpose buffers

20x Phosphate buffered saline (PBS)

2.74M NaCl
0.54M KCl
0.03M KH_2PO_4
0.16M $Na_2HPO_4.12H_2O$

Adjust to final desired volume with H_2O and check the pH of the buffer at its working concentration, before adjusting to pH 7.4 with 2N HCl. Autoclave at 121°C for 15min. Dilute 1:20 for use.

Tris-HCl Buffers

1M Tris [hydroxymethyl] aminomethane (Tris) was dissolved in distilled H_2O to give a volume of approximately 60% the final volume. Concentrated HCl was added to give a pH just above the desired value. The buffer was allowed to equilibrate overnight with mixing before final adjustments were made to the pH. The volume of the buffer was adjusted to the final amount with distilled H_2O and autoclaved at 121°C for 15min.

λ Buffer

50mM Tris-HCl pH7.5
10mM $MgSO_4.7H_2O$
Autoclave at 121°for 15min

2.7 Miscellaneous solutions

Note: All solutions were autoclaved at 121°C for 15min unless otherwise stated.

0.5M EDTA

0.5M Disodium diaminoethane tetra acetate (EDTA) was suspended in approximately 50% of the final volume of distilled H_2O, and 5M NaOH added until the EDTA just dissolved. The pH was confirmed as 8.0 and the final volume adjusted by the addition of distilled H_2O.

0.5M EGTA

0.5M ethylene glycol-bis(β-amino-ethyl ether)N,N,N',N'-tetra acetic acid (EGTA) was prepared as described for EDTA.

3M Sodium acetate pH5.2

3M sodium acetate was dissolved in distilled H_2O to a volume of 80% of the desired final volume. The pH was adjusted to 5.2 with glacial acetic acid and the final volume adjustments made with distilled H_2O. The solution was autoclaved at 121°C for 15min.

10% SDS

Sodium lauryl sulphate was dissolved in distilled H_2O to give a final concentration of $10\%^{w}/_{v}$. This solution was not autoclaved.

2.8 Storage and preservation of bacterial strains

This method is based on that described by Feltham *et al.* (1978). Bacteria to be stored were streaked onto NA plates to obtain isolated colonies. Having verified that the culture was pure, approximately 1ml of NB containing $13\%^{v}/_{v}$ glycerol was decanted onto the plate, and the bacterial growth resuspended with a sterile inoculating loop. The suspension was transferred to a 2ml sterile glass vial (Trident Ltd, London) containing 20-30 2mm diameter hollow glass beads (Ellis and Farrier, Hanover Square, London) that had been previously washed in detergent (Pril), 0.01N HCl, 3 times in warm tap water and finally in distilled water before drying, loading into the vials and autoclaving at 115°C (10psi) for 20min. The vial containing the bacterial suspension was tapped five times to dislodge air bubbles and the excess liquid removed. The vial was tapped on one corner to make the beads form a slope up the side of the vial (permits easier retrieval of the beads following freezing). An adhesive label was attached to the side of the vial with the culture collection number and strain name. In addition, the cap was painted with Tipex™ and the culture collection number written to permit rapid retrieval of the desired vials. The vials were stored at -70°C, and all strain details maintained on a computer database to permit rapid access to information regarding the strains. Retrieval of strains involved removing a single bead from the vial (avoiding unneccessary warming of the vial), and transferring to an NA plate containing antibiotics where appropriate, for incubation.

2.9 DNA manipulations

All procedures involving the manipulation of DNA utilised solutions and materials that had been previously autoclaved at 121°C for 15 min unless otherwise stated. Disposable latex gloves were worn to prevent nuclease contamination of DNA samples.

2.9.1 Buffers, solutions and reagents

40x TAE agarose gel electrophoresis buffer

1.6M	Tris
40mM	EDTA

Dissolve in distilled H_2O to obtain a volume of 70% of the final desired value. Add glacial acetic acid to obtain a pH of 7.7 (normally 6%$^{v}/_{v}$ of the final volume of buffer). Adjust the final desired volume with distilled H_2O. Dilute 1:40 for use with distilled H_2O.

0.8% agarose-TAE gels

0.8%$^{w}/_{v}$ HGT agarose (Seakem) was dissolved in 1x TAE buffer by heating in a microwave oven until all granules had dissolved. When the molten agarose had cooled to 60°C, ethidium bromide (EtBr) solution (stock dissolved in distilled water at a concentration of 5mg/ml, in a light-proof bottle) was added to a final concentration of 125pg/ml, and stored at 60°C in an oven.

5x Agarose sample buffer

12.5%$^{v}/_{v}$	40x TAE buffer
15%$^{v}/_{v}$	Glycerol
0.3%$^{w}/_{v}$	Orange G (Sigma)

TE Buffer

10mM	Tris-HCl pH7.5
1mM	EDTA

Phenol / chloroform

200ml liquified phenol and 200ml chloroform were mixed and 0.4g 8-hydroxyquinoline (Sigma) added as an antioxidant. The pH was equilibrated to 7.5 by extracting twice with 150ml 1M Tris pH7.5, followed by 1 extraction with 150ml 0.1M Tris pH7.5 and finally 150ml 0.01M Tris pH7.5. The pH was checked with pH-sensitive indicator papers (BDH). The phenol / chloroform mix was stored in a shatter-proof bottle at 4°C in the dark under 10mM Tris-HCl pH7.5.

2.9.2 Methods for manipulating DNA

2.9.2.1 Electrophoresis of DNA

Horizontal 0.8%$^{w}/_{v}$ agarose gels were prepared by pouring the molten agarose into perspex casting trays with a comb inserted in one end, manufactured at Leicester University, to a depth of 0.75-1cm, and allowing to set for approximately 30min. DNA samples were prepared by diluting in H_2O to give a final concentration of not more than

100ng/µl, and 5x TAE sample buffer added to a final concentration of 1x. The gels were submerged in electrophoresis tanks (manufactured at Leicester University) containing 1x TAE buffer. The samples were loaded into the slots created by removal of the comb, including 250ng molecular weight markers prepared from commercially prepared λ DNA digested with *Hind*III (Gibco-BRL). A voltage was applied across the gel of 6.5V/cm, ensuring that the DNA travelled towards the anode, and continued until the orange dye had travelled approximately 80% of the total gel length. The DNA was visualised by placing the gel on an ultraviolet (UV) transilluminator (UV Products Ltd), and exposing the gel to UV, 290nm. DNA bands were photographed using a Polaroid MP4 land camera, fitted with a cassette to accept 12.5x10cm Tmax film (Kodak). The molecular weights of the λ*Hind*III standard markers were 23.13kb, 9.416kb, 6.557kb, 4.361kb, 2.322kb, 2.027kb, 564bp and 125bp.

2.9.2.2 Large scale preparation (Maxi-prep) of plasmid DNA

This method is loosely based upon the method of (Birnboim and Doly, 1979).

Solutions required

Solution 1:		**Solution 2:**	**Solution 3:**	
50mM	Glucose	0.2M NaOH	3M	Potassium acetate
10mM	EDTA	1%$^{w}/_{v}$SDS	11.5%$^{w}/_{v}$	Glacial acetic acid
25mM	Tris-HCl pH8.0			

4.4M LiCl

3%$^{w}/_{v}$ N-lauryl sarcosine (sodium salt) (Sigma). This solution was not autoclaved.

CsCl saturated butan-2-ol. CsCl was added in slight excess to approximately 50ml H_2O. Approximately 150ml butan-2-ol was added to the CsCl saturated water and shaken. Two phases separated, the CsCl saturated butan-2-ol forming the upper phase.

1 Liter of the strain containing the plasmid for preparation was grown overnight at 30 or 37°C with vigourous shaking in NB containing the appropriate antibiotic. The bacterial cells were harvested by centrifugation in a GS3 rotor of a Sorval centrifuge, at 3580g (5000 RPM) for 10min at 4°C.

The bacterial pellets were resuspended and pooled in 24ml Solution 1, which was then divided between two 55ml Falcon centrifuge tubes and kept on ice. 24ml of Solution 2 was added to each tube and mixed gently. The tubes were left on ice for 5min to allow complete lysis to take place. 18ml of ice cold Solution 3 was added to each tube, mixed gently and incubated on ice for a further 5min. The tubes were centrifuged at 3200g (4000 RPM) in a Heraeus Megafuge 1R refrigerated centrifuge at 4°C for 10min. The

supernatant was transferred to four Falcon tubes (25ml in each), and an equal volume of propan-2-ol added. The tubes were incubated on ice for 30min followed by centrifugation at 3200g, 4°C for 15min. Following removal of the supernatant, the pellets were shaken gently in 20ml 70%$^{v}/_{v}$ ethanol and centrifuged for a further 5min at 3200g, 4°C. The supernatant was again discarded, and the pellets dried in a vacuum desicator. To precipitate much of the RNA and protein, the pellets were pooled and dissolved in 8ml distilled water followed by 10ml of 4.4M LiCl, and incubated on ice for 60min. The tubes were centrifuged at 3200g, 4°C for 10min and the supernatant transferred to a fresh tube. 2.5 volumes of -20°C 100% ethanol was added, and the tube incubated at -20°C for 30min. The plasmid DNA was pelleted by centrifuging at 3200g, 4°C for 15min. The supernatant was discarded and replaced by 15ml 70%$^{v}/_{v}$ ethanol and centrifuged for a further 5min. The pellet was dried as before, dissolved, initially in 1ml distilled H_2O and the volume adjusted to 1.55ml with distilled H_2O. An equal volume of 3%$^{w}/_{v}$ N-lauryl sarcosine was added, followed by 3.52g CsCl and 120µl 10mg/ml EtBr. Two Beckman 11x32mm Quickseal™ tubes (ref. 344625) were loaded, balanced to within 5mg, sealed, and placed in the TLA100.2 rotor of a Beckman TLA100 ultracentrifuge. The tubes were centrifuged at 250000g, at 20°C overnight. After removing the top of the Quickseal™ tube with a heated scalpel blade, the plasmid DNA (lower band) was recovered from the CsCl gradient by aspirating through a 27G 20mm syringe needle attached to a 1ml syringe and transferred to a 1.5ml Eppendorf tube.

The EtBr was removed by adding approximately 300µl of CsCl saturated butan-2-ol, vortexing vigorously, removing and safely discarding the top phase in which the EtBr preferentially partitioned. This step was repeated until no further colouration was noted in the plasmid-containing lower phase. The volume of plasmid containing solution was noted and the DNA precipitated at room temperature by the addition of distilled water and propan-2-ol in the ratio 0.54: 0.5: 0.4 of distilled water: propan-2-ol: DNA solution, respectively. The tube was centrifuged at 13000g for 10min at room temperature, the supernatant discarded and replaced by 1ml 70%$^{v}/_{v}$ ethanol. After a further 5min spin at 13000g, the ethanol was removed and the DNA pellet dried as described above. The pellet was resuspended in 200µl H_2O and the DNA concentration determined as described below in section **2.9.2.5**. The concentration of plasmid DNA was then adjusted by a further addition of H_2O to obtain a concentration of 0.5µg/ml.

2.9.2.3 Small scale preparation (Mini-prep) of plasmid DNA

This method is based upon the method of (Birnboim and Doly, 1979).

Solutions and reagents required:

Solutions 1, 2 and 3 from the Maxi-prep method (see above).

Phenol / chloroform (see above)

10mg/ml RNAse A. RNAse A (Sigma) was dissolved in distilled H_2O to a final concentration of 10mg/ml and aliquoted into Eppendorf tubes. The tubes were boiled for 15min to inactivate DNAses and stored at -20°C.

5ml overnight cultures of strains containing the plasmids for purification were grown in NB containing appropriate antibiotics. Approximately $4.0A_{600}$ units of each culture was transferred to 1.5ml Eppendorf tubes and centrifuged at 13000g for 2 min. The supernatant was aspirated from each tube and replaced by 100µl of Solution 1. The bacterial pellets were vigorously resuspended by vortexing and transferred to ice. 200µl of Solution 2 was added to each tube to lyse the bacteria and mixed by inverting the tubes 5 times before replacing on ice. 150µl of ice cold Solution 3 was added to denature the proteins and chromosomal DNA in each tube, mixed gently by inversion and centrifuged at 13000g for 5min at 4°C. The supernatant was transferred to fresh, sterile Eppendorf tubes and 400µl of phenol / chloroform added to denature and precipitate any remaining proteins. Each mixture was vortexed vigorously and then centrifuged at 13000g, room temperature for 5min. 85% of the upper phase was transferred to fresh, sterile tubes and the plasmid DNA precipitated by the addition of 2.5 volumes of 100% ethanol. The tubes were incubated at -20°C for 30min, centrifuged at 13000g, 4°C for 10min and the supernatant discarded by aspiration. The pellets were washed by the addition of 500µl 70% ethanol, centrifuged for a further 5min and the pellets dried in a vacuum dessicator following removal of the 70% ethanol. Finally, each pellet was resuspended in 30-50µl distilled water containing 20ng/ml RNAseA and incubated for 15min at 37°C to remove tRNA remaining in the plasmid preparations.

2.9.2.4 Preparation of chromosomal DNA

This method is taken from (Ausubel *et al.*, 1992).

Solutions and reagents required:

TE Buffer

10%$^{w}/_{v}$ SDS

20mg/ml Proteinase K Proteinase K (Sigma) was dissolved in distilled water just prior to use, at a concentration of 20mg/ml.

5M NaCl

24:1 Chloroform: isoamyl alcohol

Phenol / chloroform
Propan-2-ol
10mg/ml RNAse A
CTAB/NaCl solution Dissolve 4.1g NaCl in 80ml H_2O, followed by the slow addition of CTAB (hexadecyltrimethyl ammonium bromide) (Sigma), whilst heating and stirring. Adjust final volume to 100ml. This solution was not autoclaved.

10ml of overnight cultures were grown in NB ($A_{600} \approx 4.0$), transferred to 55ml Falcon centrifuge tubes and centrifuged for 10min in a Heraeus Megafuge 1R at 3200g (4000 RPM), 4°C. The supernatant was discarded, and the cells resuspended in 11.4ml TE buffer. 600µl of 10% SDS and 60µl of 20mg/ml Proteinase K were added and mixture incubated for 60min at 37°C. 2.01ml of 5M NaCl was gently mixed into the lysate, followed by 1.6ml CTAB/NaCl solution and incubated at 68°C for 10min. 15.6ml chloroform / isoamyl alcohol was added and gently mixed. The tubes were then centrifuged at 3200g for 10min at 4°C. 80-90% of the upper phase was carefully transferred to a fresh Falcon tube and 5ml phenol / chloroform added. Again, the mixture was gently mixed, centrifuged for 10min and the upper phase transferred to a clean tube. A second phenol / chloroform extraction was performed, and after collection of the upper phase, 0.6 volumes of propan-2-ol was added to precipitate to chromosomal DNA. The chromosomal DNA was collected by stirring a heat-sealed Pasteur pipette in the tube and allowing the DNA to stick to the pipette. The DNA was washed by swirling the pipette with the attached DNA in a sterile tube containing 15ml 70% ethanol and then transferring the DNA to a second tube also containing 70% ethanol. After gently scraping the chromosomal DNA from the Pasteur pipette into the ethanol, the tube containing the DNA was centrifuged at 3200g for 5min, the supernatant discarded and the DNA pellet dried. Finally, the chromosomal DNA was resuspended in 500µl distilled water containing 20µg/ml RNAseA and ready for restriction digestion.

2.9.2.5 Determination of DNA concentration in a solution

1µl of the DNA solution to be tested was diluted in 500µl H_2O and transferred to a 0.5ml quartz cuvette. The absorbance was measured at 260 and 280nm using a Pharmacia Ultraspec III spectrophotometer, and the concentration calculated on the basis that 1.0 A_{260} unit corresponds to 50µg/ml of double stranded DNA. The ratio of A_{260} /A_{280} was also determined inorder to estimate the purity of the DNA, which should ideally be 1.8.

2.9.2.6 Ethanol precipitation of DNA

The DNA in a solution can be quickly precipitated and washed to remove salts as follows: add 0.1 volumes of 3M sodium acetate pH5.2 and 2.5 volumes of 100% -20°C ethanol. Store at -20°C for 20-30min followed by centrifuging at 13000g for 10min at 4° C. Remove all the ethanol and replace with 500µl 70%$^{v}/_{v}$ ethanol. Centrifuge for 5min at 13000g at 4°C, remove all ethanol and dry the pellet in a vacuum desicator. The DNA can then be resuspended in distilled water of the desired volume. If the initial DNA concentration is very low, the addition of 1-2µl of 10mg/ml tRNA (type XX from *E. coli*, Sigma) prior to the addition of sodium acetate and ethanol greatly improves the efficiency of DNA precipitation.

2.9.2.7 Restriction endonuclease digestion of DNA

For most restriction endonuclease reactions, 0.2-1µg of DNA was digested. All the restriction enzymes used in this work were supplied by Gibco-BRL at a concentration of 8 units/µl, and were accompanied by a 10x reaction buffer that had been optimised for each enzyme. DNA to be digested was mixed with distilled H_2O, 0.1 volumes of 10x buffer, and 2 units of restriction enzyme and incubated at the temperature regarded as appropriate for the enzyme, normally for a period of 60min. In cases where larger amounts of DNA were digested, all the components of the reaction were scaled up accordingly. To terminate the reaction, 5x TAE sample buffer was added to obtain a final 1x concentration. The digested DNA was loaded into the slot of a 0.8%$^{w}/_{v}$ TAE-agarose gel, the DNA fragments resolved by electrophoresis and visualised as described earlier.

2.9.2.8 Purification of DNA fragments from agarose gels

2.9.2.8.1 Purification of DNA with polyallomer wool

Blocks of agarose containing the DNA fragment to be purified were excised from the gel and transferred to a 0.5ml Eppendorf tube that had been pierced in the base with a syringe needle, and contained a few strands of polyallomer wool (Interpret™; obtained from a local tropical fish stockist. The wool is used in the filtration units of tropical fish aquaria). The 0.5ml tube was placed inside a 1.5ml Eppendorf tube with its cap removed, and centrifuged at room temperature at 13000g for 20min. The liquid collected in the 1.5ml tube was transferred to a fresh tube, and the tubes containing the polyallomer wool and agarose centrifuged for a further 5min. The liquid collected was pooled with the first fraction and extracted with an equal volume of phenol / chloroform (see Mini-prep method). Following recovery of approximately 80% of the upper phase, residual DNA remaining with the phenol / chloroform was recovered by re-extraction with distilled H_2O.

This involved adding a volume of sterile distilled H_2O equal to the volume of DNA-containing solution that had been transferred to the clean tube, and processing as before. Following centrifugation, 80% of the upper phase was pooled with the first fraction and the volume of DNA solution estimated. The DNA was precipitated, washed and resuspended as described in section 2.9.2.3.

2.9.2.8.2 Recovery of DNA from agarose using the Qiagen Qiaex™ DNA gel extraction kit

This method relies upon the natural affinity that DNA has for silica and glass in the presence of high concentrations of salt. The buffers supplied in the kit were composed as follows:

QX1: 3M NaI
4M $NaClO_3$
5mM Tris-HCl, pH7.5
0.1%$^{w}/_{v}$ Na_2SO_3

QX2: 8M $NaClO_3$

QX3: 70%$^{v}/_{v}$ ethanol
100mM NaCl
10mM Tris-HCl, pH7.5
1mM EDTA

The weight of DNA-containing agarose to be treated was determined and 300µl of QX1 added per 100mg of agarose. The tube was heated at 50°C with occasional mixing until the agarose had fully dissolved. 10µl of thoroughly resuspended Qiaex™ matrix was added to the tube of dissolved agarose and incubated at 50°C for a further 10min, with regular mixing every 2min. The tube was centrifuged at 13000g for 20s, the supernatant discarded, and the pellet resuspended in 500µl QX2 by vigorous vortexing. The suspension was again centrifuged for 20s and respended in a further 500µl QX2. The pellet was then resuspended and washed twice in QX3 (as for QX2). After removal of the supernatant from the second QX3 wash, the tube was again briefly centrifuged to collect residual droplets of buffer, which were discarded. The pellet was air dried for a few minutes, resuspended in 20µl H_2O and heated at 55°C for 5min to elute the bound DNA. The tube was centrifuged for 60secs at 13000g and the supernatant transferred to a fresh tube. A further 20µl H_2O was added to the Qiaex™ pellet, the pellet resuspended, heated and centrifuged as before, and the supernatant pooled with the first eluate. 1µl of the eluate was loaded onto a mini-agarose gel prepared on a microscope slide to estimate

approximately, the concentration of DNA by comparing with known amounts of λ DNA digested with *Hind*III.

2.9.2.9 Dephosphorylation of vector DNA

In cases where a fragment of DNA was to be cloned into a vector that had been digested to yield homologous ends, the vector was dephosphorylated with calf intestinal alkaline phosphatase (CIP) (Pharmacia) to prevent self ligation during the ligation reaction. Following digestion of the vector, the DNA was ethanol precipitated (see section **2.9.2.6**) and resuspended in 44µl sterile distilled H_2O. 5µl of 10x CIP buffer (10mM $ZnCl_2$, 10mM $MgCl_2$, 100mM Tris-HCl; pH8.3) and 1µl (1 unit) of 1000 units/ml CIP (Pharmacia) was added and incubated for 30min at 37°C. In instances where the vector had blunt ends, a further 1µl of CIP was added after 30min incubation, and incubated for another 30min. To terminated the reaction, 1µl of 0.5M EDTA and 150µl of distilled H_2O were added, extracted with 200µl of phenol / chloroform, and re-extracted (see section **2.9.2.8.1 polyallomer wool method**). Finally, the DNA was ethanol precipitated and resuspended in 21µl of H_2O. 1µl of the dephosphorylated vector was loaded on a mini-agarose gel to estimate the concentration as described above.

2.9.2.10 Ligating and cloning of DNA

Prior to cloning a fragment of DNA into a vector, 1µl of vector and insert DNA were loaded onto a mini-agarose gel on a microscope slide in order to determine the approximate concentrations of each (see section **2.9.2.8.2**). In a 10µl ligation reaction, equimolar concentrations of vector and insert DNA were mixed together with 1µl of 10mg/ml ATP, 2µl 5x ligation buffer (Pharmacia) and 1µl (5 units) T4 DNA ligase (Pharmacia). The ligation mixture was incubated overnight at 16°C and then ethanol precipitated in the presence of tRNA (see section **2.9.2.6**). Ligation controls were always included and these consisted of phosphorylated vector alone, dephosphorylated vector alone and insert DNA alone.

2.9.3 Transformation of plasmid DNA into *E. coli*

2.9.3.1 Calcium chloride method

This method was used where a high transformation efficiency was not required. A 5ml culture of the strain to be transformed was incubated overnight (A_{600}≈4.0) and then transferred to a 55ml Falcon tube and centrifuged for 5min at 3200g, 4°C. The bacterial pellet was gently resuspended in 1ml sterile ice cold 0.1M $CaCl_2$, transferred to an Eppendorf tube and centrifuged for 30secs. The supernatant was discarded, the pellet

again gently resuspended in 1ml ice cold $CaCl_2$ and incubated on ice for 1-2h. 100µl aliquots of cells were transferred to fresh Eppendorf tubes with ligated DNA or previously prepared plasmid. Each tube was incubated on ice for a further 20min, the bacteria heat-shocked at 42°C for 90s, 1ml NB added and incubated at 30 or 37°C for 60min. The cells were pelleted by centrifuging at 13000g for 15-20s, the supernatant decanted from the tubes and the cells resuspended in the residual liquid remaining in the tubes. The transformed cells were then inoculated onto NA plates containing antibiotics where appropriate, and the plates incubated overnight at 30 or 37°C. A transformation efficiency of 10^4-10^5 transformants / µg plasmid was normally obtained.

2.9.3.2 Electroporation

This method yields much higher numbers of transformants than the $CaCl_2$ method. A 1:100 dilution of an overnight culture was prepared in 100ml prewarmed NB and shaken vigorously until the A_{600} reached 0.6. The flask was transferred to a container filled with ice and swirled continuously in order to chill the bacteria as rapidly as possible. The culture was transferred to two 55ml Falcon centrifuge tubes and centrifuged for 10min at 3200g, 2°C. The supernatant was discarded, the pellets resuspended and pooled in 25ml ice cold distilled H_2O and centrifuged for 10min. The supernatant was discarded and the cells washed a further three times as described above. After the fourth wash, the pellet was resuspended in 0.5ml ice cold distilled H_2O and stored on ice until ready for transformation. It is most important that all salt is removed from the DNA (and cells) prior to electroporation. As indicated in section **2.9.2.10**, after ligations had been completed, the DNA was ethanol precipitated in the presence of tRNA to remove the salt from the ligation reaction. The precipitated DNA was resuspended in 10µl distilled H_2O and 5µl transferred to a fresh tube. 50µl of the washed *E. coli* cells were added to the 5µl of DNA to be transformed and transferred to a sterile, ice cold electroporation cuvette (BioRad) with a 0.5mm electrode gap. The cuvette was transferred to a cuvette holder attached to a BioRad Gene Pulser, programmed to apply an electrical discharge of 1.5kV with a capacitance of 25µF and a resistance load of 1000Ω. After electroporation of the cells, the time constant was noted on the Gene Pulser unit - a value of less than 18 indicated trace contamination with salts which would reduce the efficiency of transformation. As soon as possible after electroporation, 1ml of SOC medium (see section 2.4) was added to the cuvette and the transformed cells transferred to a large culture tube for incubation at 30 or 37°C for 60min. Finally, the cells were transferred to an Eppendorf tube, centrifuged at 13000g for 15s, the supernatant decanted and the pelleted cells resuspended in the residual medium, remaining in the tubes. The resuspended cells were then inoculated onto NA

containing appropriate antibiotics and incubated overnight at 30 or 37°C. Controls were always included and these consisted of a sterility control, where 5µl of distilled H_2O was added to the cells, and a positive control where 1µg of uncut vector DNA was added. Typically, a transformation efficiency of 10^9-10^{10} transformants / µg DNA was obtained.

2.10 Purification of λ bacteriophage DNA from the Kohara library

2.10.1 Preparation of λ phage stocks

A 5ml overnight culture of *E. coli* 5K was grown in LUB containing 10mM $MgSO_4$ and 0.2%$^{w}/_{v}$ maltose. The culture was diluted 1:100 in 10ml prewarmed LUB, again containing 10mM $MgSO_4$ and 0.2%$^{w}/_{v}$ maltose and grown to an A_{600} of 1.0. 200µl of the bacterial culture was transferred to small, sterile test tubes and 2µl of λ phage suspensions from the Kohara library added. The tubes were incubated at 37°C for 15min, 3ml molten (60°C) Soft nutrient agar (SNA) containing 10mM $MgSO_4$ and 0.2%$^{w}/_{v}$ maltose added, and the suspension quickly overlayed onto LUA plates. The LUA plates were incubated overnight at 37°C. The following day, isolated plaques were stabbed with sterile Pasteur pipettes and the phage resuspended in 0.5ml λ buffer.

2.10.2 Preparation of λ DNA

25µl of each phage suspension was added to 200µl of *E. coli* 5K cells as described above, incubated for 15min, mixed with molten SNA and overlayed onto LUA plates. The plates were incubated at 42°C until the plaques became virtually confluent. 8ml of λ buffer was added to each plate and rocked gently at 4°C overnight. The phage suspensions were transferred to 35ml Corex™ tubes (DuPont Instruments), mixed with 100µl chloroform and vortexed for 15s. The tubes were centrifuged for 10min at 9220g (10,000 RPM), 4°C in a Sorvall SS34 rotor, and the supernatant transferred to 55ml Falcon centrifuge tubes. Contaminating *E. coli* DNA and RNA was removed by the addition of 20µg/ml DNAseI and RNAseA and incubated at room temperature for 30min. The volumes of each phage suspension were adjusted to 10ml with λ buffer and 1ml of Tris-HCl pH8.0, 1ml 0.5M EDTA and 400µl 5M NaCl added. The phage coats were removed by the addition of 0.5%$^{w}/_{v}$ SDS and 1mg/ml proteinase K (Sigma). The tubes were incubated for 30min at 37°C and then twice extracted with 5ml phenol / chloroform, followed by re-extraction with distilled H_2O. The DNA was ethanol precipitated and resuspended in 100µl H_2O.

2.11 Nucleic acid labelling and detection

Two commercial systems were used for the non-radioactive labelling and detection of DNA and RNA. The DIG system manufactured by Boehringer-Mannheim involved

labelling the probe with digoxigenin-11-dUTP whereas the Gene Images™ system manufactured by Amersham International PLC used latterly in this project incorporated fluorescein-11-dUTP into the probe. In both cases, the probe was detected by the use of a specific monoclonal antibody-alkaline phosphatase conjugate. In the DIG system, visualisation of the hybridised probe was facilitated by the occurence of a chromogenic reaction on the filter. The Amersham system utilises chemiluminescence as the signal, which is detected by X-ray film.

2.11.1 Transfer of DNA from agarose gels to a nylon membrane for Southern blot analysis

20x SSC	3M NaCl 0.3M Sodium citrate pH7.0
Denaturing solution	1.5M NaCl 0.5M NaOH
Neutralising solution	1M Tris-HCl 1.5M NaCl pH8.0

Following electrophoresis of the DNA, the EtBr stained gel was photographed with a ruler alongside the λ*Hind*III molecular weight markers. The DNA was then denatured by soaking the gel twice in 200ml denaturing solution for 20min each time, followed by neutralisation in two 20min treatments with neutralisation solution. The gel was then placed upside down onto the 3MM paper (soaked in 10x SSC) of the transfer apparatus. The transfer apparatus consisted of a tray containing 500ml 10x SSC with a glass plate placed across the tray. A sheet of 3MM chromatography paper (Whatman) was placed over the glass plate with the ends immersed in the 10x SSC, and allowed to become saturated in 10x SSC. A piece of Nylon membrane (Hybond N, Amersham International PLC) that had been presoaked in 2x SSC, was placed over the gel, avoiding the introduction of bubbles, followed by two pieces of 3MM paper of the same size as the membrane. To draw the buffer through the gel and transfer the DNA onto the membrane, a stack of 10 sheets of Quickdraw™ (Sigma) absorbent paper was placed over the two sheets of Whatman 3MM paper, with a 500ml bottle of water placed on the top to apply pressure to the assembly. After 3h, the nylon membrane was removed from the assembly, washed briefly in 5x SSC and air dried. The DNA was crosslinked to the membrane by wrapping the membrane in Saran Wrap™ and exposing to ultraviolet light at an intensity of 70mJ/cm^2, using an Amersham UV crosslinker. The filter was then ready for hybridisation.

2.11.2 Slot blot DNA hybridisation analysis

DNA to be analysed by slot blot analysis was diluted to a concentration of 1pg/µl in the dilution buffer supplied with the Boehringer-Mannheim kit (50mg/ml Herring sperm DNA, 10mM Tris-HCl and 1mM EDTA; pH8.0), to a final volume of 30µl. The slot blot apparatus (BRL-Gibco) was assembled by firstly pretreating the nylon membrane (Hybond-N, Amersham International PLC). Pretreatment of the 3.5x11.5cm membrane involved soaking in distilled water for 5min followed by soaking in 10x SSC for 15min and then allowing to dry. A piece of 3MM chromatography paper (Whatman) of the same size was soaked in 20x SSC and placed in the slot-blot apparatus, with the nylon membrane placed on top. The blotting unit was reassembled and connected to a vacuum pump. The diluted DNA samples were boiled for 10min and then applied to the wells of the slot blot apparatus. Following transfer of the samples to the membrane, the membrane was gently floated on 3M sodium acetate, pH5.2 for 5min, then floated on 2x SSC for 10min, and subsequently briefly immersed. Finally, the filter was air dried and UV cross-linked as described in section 2.11.1. Hybridization was performed as described below, using the Boehringer-Mannheim DIG kit.

2.11.3 Non-radioactive labelling and detection of DNA

2.11.3.1 Labelling and the detection of DNA, labelled with digoxigenin

The DNA to be used as probes was prepared from plasmids digested as indicated in the results chapters of this report, and purified using the polyallomer wool method described in section 2.9.2.8.1. The procedures for the labelling and detection of hybridized probes were as described in the protocols supplied by Boehringer-Mannheim.

2.11.3.1.1 Labelling of probes

Approximately 1µg of gel-purified DNA was made upto a volume of 15µl with distilled H_2O in an Eppendorf tube, boiled for 10min, chilled on ice and mixed with 2µl of random hexanucleotide primer mix and 2µl of dNTP mix. 1µl (2U/µl) of Klenow enzyme was added and the tube incubated for 1-2h. The reaction was terminated by the addition of EDTA to a final concentration of 10mM, and the labelled probe precipitated by the addition of 2.5µl of 4.4M LiCl and 75µl ethanol. The DNA was pelleted, washed and dried as described earlier in section 2.9.2.6. Finally, the probe was resuspended in 50µl H_2O.

2.11.3.1.2 Hybridization of probe to immobilised target DNA

Nylon membrane (Hybond N, Amersham International PLC) containing the cross-linked, denatured DNA to be probed was prehybridised at 68°C for 60min in 50ml hybridisation buffer (5x SSC, 0.1%$^{w}/_{v}$ N-lauryl sarcosine (sodium salt, Sigma), 0.02%$^{w}/_{v}$ SDS, 1%$^{w}/_{v}$ blocking reagent (freshly dissolved by gentle heating)) in a Hybaid dual hybridization oven. The hybridization buffer was discarded from the filter and replaced with 20ml of fresh hybridization buffer. Boiled probe was added to the hybridization buffer to attain a probe concentration of approximately 10ng/ml and allowed to hybridize overnight. The following day, the filter was washed twice for 5min in 1x SSC, 0.1%$^{w}/_{v}$ SDS at room temperature, followed by two 15min washes in 0.1x SSC, 0.1%$^{w}/_{v}$ SDS, at 68°C.

2.11.3.1.3 Detection of bound probe

The filter was washed briefly in washing buffer (100mM Tris-HCl, 150mM NaCl, pH7.5) and blocked for 30min in 100ml washing buffer containing 0.5%$^{w}/_{v}$ blocking reagent. The filter was again briefly washed in washing buffer and then incubated with 25ml of a 1:5000 dilution of anti-digoxigenin-alkaline phosphatase conjugate diluted in washing buffer, for 30min. Unbound antibody was removed by washing the membrane twice for 15min in 100ml washing buffer and then equilibrated by briefly washing in developer buffer (100mM Tris-HCl, 100mM NaCl, 1mM $MgCl_2$; pH9.5). Detection of the bound probe was performed by incubating the membrane in 10ml developer buffer containing 45µl of 75mg/ml nitroblue tetrazolium (NBT; 2,2'-di-p-nitrophenyl-5,5'-diphenyl-3,3'-[3,3'-dimethoxy-4,4'-diphenylene]-ditetrazolium chloride, Sigma) and 35µl of 50mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma). The coloured bands were allowed to develop in the dark and when complete, the reaction was terminated by the addition of 10mM Tris-HCl pH7.5, 1mM EDTA and the blot photographed.

2.11.3.2 Labelling and detecting DNA labelled with fluorescein

2.11.3.2.1 Labelling the probe with fluorescein-11-dUTP

The methodology behind the Gene Images™ system (Amersham International PLC) is very similar to the Boehringer-Mannheim system. Typically, 2µg of DNA to be labelled, was added to distilled H_2O to obtain a final volume of 39µl. The DNA was boiled for 10min and quickly chilled on salt-ice. 10µl of labelling mix (dNTPs) and 5µl of hexanucleotide primers were added to the denatured DNA, mixed and finally 1µl of Klenow enzyme added. The probe was labelled for 60min at 37°C and the reaction terminated by the addition of EDTA at a final concentration of 20mM.

2.11.3.2.2 Hybridization of the fluorescein-labelled probe with the immobilised DNA or RNA

The nylon membrane containing the immobilised DNA or RNA was prehybridized in 30ml of hybridization buffer (5x SSC, 0.1%$^{w}/_{v}$ SDS, 5%$^{w}/_{v}$ dextran sulphate (Sigma) and $^{1}/_{20}$ volume liquid block (Amersham) at 65°C for 60min. The fluorescein-labelled probe was boiled for 10min and added to the hybridization buffer to give a final concentration of 10ng/ml. Hybridization was allowed to take place overnight at 65°C. The following day, the filters were washed using the conditions specified in the results chapters.

2.11.3.2.3 Detection of the hybridized probe

Following the stringency washes, the filter was briefly washed in 100ml sterile washing buffer (100mM Tris-HCl, 300mM NaCl; pH7.5). The filter was then blocked for 60min in 50ml washing buffer containing $^{1}/_{10}$ dilution of liquid blocking reagent. The blocking solution was replaced by 100ml washing buffer for 3min and then by 25ml 1:5000 dilution of anti-fluorescein alkaline phosphatase conjugate in washing buffer containing 0.5%$^{w}/_{v}$ bovine serum albumin (BSA; fraction VIII, Sigma). The filter was gently rocked with the antibody for 60min and the unbound antibody removed with three 15min washes of 100ml washing buffer containing 0.3%$^{v}/_{v}$ Tween20 (Sigma). Finally, the filter was washed briefly in washing buffer, all excess liquid allowed to run off and then placed on Saran Wrap™. The membrane was sparingly sprayed with Dioxetane reagent, the spray evenly distributed by folding the Saran Wrap™ over the membrane and running gloved hands over the filter. The Saran Wrap™ was opened, the filter suspended in the air for 2min to allow excess liquid to collect at the bottom, and gently removed by touching the bottom of the membrane on the Saran Wrap™. The filter was then placed into a clean, clear plastic bag in an autoradiograph cassette and exposed to Fuji RX X-ray film overnight.

2.11.4 Northern blot analysis

2.11.4.1 Preparation of bacterial RNA

The method for preparing RNA is based upon the protocol described in Ausubel *et al* (1992).

Solutions, buffers and reagents:

All materials and equipment were soaked in a 0.1%$^{v}/_{v}$ solution of diethyl pyrocarbonate (DEPC; Sigma) for 2h and autoclaved at 121°C for 15min. Where possible, all solutions were treated by the addition of 0.1%$^{v}/_{v}$ final concentration of DEPC, shaken vigorously and autoclaved, with the exception of Tris- and EDTA based buffers where the

DEPC would have reacted with the amine groups in these compounds, therefore, these buffers were autoclaved only.

Protoplasting buffer

15mM	Tris-HCl pH8.0
0.45M	Sucrose
8mM	EDTA

Autoclave at 121°C for 15min

Lysis buffer

10mM	Tris-HCl
10mM	NaCl
1mM	Sodium citrate
1.5%$^{w}/_{v}$	SDS

Autoclave at 121°C for 15min

Phenol / chloroform (see section 2.9.1)

50mg/ml Lysozyme (Sigma) in protoplasting buffer

Saturated NaCl

40g	NaCl
100ml	H_2O
100µl	DEPC

Autoclave at 121°C for 15min.

5ml overnight cultures of the strains to be tested were diluted 1:100 in 10ml prewarmed NB (30 or 37°C) and grown to mid-exponential phase (0.4-0.5). 4.0 A_{600} units of each culture were transferred to 55ml Falcon centrifuge tubes and centrifuged for 5min at 3200g, 4°C. The supernatant was discarded, the pellets resuspended in 1ml protoplasting buffer and transferred to Eppendorf tubes. 80µl Lysozyme solution was added to each suspension and the tubes incubated on ice for 15min. The protoplasts were centrifuged for 15-20 s, the supernatant decanted, and the pellet resuspended in the remaining 40-50µl of protoplasting buffer by gentle vortexing. The protoplasts were lysed by the addition of 0.5ml lysis buffer containing 3%$^{v}/_{v}$ freshly added DEPC and incubated at 37°C for 5min. The tubes were transferred to ice, 250µl saturated NaCl added, mixed gently and incubated for 15min on ice. The tubes were centrifuged at 13000g for 10min at 4°C and the supernatant transferred to fresh tubes. To each tube, 400µl phenol / chloroform was added, vortexed vigorously and centrifuged for 5min. 80% of the upper phase was transferred to fresh tubes and the RNA precipitated by the addition of 2.5 volumes of ethanol and processed as for DNA precipitations. The dried RNA pellets were resuspended in 50µl of DEPC treated distilled water, 2µl were checked on a TAE agarose

gel and the remainder used in Northern hybridization analyses. The RNA concentration was estimated at 5μg/μl.

2.11.4.2 Preparation of the RNA samples for electrophoresis, the formaldehyde denaturing gel, and blotting of the RNA

This method is based upon the method described in Ausubel *et al* (1992)

Distilled water Five1litre bottles of 0.1%$^{v}/_{v}$ DEPC treated water
One bottle containing 800ml DEPC treated water.

5x Running buffer 700ml Distilled H_2O
40mM Sodium acetate pH5.2
5mM EDTA
0.8ml DEPC

Shake vigorously and autoclave at 121°C for 15min. Add 3-[N-morpholino] propane sulphonic acid (MOPS, Sigma) to obtain a final concentration of 0.1M, from a previously unopened container and mix well (the MOPS was weighed in a glass beaker that had been previously treated with DEPC-water and autoclaved). To adjust the pH of the 5x buffer stock, 300μl aliquots of the buffer were withdrawn and diluted in approximately 50ml distilled water, and the pH measured. DEPC treated 2M NaOH was added to the 5x stock buffer on the basis of the pH measurements obtained from the diluted buffer. All additions of NaOH were noted and when the desired pH attained, the volume of buffer calculated, and DEPC treated water added to give a final volume of 1litre. The buffer was stored at room temperature in a foil-wrapped bottle.

RNA Sample buffer

50%$^{v}/_{v}$ Glycerol
1mM EDTA
0.25%$^{w}/_{v}$ Bromophenol blue (Sigma)
0.25%$^{w}/_{v}$ Xylene cyanol (Sigma)
0.1%$^{v}/_{v}$ DEPC
Autoclave at 121°C for 15min.

1.2%$^{w}/_{v}$ Agarose

2.1g Agarose LE (Seakem)
134ml Distilled H_2O
134μl DEPC (final concentration = 0.1%$^{v}/_{v}$ DEPC)

Autoclave at 121°C for 15min. Allow to cool to 60°C, add 35ml 5x Running buffer and 5.25ml 37-41%$^{w}/_{v}$ formaldehyde. The molten agarose was poured into a casting plate with comb that had been soaked overnight in 5%$^{v}/_{v}$ H_2O_2, and allowed to set for 30min in a fume hood.

1x Running buffer Add 200ml 5x buffer to the bottle containing 800ml DEPC treated, autoclaved H_2O.

The RNA samples were prepared by adding 4.5µl RNA sample, 2µl 5x running buffer, 3.5µl formaldehyde and 10µl formamide and heating at 65°C for 15min. The samples were than chilled on ice for 2min, centrifuged briefly and 2µl of RNA sample buffer added. While the RNA samples were being prepared, the gel was pre-run at 5V/cm. The gel was loaded with each of the samples including RNA calibration markers (Promega) comprising the following sizes: 9488, 6225, 3911, 2800, 1898, 872, 562 and 363 nucleotides.

The gel was run at 5V/cm until the bromophenol blue band had travelled 85% of the entire gel length. The gel was removed, soaked in 10x SSC for 60min and blotted onto Hybond N nylon membrane (Amersham International PLC) for 3h as described in section **2.11.1**. The filter was briefly washed in 6x SSC, air dried and UV crosslinked as described earlier. To check that the RNA had transferred successfully, the membrane was stained with methylene blue (0.04% methylene blue (Sigma), 0.5M sodium acetate pH5.2) for 5min and destained in distilled water. The section of the membrane containing the RNA size standards was separated from the remainder of the membrane, and kept in the dark for future reference. The membrane was probed using fluorescein labelled probes and the Amersham Gene Images™ kit as described in sections **2.11.3.2.1-3**.

2.12 Sequencing the *verA* locus

The dideoxy nucleoside triphosphate chain-terminating method of Sanger *et al* (1977) lends itself well to M13 single stranded DNA sequencing. The following sections cover the growth and handling of M13, cloning of the *verA* locus into M13, preparation of the nested deletions of the M13-*verA* subclones, preparation of single stranded DNA (ssDNA) template, labelling of the DNA and electrophoretic analysis of the labelled DNA.

2.12.1 Preparation of DNA for sequencing

2.12.1.1 Culturing M13

Since M13 is a so-called 'male specific' bacteriophage, the host strain must encode the genes for the synthesis of F-pili. The strain chosen for these procedures was XL-1 Blue (see section **2.1**). The infectious M13 phage can be maintained in λ buffer (see section **2.6**) at 4°C for long periods. Since M13 does not kill the host, strains containing the recombinant M13 clones can also be maintained on NA plates. *E. coli* can be infected with M13 either by adding phage stock in λ buffer or by stabbing an inoculating loop into

a phage plaque and transferring the phage to NB + tetracycline containing XL-1 Blue, and incubating overnight at 37°C.

2.12.1.2 Cloning the 3.17kb *Hpa*I-*Hpa*I region from plasmid pLG701 into M13

As indicated in Chapter 4, the *Hpa*I-*Hpa*I region from plasmid pLG701 was found to complement temperature sensitivity in the *verA1* mutant, and was therefore cloned in both orientations into the SmaI site of M13mp18 by the methods described above. Following electroporation of the ligated DNA and incubation of the host strain in SOC for 60min, the 1ml SOC culture was added to 3ml of molten (55°C) soft nutrient agar (SNA). 12.5µl of 50mg/ml X-Gal (5-bromo-4-chloro-3-indolyl phosphate, dissolved in dimethyl formamide) and 12.5µl of 50mg/ml IPTG (isopropyl thio-β-D-galactoside, dissolved in H_2O) were added to the SNA, briefly vortexed and poured onto an LUA plate containing tetracycline. The overlayed plates were allowed to set for 5min and incubated overnight at 37°C. Recombinant M13 plaques were identified as colourless (white) plaques, compared with the self-ligated M13 which formed blue plaques. White plaques were screened to confirm successful construction of the recombinant plasmid by taking stabs of a selection of white plaques and infecting 5ml cultures of XL-1Blue (as indicated above), performing Mini-prep DNA extractions on the RF form of the phage and checking that the restriction profiles agreed with plasmid pLG702 (see **Chapter 3**). The M13 constructs with the two orientations of the 3.17kb insert were named pLG712f and pLG712r.

2.12.1.3 Preparation of nested deletions of pLG712f and pLG712r.

10x Klenow buffer

0.5M	Tris-HCl pH7.5
0.1M	$MgCl_2$
1mM	dithiothreitol

10x Thionucleotide mix

400µM of dATPαS, dGTPαS, dTTPαS and dCTPαS

6x ExonucleaseIII buffer

400mM	Tris-HCl, pH8.0
4mM	$MgCl_2$

0.3M NaCl

S1 nuclease buffer

150mM	Potassium acetate, pH4.6
1.25M	NaCl
5mM	$ZnSO_4$
25%$^{v}/_{v}$	Glycerol

S1 Stop mix

303mM	Tris base
50mM	EDTA

5x Ligase mix

250mM	Tris-HCl, pH7.6
50mM	$MgCl_2$
4.55mM	ATP
50mM	Dithiothreitol
2.5mM	Spermidine-HCl
50%$^{v}/_{v}$	Glycerol
0.13U/µl	T4 DNA ligase

By producing a series of nested deletions in plasmids pLG712f and pLG712r, the entire insert could be quickly and methodically sequenced, using a single primer. The method of Henikoff (1984) was used, and was carried out using the double stranded Nested Deletion Kit manufactured by Pharmacia (ref. 27-1691-01). **Fig. 15**, Chapter 4 summarises the stages involved in the preparation of the nested deletions. 3µg of pLG712f and pLG712r were digested with *Hind*III and ethanol precipitated. The DNA was resuspended in 20µl distilled H_2O and the *Hind*III cut ends filled in with thionucleotides to protect from Exonuclease III (*Exo*III) digestion as follows: 10µl of DNA (2µg) was mixed with 1µl of 10x Klenow buffer, 1µl 10x dNTPαS thionucleotide mix, 1µl Klenow fragment (5-10 units/µl) and incubated at 37°C for 15min. The enzyme was inactivated by incubating at 65°C for 20min and ethanol precipitated (see section 2.9.2.6). The DNA was resuspended in 17µl H_2O and digested with *Xba*I in a reaction volume of 20µl. 2x *Exo*III buffer was prepared by mixing 8µl of 6x *Exo*III buffer, 4µl 0.3M NaCl and 12µl H_2O, and the S1 nuclease reaction mixture prepared with 33µl S1 buffer, distilled H_2O and 1µl (40-60 units) S1 nuclease. 3µl aliquots of the S1 reaction mix were transferred to 19 tubes, labelled 0-18 and stored on ice.

Following *Xba*I digestion of plasmids pLG712f and pLG712r, 20µl of 2x *Exo*III buffer was added to each tube, mixed and the tubes preincubated at 30°C for 5min. A 2µl aliquot was removed from each plasmid-containing tube and transferred to the tubes labelled '0', containing S1 reaction mix, and stored on ice. The *Exo*III reactions were started by adding 1µl (90-130 units/µl) of *Exo*III to each plasmid-containing tube with continued incubation at 30°C. 2µl aliquots were removed at 3min intervals and transferred to the appropriate S1 nuclease tubes on ice. After completion of the *Exo*III stage, all the S1 nuclease tubes were incubated at room temperature for 30min to remove ssDNA ends resulting from the *Exo*III digestion. The S1 reactions were terminated by the addition of 1 µl S1 Stop mix. A 3 µl aliquot was taken from each time-point and loaded onto an

agarose gel to check that the reaction had been performed successfully. A stock ligase mix was prepared by adding 85µl 5x ligase mix, 85µl 25%$^{w}/_{v}$ polyethylene glycol and 195µl distilled H_2O. 17µl aliquots of the stock ligation mix were added to the remaining 3µl of S1 treated DNA from each time point and incubated for 2h, at room temperature. The ligated DNA was then transformed into XL-1 Blue by the $CaCl_2$ method, plaques isolated, M13RF DNA mini-prepped and the cloned inserts sized by restriction analysis and agarose gel electrophoresis. M13 phage stocks were prepared from suitable clones by stabbing the plaques with an inoculating wire and resuspending the phage in 1ml aliquots of λ buffer, which were then stored at 4°C.

2.12.1.4 Synthesis of single stranded DNA (ssDNA) template for sequencing the *verA* locus

Having selected a range of M13 clones containing suitable deletions that would permit sequencing of the cloned 3.17kb region, ssDNA was prepared from each of the clones to act as a template for the sequencing reactions. To infect *E. coli* with M13 for producing ssDNA, an overnight culture of XL-1 Blue was diluted 1:100 in 10ml prewarmed NB (37°C) and shaken vigorously for 30min. 600µl of phage stock was added to the culture and the culture then incubated for a further 5.5h. The cultures were then transferred to SS34 centrifuge tubes (Sorvall) and centrifuged at 14400g (12,500 RPM) for 10min, at 4°C. The supernatant was transferred to 35ml Corex™ tubes (DuPont Instruments), 2ml PEG/NaCl (10$^{w}/_{v}$ polyethylene glycol $(PEG)_{6000}$, 2.5M NaCl) added and incubated at 16°C for 15min, to precipitate the phage particles. The phage were harvested by centrifuging at 14400g for 10min, at 4°C. All traces of PEG/NaCl were removed by briefly centrifuging the tube and collecting the drops with a Gilson pipette, the phage pellet resuspended in 200µl 3M sodium acetate, pH5.3 and transferred to Eppendorf tubes. An equal volume of phenol / chloroform was added and the tubes very vigorously mixed by dragging the tubes over an Eppendorf rack, to disrupt the phage coats. Following centrifugation at 13000g for 5min, the upper phase was transferred to a fresh tube, and the residual DNA recovered by re-extraction with H_2O (see section 2.9.2.8.1). The DNA was phenol / chloroform extracted and back extracted for a second time and then ethanol precipitated. The dried DNA pellet was resuspended in 30µl H_2O, 2 µl checked on an agarose gel and then used in sequencing reactions. A concentration of 1 µg ssDNA /µl was normally obtained.

2.12.2 Labelling of DNA for sequencing

As indicated earlier, the dideoxy nucleoside triphosphate chain-terminating method of Sanger *et al* (1977) was used for labelling the DNA for sequencing. The solutions and buffers used to label the DNA are indicated below:

5x T7 DNA polymerase reaction buffer

200mM Tris-HCl, pH7.5
100mM $MgCl_2$
250mM NaCl

5x Labelling mix (dGTP)

7.5µM dGTP (Pharmacia)
7.5µM dCTP (Pharmacia)
7.5µM dTTP (Pharmacia)

ddGTP Termination mix

80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP,
8µM ddGTP
50mM NaCl

ddATP Termination mix

80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP,
8µM ddATP
50mM NaCl

ddTTP Termination mix

80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP,
8µM ddTTP
50mM NaCl

ddCTP Termination mix

80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP,
8µM ddCTP
50mM NaCl

Enzyme dilution buffer

10mM Tris-HCl, pH7.5
5mM Dithiothreitol (Sigma)
0.5mg/ml Bovine serum albumin (BSA)

Stop solution

95%$^{v}/_{v}$ Formamide
20mM EDTA
0.05%$^{w}/_{v}$ Bromophenol blue
0.05%$^{w}/_{v}$ Xylene cyanol FF

M13 Primer

5'-G T T T T C C C A G T C A C G A C-3'

2μg of template DNA was mixed with distilled H_2O to give a final volume of 7μl. 2 μl of 5x reaction buffer and 1μl of 0.5pmol/μl M13 forward primer were added to the template, the tube incubated at 65°C for 2min and then transferred to a beaker containing approximately 150ml of H_2O at 65°C, which was allowed to gradually cool to 30°C. The tubes were briefly centrifuged and then the following were added: 1μl 0.1M dithiothreitol, 2μl 5x labelling mix, 0.5μl (0.185MBq) [α-^{35}S]-dATP (Amersham International PLC) and 2μl (2 units) T7 DNA polymerase (Pharmacia), (stock enzyme diluted 1:8 in enzyme dilution buffer supplied with enzyme). The mixture was incubated at room temperature for 3min. 3.5μl aliquots were then transferred to 4 tubes at 37°C containing 2.5μl of the four termination mixes, and incubated for 5min. Finally, the reactions were stopped by the addition of 4μl of Stop mix, and frozen at -20°C until ready to load the sequencing gel.

2.12.3 Preparation of the denaturing sequencing gel

10x TBE buffer

0.89M	Tris base
0.89M	Boric acid
20mM	EDTA

6%$^{V}/_{V}$ Denaturing polyacrylamide gel mix

75g	Ultrapure urea (Serva)
7.5ml	10x TBE
22.5ml	40:0.08 Acrylamide : Bisacrylamide (National Diagnostics)

H_2O to 150ml. Filter and de-gas for 20min.

Add 0.7ml 10%$^{W}/_{V}$ ammonium persulphate (APS, Sigma) (stored at 4°C), followed by 48μl N,N,N',N',-tetramethylethylenediamine (TEMED, Sigma) (stored at 4°C) to be added just prior to casting the gel. This gel was sufficient to prepare a 30x40cm wedge gel with a thickness of 0.4mm at the top, and 1.2mm at the base. The sequencing apparatus and accessories used were for the S2 system, manufactured by Gibco-BRL. After pouring the gel mixture, two 0.4mm, 24-slot sharkstooth combs were inserted upside-down into the top of the gel prior to polymerisation. The gels were poured the day before use, and wrapped in Saran Wrap™ to prevent dehydration. Prior to assembling the gel in the electrophoresis unit, the combs were removed and deionized water run into the slot formed by the combs, to remove traces of unpolymerised acrylamide. The combs were then replaced, teeth facing into the gel, and the apparatus assembled. The upper and lower buffer chambers were filled with 0.5x TBE and the gel pre-run to raise the temperature to 50°C, using a BioRad 3000Xi power supply, set to deliver a constant power of 65W. When the temperature of the gel reached 50°C, the labelled DNA samples were denatured by heating to 75-80°C for 5min, and 3μl of each sample loaded, in the order A G T C. To

maximise the amount of sequence obtained from a given M13 clone, extension gels were routinely performed. This involved loading upto 6 samples (6 x 4) into the slots of one comb and running the gel until the bromophenol blue marker reached a distance of approximately 2cm above the base of the gel. The samples were boiled and loaded again, in exactly the same order in the other half of the gel. When the xylene cyanol FF dye reached the base of the gel, the power was switched off, the gel apparatus dismantled and the electrophoresis plates prised apart. During preparation of the plates, prior to casting the gel, one plate was treated with Acryl-Glide (Amresco) to ensure that the gel separated readily from the treated plate. The gel, supported by the untreated plate, was transferred to a large tray containing 2l 10%$^{v}/_{v}$ glacial acetic acid, 12%$^{v}/_{v}$ methanol and fixed for 20min. The fixer was discarded and replaced by a further 2l of fixer and treated for another 20min. After fixation, the gel was transferred to a sheet of 3MM chromatography paper (Whatman) and dried using a BioRad gel drier, programmed to dry at 80°C for 3h. The gel was then transferred to an autoradiograph cassette and exposed to Fuji RX film overnight. Typically, 320-350bp of sequence was reliably obtained per M13 clone.

2.13 P1 transduction

The methods for performing P1 transduction were taken from Silhavy *et al* (1984). Stocks of $P1_{vir}$ were kindly supplied by Dr R. Cooper, Dept of Biochemistry, Univ. of Leicester, UK.

2.13.1 Preparation of P1 phage stocks

5ml overnight cultures of the strains to be used for preparing P1 stocks were diluted 1:100 in 10ml fresh LUB containing 0.2%$^{w}/_{v}$ glucose and 5mM $MgCl_2$, and shaken vigorously for 30min at 37°C. 0.1ml of $P1_{vir}$ phage stock was added to the culture and shaken for a further 3h. The cultures were transferred to 35ml Corex™ tubes (DuPont Instruments), 100µl of chloroform added and vortexed vigorously. The tubes were then centrifuged at 10,000g (10,000 RPM) in a Sorvall SS34 rotor for 10min and the supernatant transferred to a sterile glass bottle. A further 100µl of chloroform was added, the bottle vortexed and the P1 suspension stored at 4°C until required.

2.13.2 Titration of P1 phage stocks

A 5ml overnight culture of W3110 was centrifuged at 3200g for 5min at room temperature, and the pellet resuspended in 2.5ml 10mM $MgSO_4$, 5mM $CaCl_2$. Two 100µl aliquots of resuspended W3110 were added to two 3ml aliquots of BTL agar containing 10mM $MgSO_4$, 5mM $CaCl_2$ and overlayed onto LUA plates. The plates were incubated at

42°C without their lids, to dry the agar surfaces. The P1 phage stocks were diluted in serial 10-fold dilutions from 10^{-1} to 10^{-8} by transferring 100µl of phage stock in 900µl λ buffer, mixing and transferring 100µl to the next tube containing 900µl λ buffer, and repeating this procedure for the remainder of the dilution series. For each transfer, a fresh, sterile Gilson P200 pipette tip was used. The LUA plates overlayed with BTL containing the indicator bacteria were divided into 6 sectors, and a duplicate 20µl drop of the dilutions ranging from 10^{-8} to 10^{-3} deposited on equivalent sectors of the 2 plates. After overnight incubation, the plaques were counted on the two plates and a mean value obtained from the highest dilutions giving countable plaques. Typically, titres of 10^{9}-10^{10} phage /ml were obtained.

2.13.3 P1 transduction

A 5ml overnight culture of the strain to be transduced was centrifuged at 3200g for 5min at room temperature, and the pellet resuspended in 2.5ml 10mM $MgSO_4$, 5mM $CaCl_2$. Five sterile Eppendorf tubes were prepared, containing the following:

Tube no.	Volume of cell suspension	Volume of P1 stock
1	0.1	0
2	0.1	10µl
3	0.1	50µl
4	0.1	100µl
5	0	100µl

Table 2. Volumes of bacterial suspension and P1 bacteriophage used in P1 transduction.

The tubes were incubated for 30min at 37°C, followed by the addition of 0.1ml 1M sodium citrate. Finally, the suspensions were inoculated onto LUA plates containing appropriate antibiotics for the selection of P1 transductants and incubated overnight.

2.14 Determination of the minimal inhibition concentration (MIC) of a drug

Overnight cultures of the strains to be tested were grown in 5ml M9 minimal medium. A series of 10 tubes were prepared for each strain to be tested, 9 with 5ml M9 medium and one with 10ml M9 medium, containing the highest concentration of drug to be tested. 5ml of the 10ml drug-containing medium was transferred to the second tube in the series, containing 5ml medium and mixed. A 5ml aliquot of this medium was transferred, using a fresh, sterile pipette to the third tube in the series and mixed as before. This procedure was continued to tube 8 in the series. Tubes 9 and 10 contained no drug.

20µl (~10^5 bacteria) of the bacterial culture was added to tubes 1 to 9, tube 10 remained uninoculated, to act as a sterility control. Tube 9 functioned as a growth control. The tubes were incubated overnight at 30°C.

2.15 Polymerase chain reaction (PCR)

10x PCR buffer

500mM	KCl
100mM	Tris-HCl, pH8.3
1mg/ml	Bovine serum albumin (DNAse free, Pharmacia)
40mM	$MgCl_2$

10x dNTP mix

10mM	Ultrapure dATP (Pharmacia)
10mM	Ultrapure dTTP (Pharmacia)
10mM	Ultrapure dCTP (Pharmacia)
10mM	Ultrapure dGTP (Pharmacia)

Both the 10x buffer and 10x nucleotide mix were stored at -20°C.

In a typical 10µl PCR reaction, the following constituents were added:

Template (10pg/µl)	1µl
1µM Primer 1	1µl
1µM Primer 2	1µl
10x dNTP mix	1µl
10x PCR buffer	1µl
H_2O	4µl
Taq DNA polymerase (Applied Biotechnology)	1µl

Overlay the reaction mixture with 50µl of mineral oil (Sigma).

The PCR reactions were performed in a Hybaid OmniGene thermal cycler. Conditions for individual experiments are indicated in relevant chapters. Following completion of the PCR reaction, 2µl of agarose sample buffer was added and the reaction mixture analysed by agarose gel electrophoresis.

2.16 SDS-Polyacrylamide gel electrophoresis

2.16.1 Preparation of Total protein extracts from *E. coli*

4x SDS PAGE Sample buffer

0.5M	Tris-HCl, pH6.8
4%$^{w}/_{v}$	SDS
20%$^{v}/_{v}$	Glycerol
1.43M	β-mercaptoethanol (Sigma)
0.1%$^{w}/_{v}$	Bromophenol blue (Sigma)

To prepare total protein extracts from *E. coli*, 1.0 A_{600} unit of culture was transferred to an Eppendorf tube and centrifuged at 13000g for 30sec. The supernatant was discarded and the pellet resuspended in 200µl 1x PBS. 50µl of 4x sample buffer was added to the suspended cells and the sample boiled prior to loading the gel. For a mini-PAGE gel, 10-15µl of sample was loaded; for a large gel, 25-30µl of sample was loaded.

2.16.2 Preparation of heat-stable protein fractions from *E. coli*

2.0 A_{600} units of culture were centrifuged at 13000g for 30s and the pellet resuspended in 200µl TEK buffer (50mM Tris-HCl, 1mM EDTA, 100mM KCl, pH8.0). The resuspended cells were boiled for 10min, centrifuged at room temperature for 5min and the supernatant transferred to a fresh tube. 50µl of 4x sample buffer was added and the samples boiled for 5min prior to loading 10-15µl on a mini-gel or 25-30µl on a large gel.

2.16.3 Preparation of SDS-polyacrylamide gels

The discontinuous buffering system of Laemmli (1970) was used. All gels were of 0.75mm thickness. Two systems were used for SDS-PAGE, a mini-gel system, Mini-Protean II (BioRad), which produced gels with 7.2x10.2cm dimensions and a large Protean II system producing gels with dimensions of 16x16cm. Recipes for different gel strengths using the two systems are indicated below. For mini-gels, a stacking gel of approximately 0.75cm depth was used; for the large gels, the stacking gel was increased to 1.5cm depth. During electrophoresis, mini-gels were run with a constant current of 30mA, large gels were run at 40mA, the system being cooled by passing tap water through the central core of the unit. In all cases, a 5%$^{v}/_{v}$ acrylamide stacking gel was used, the acrylamide mix was purchased in a ready mixed form (National Diagnostics), in the ratio of 30:0.8 acrylamide : bis-acrylamide.

2x Buffer A

0.75M	Tris-HCl, pH8.8
0.2%$^{w}/_{v}$	SDS

2x Buffer B

0.25M	Tris-HCl, pH6.8
0.2%$^{w}/_{v}$	SDS

10mg/ml Ammonium persulphate (APS, Sigma)

N,N,N',N',-tetramethylethylenediamine (TEMED, Sigma)

10x SDS PAGE running buffer

0.25M Tris
1.92M Glycine
1.9%$^{w}/_{v}$ SDS

Dissolve in distilled H_2O and check that the pH is between 8.3 and 8.6. Dilute 1:10 for use, with distilled H_2O.

	Mini-gel	**Large gel**
Distilled H_2O	650µl	6.5ml
Buffer B	1ml	10ml
30% Acrylamide mix*	350µl	3.5ml
10mg/ml APS	50µl	500µl
TEMED	4µl	40µl

Table 3. Ingredients for 5% stacking gels

Mini-gels				**Large gels**		
11%	**13%**	**14%**		**11%**	**13%**	**14%**
560µl	220µl	10µl	Distilled H_2O	2.8ml	1.1ml	50µl
2.7ml	2.7ml	2.7ml	Buffer A	13.5ml	13.5ml	13.5ml
2.0ml	2.34	2.55ml	30% Acrylamide mix*	10ml	11.7ml	12.75ml
190µl	190µl	190µl	10mg/ml APS	950µl	950µl	950µl
4µl	4µl	4µl	TEMED	75µl	75µl	75µl

Table 4. Ingredients for separating gels

* Acrylamide mix = 30:0.8 acrylamide : bis acrylamide

2.16.4 [^{14}C]-methylated protein molecular weight standards (Amersham International PLC)

Molecular weight (Daltons)	**Protein**
14,300	Lysozyme
30,000	Carbonic anhydrase, bovine erythrocytes
46,000*	Ovalbumin
69,000	Bovine serum albumin
97,400*	Phosphorylase-b, rabbit muscle
205,000	Myosin, rabbit muscle

*Ovalbumin often appears as two bands of 46,000 and 50,000Da, and phosphorylase-b often appears as two bands of 97,400 and 100,000Da. These markers were not boiled prior to loading on the gel. 5µl of markers were loaded onto mini-gels, 10µl on large gels.

2.16.5 Coomassie brilliant blue stainable protein molecular weight standards

MW-SDS-200 kit, Sigma.

Molecular weight (Daltons)	Protein
29,000	Carbonic anhydrase, bovine erythrocytes
45,000	Ovalbumin
66,000	Bovine plasma albumin
97,000	Phosphorylase-b, rabbit muscle
116,000	β-galactosidase, *E. coli*
205,000	Myosin, rabbit muscle

The stainable molecular weight standards were boiled prior to loading the gel. 5µl were loaded onto mini-gels, 10µl on large gels.

2.16.6 Coomassie brilliant blue staining of SDS-PAGE gels

Dissolve 0.25g of Coomassie brilliant blue R250 (Sigma) in 45ml methanol, 45ml H_2O and 10ml glacial acetic acid. The stain was filtered through Watman no.1 filter paper to remove residual particulate matter. Following electrophoresis, the gel was transferred to a dish containing the stain, and gently rocked. Mini-gels were stained for 60min and large gels for a minimum of 6h (preferably overnight). Following staining of the gel, the stain was returned to its storage bottle for reuse. The gels were destained in 40%$^{v}/_{v}$ methanol, 10%$^{v}/_{v}$ glacial acetic acid, rehydrated in distilled H_2O, photographed and dried onto 3MM chromatography paper (Whatman), using a BioRad gel drier, programmed to dry at 80°C for 90min.

2.16.7 Fluorography of [^{35}S]-labelled protein gels

Large gels were fixed in destain (40%$^{v}/_{v}$ methanol, 10%$^{v}/_{v}$ glacial acetic acid) for 45min, small gels for 20min, by gentle agitation. The gels were then washed in two changes of distilled H_2O for 45 and 20min respectively, followed by soaking the gels in 1M sodium salicylate solution (1M sodium salicylate, 5%$^{v}/_{v}$ methanol and 1%$^{v}/_{v}$ glycerol) for 60min. Finally the gels were transferred to 3MM chromatography paper (Whatman) and dried for 90min on a BioRad gel drier, at 80°C. The dried gels were exposed overnight to Fuji RX film.

2.17 [^{35}S]-Labelling of *E. coli* proteins, and the preparation of extracts of heat stable protein fractions.

5ml overnight cultures of *E. coli* were grown in M9 or TCG minimal medium at 30° C and diluted 1:100 in 75ml prewarmed minimal medium. The cultures were shaken vigorously until the A_{600} reached 0.4. Aliquots of the cultures were transferred to 50ml

flasks containing where appropriate, quantities of drugs, $CaCl_2$ or EGTA, and after 5min, 2.22MBq (60μCi) of [^{35}S-] L-methionine (Amersham International PLC) added. The cultures were incubated for a further 30min before being transferred to 55ml Falcon centrifuge tubes. The labelled cells were pelleted by centrifuging at 3600g for 10min, at 4° C, in a Heraeus Megafuge 1R centrifuge, resuspended in 1ml PBS buffer and transferred to Eppendorf tubes. The cells were washed by centrifuging at 13000g for 30s and resuspending the pellets in 1ml fresh PBS buffer 3 times. After the final spin, the pellets were resuspended in 500μl TEK buffer (50mM Tris-HCl, 1mM EDTA and 100mM KCl; pH8.0). 100μl of each sample was transferred to fresh tubes containing 0.25 volumes of 4x PAGE sample buffer (Total protein samples). Heat stable protein extracts were prepared by boiling the remaining cell suspensions for 10min, followed by centrifuging at 13000g for 10min. The supernatants were transferred to clean tubes and the heat stable proteins precipitated by the addition of $^1/_{10}$vol 100%$^w/_v$ trichloracetic acid (TCA), and incubated on ice for 30min. The tubes were centrifuged for 10min at 13000g, the supernatants discarded, and the pellets resuspended in 73μl PBS. The TCA was neutralised by the addition of 2μl saturated Tris and 4xPAGE buffer added to each sample to give a final volume of 100μl.

2.18 Western blotting

Electroblotting buffer

0.027M	Tris base
0.192M	Glycine

10x Ponceau S stain

2%$^w/_v$	Ponceau S (Sigma) (3-hydroxy-4-(2-sulpho-4-[4-sulphophenylazo]-phenylazo)-2,7-naphthalenedisulphonic acid)
30%$^w/_v$	Trichloracetic acid
30%$^w/_v$	Sulphosalicylic acid

Dissolve in H_2O. Store in foil-wrapped bottle

Blocking solution

0.3%$^w/_v$	Marvel™ fat-free dried milk powder (available from any supermarket)
0.05%$^v/_v$	Tween 20 (polyoxyethylene-sorbitan-monolaurate, Sigma)
20ml	1x PBS

Alkaline phosphatase buffer

100mM	Tris-HCl, pH9.5
100mM	NaCl
5mM	$MgCl_2$

NBT Stock

5%$^{w}/_{v}$ Nitroblue tetrazolium (Sigma) dissolved in 70%$^{v}/_{v}$ dimethyl formamide, stored at -20°C in a light-proof bottle.

BCIP Stock

5%$^{w}/_{v}$ 5-Bromo-4-chloro-3-indolyl phosphate (toluidine salt, Sigma) dissolved in 100% dimethyl formamide, stored at -20°C in a light-proof bottle.

Heat-stable protein extracts were prepared from *E. coli* as described in section 2.16.2. 25µl of each protein sample and 5µl of stainable markers were loaded onto a 13% acrylamide mini-gel. Following completion of electrophoresis, the mini-gel kit was dismantled and two sheets of 3MM chromatographic paper (Whatman), the same size as the gel, soaked in electroblotting buffer, placed over one surface of the gel, taking care to eliminate air bubbles. The gel, with the two pieces of 3MM paper on one surface, and a mini-gel glass plate on the other surface, was inverted and the glass plate removed. A sheet of 0.45µm pore-size nitrocellulose (Schleicher and Schuell) of the same dimensions as the gel, was soaked in electroblotting buffer and placed onto the gel, again avoiding the introduction of air bubbles. Finally, two more pieces of 3MM paper, soaked in buffer were placed over the nitrocellulose membrane and the 'sandwich' inserted into a BioRad mini-Western blotting apparatus, filled with electroblotting buffer, with the nitrocellulose covered surface of the gel facing towards the anode. The blotting apparatus was placed in a cold room at 4°C and a voltage of 60V passed through the unit for 30min. The unit was dismantled and successful transfer of proteins verified by staining briefly in 1x Ponceau S. The unbound stain was washed off with several changes of distilled H_2O. The membrane containing the stainable molecular weight standards was cut off, dried and preserved in the dark for later comparison. The remainder of the membrane was blocked for 60min at room temperature or overnight at 4°C in 20ml blocking solution. The blocking solution was replaced by 20ml blocking solution containing the appropriate dilution of rabbit derived primary antibody, as indicated in the results chapters, and incubated for 60min at room temperature. Unbound antibody was removed by three 10min washings in 50ml PBS. After the final wash, the membrane was incubated for 60min with 20ml blocking solution containing 1:1000 dilution of goat derived anti-rabbit alkaline phosphatase conjugate (DakoPats). Unbound conjugate was removed by washing three times in 50ml PBS. The membrane was then washed briefly in 50ml alkaline phosphatase buffer and the bound antibody visualised by the addition of 20ml alkaline phosphatase buffer containing 132µl NBT and 66µl BCIP stock solutions and incubated in the dark. When colour development

had been completed, the developer was discarded and the filter washed in 50ml PBS containing 1mM EDTA. The membrane was immediately photographed and stored in foil to prevent fading or discolouration.

2.19 Conjugation of the pCVD442 suicide vector from SM10λ*pir* into N43*verA1*

Overnight cultures of the donor strain SM10 λ*pir* containing the suicide plasmid pCVD442 and its derivatives, and the recipient strain N43*verA1* were diluted 1:100 in 5ml prewarmed NB and grown at 30°C to mid-exponential phase (A_{600}≈0.4) (the N43*verA1* culture was incubated 60min before the donor strains, due to its comparatively slow growth rate). 2ml of donor and recipient cultures were transferred to 25ml flasks and incubated by rocking gently at 45 RPM in an Innova 4000 incubator shaker for 90min at 30°C. 100µl of each mating mixture was diluted in 900µl PBS buffer, vortexed vigourously for 30s, and serial 10-fold dilutions prepared down to a dilution factor of 10^{-6}. To enumerate the number of recipients in the mating mixture, duplicate 10µl aliquots of each dilution were inoculated onto NA+streptomycin plates. Three 200µl aliquots of each neat mating mixture and 10^{-1} dilution were spread onto NA plates containing ampicillin and streptomycin, and incubated overnight at 30°C.

2.20 Assay of β-galactosidase

The method of assaying β-galactosidase in *E. coli* is taken from Miller (1972).

Z buffer

0.06M	$Na_2HPO_4.12H_2O$
0.04M	$NaH_2PO_4.2H_2O$
0.01M	KCl
1mM	$MgSO_4$
0.05M	β-mercaptoethanol

1M Na_2CO_3

ONPG solution

Dissolve 4mg/ml o-nitrophenyl β-D-galactopyranoside (Sigma) in M9 minimal medium and store in a sterile, foil-wrapped container.

0.5%$^{w}/_{v}$ SDS

When measuring the β-galactosidase activity of a culture, under a specific set of conditions, measurements were always made on triplicate cultures, grown in M9 medium to reduce error in assay measurements. Prior to commencing the experiments, tubes were prepared for performing the assays. Each tube contained 0.9ml Z buffer, 2 drops of chloroform and 2 drops 0.5%$^{w}/_{v}$ SDS from a Gilson P200 pipette. To assay β-

galactosidase activity, two 100µl aliquots were sampled from each culture, added to tubes containing Z buffer and vortexed very vigorously for 10secs. After equilibrating the tubes at 28°C for 5min, 200µl ONPG solution was added and upon appearance of a yellow colour (o-nitrophenol), the reaction was stopped by the addition of 0.5ml 1M Na_2CO_3. The time (t) taken for the colour to develop was recorded. The absorbance of the β-galactosidase assay tubes was measured simultaneously at 420 and 550nm on a Pharmacia Ultraspec III specrophotometer. In addition, a 700µl aliquot of each culture was also sampled, transferred to a 1ml cuvette, and the optical density of the culture measured at 600nm. The β-galactosidase activity of a culture was calculated using the following formula:

β-galactosidase activity (Miller units) = $1000 \times (A_{420}-(1.75 \times A_{550}))/OD_{600} \times Vol \times t$ (Miller, 1972)

where A_{420} represents the absorbance of the assay at 420nm;
A_{550} represents the absorbance of the assay at 550nm, and is used to correct for light scattering caused by the bacterial suspensions;
OD_{600} represents the optical density of the culture, measured at 600nm;
Vol. represents the volume of culture used in the assay, in ml; and
t represents the time in mins taken for the yellow colour to develop.

For each set of triplicate cultures, the A_{420} and A_{550} values were averaged, and the mean β-galactosidase activities and standard deviations calculated.

2.21 *In-vitro* gene expression experiments

2.21.1 *In-vitro* coupled transcription-translation experiments (Zubay)

A commercial *E. coli* S30 extract system, manufactured by Promega was used to perform the Zubay experiments. The DNA templates used were purified by CsCl isopycnic density gradient centrifugation (see section **2.9.2.2**), thereby avoiding the need for RNAse A treatment (highly detrimental to the procedure).

All the quantities recommended in the protocol supplied with the kit were reduced by 75%. The basic reaction was comprised as follows:

0.5µg	DNA template
5µl	Premix (lacking methionine) (Promega)
3.25µl	S30 extract (Promega)
0.25µl	[^{35}S-] L-methionine (37TBq/mmol)
	H_2O to 12.5µl.

Where required, everted inner membrane vesicles were added to the Zubay reaction mixture, and the amount of H_2O adjusted accordingly. The labelling reaction was incubated at 37°C for 60min, and then the proteins were precipitated by the addition of 50 µl acetone and incubated on ice for 15min. The tubes were centrifuged for 5min at 13000g, the supernatant discarded, and the pellet dried in a vacuum dessicator for 5min. Finally, the pellets were resuspended in 50µl 1x PAGE sample buffer, boiled for 5min and 10µl loaded on an 11% polyacrylamide gel. The gel was then processed for fluorography as described in section **2.16.7**.

2.21.2 Production of everted inner membrane vesicles

Membrane buffer A

50mM	Triethanolamine (Sigma) (**NB** Stock = 7.506M)
5mM	Magnesium acetate
1mM	Dithiothreitol (DTT)
pH7.5	

The triethanolamine and magnesium acetate were dissolved in 75% of the final volume of H_2O. The pH was adjusted to 7.5 with glacial acetic acid and the volume made up to 99% of the final desired volume. The buffer was autoclaved at 121°C for 15min, and after cooling to room temperature, filter sterilised 100mM DTT was added.

Membrane buffer B

50mM	Triethanolamine
10mM	EDTA
1mM	DTT
pH7.5	

This buffer was prepared as for membrane buffer A.

A 50ml culture of MC4100 was grown overnight in NB at 37°C. 10ml aliquots of overnight culture were transferred to four prewarmed 2 litre baffled flasks containing 1l brain-heart infusion, and shaken vigorously at 37°C until the A_{600} of the culture reached 0.6-0.8. The flasks were chilled rapidly by placing in large buckets containing a mixture of water and ice. The bacteria were harvested by centrifuging at 3580g (5,000RPM) in the GS3 rotor of a Sorvall Superspeed RC5B centrifuge (DuPont Instruments), at 4°C for 10min. The bacteria were washed by resuspending and pooling in 200ml ice cold PBS and

centrifuging at 8540g (10,000RPM) in a Sorval GSA centrifuge rotor, at 4°C for 10min. The supernatant was discarded, the pellet resuspended in a final volume of 60ml ice cold membrane buffer A and split into two aliquots in 50ml sterile beakers, kept on ice.

Each aliquot of cells was sonicated, using a 19mm probe attached to an MSE Soniprep sonicator set at medium power, with an amplitude of 3μm. The cells were sonicated for ten 30sec pulses, interspersed by ten 30sec pauses. During the entire procedure, the sonicated cells were kept chilled on ice. The sonicates were transferred to SS34 centrifuge tubes (DuPont Instruments) and centrifuged at 9220g (10,000RPM), at 4 °C for 10min. The supernatant was carefully decanted into fresh SS34 tubes on ice. Six SW40Ti (Beckman) thin walled, clear tubes were loaded with 2ml membrane buffer A containing 20%$^{w}/_{v}$ sucrose (sucrose cushions) and kept on ice. The sonicated extracts were carefully overlayed onto the surose cushions, loaded into the SW40Ti centrifuge rotor and centrifuged at 65650g (23,000RPM), 4°C for 60min in a Beckman L5-65 centrifuge. The supernatant was discarded and the 6 pellets resuspended and pooled in 1ml membrane buffer A.

Four sucrose step gradients were prepared in SW40Ti tubes, consisting of 1ml 60%, 2ml 55%, 2ml 50%, 2ml 45%, 2ml 40% and 2ml 35%$^{w}/_{v}$ sucrose in membrane buffer A, as follows:

%$^{w}/_{v}$ sucrose required	**Vol. of 60%$^{w}/_{v}$ sucrose (ml)**	**Vol. membrane buffer A (ml)**
55	9.17	0.83
50	8.33	1.67
45	7.5	2.5
40	6.67	3.33
35	5.83	4.17

Table 5. Quantities of 60%$^{w}/_{v}$ sucrose stock solution (dissolved in membrane buffer A) and diluted with membrane buffer A to obtain the required concentrations of sucrose for the step-gradients.

The different sucrose solutions were added using a Gilson P1000 pipette, with the end of the blue disposable tip cut off, to reduce the risk of disrupting the gradient. Marks were made on the sides of the tubes corresponding to the interface of each sucrose solution. The 1ml of resuspended membrane pellet was passed through a 5ml syringe attached to a narrow bore needle, to break up particulate matter and made up to a final volume of 4ml. 1ml was loaded onto each gradient and the gradients centrifuged overnight at 65650g, 4° C. The 40 and 45%$^{w}/_{v}$ sucrose fractions were collected and transferred to an SS34 tube

on ice. 3 volumes of membrane buffer B were added, mixed with the sucrose fractions and overlayed onto six 20%$^{w}/_{v}$ sucrose cushions. The tubes were centrifuged in the SW40Ti rotor for 2h at 65650g, the supernatant discarded and the pellets resuspended and pooled in 100µl membrane buffer A. The resuspended membranes were aliquoted in 5µl amounts and stored at -20°C.

2.21.3 *In vitro* gene expression using minicells

This method is taken from Stoker *et al* (1984).

2.21.3.1 Preparation of sucrose gradients

M9 minimal medium was added to 180g sucrose to obtain a final volume of 900ml. When the sucrose had completely dissolved, it was filter sterilised and 35ml aliquoted into autoclaved SS34 polycarbonate tubes. The tubes were frozen at -70°C for 60min and allowed to thaw overnight at 4°C. The resulting gradients ranged from 10-30%$^{w}/_{v}$ sucrose.

M9 medium + 30%$^{v}/_{v}$ glycerol.

Add 60ml glycerol to 100ml distilled H_2O and autoclave at 121°C for 15min. Add 40ml 5x M9 salts, 2ml 40%$^{w}/_{v}$ glucose, 10µg/ml thiamine and 10mM $MgSO_4$.

10.5%$^{w}/_{v}$ Methionine assay medium

Dissolve 5.25g Methionine assay medium (Difco) in 50ml distilled H_2O. Autoclave at 115°C for 20min.

2.21.3.2 Purification of minicells

400ml of overnight cultures of DS410 were grown in NB containing appropriate antibiotics, harvested by transferring to GS3 centrifuge pots (DuPont Instruments) and centrifuging in a Sorvall Superspeed RC5B centrifuge at 9150g (8,000RPM) at 4°C for 15min. The pellets were resuspended in 6ml M9 medium and transferred to a sterile SS34 tube, on ice. The thawed sucrose gradients were placed on ice and the resuspended cells carefully layered onto the top of 2 gradients per minicell preparation. The gradients were placed into an HB4 rotor (DuPont Instruments) and centrifuged at 3160g (5000RPM) for 18mins, at 4°C. Sterile Pasteur pipettes were used to recover the upper 65% of the minicell band that had formed; the larger normal cells, formed a pellet at the bottom of the tube. The minicells were transferred to a sterile SS34 tube and kept on ice. The volume of minicells was determined, and an equal volume of M9 medium added. The tubes were centrifuged at 10,000g (10,000 RPM) for 10min at 4°C. After discarding the supernatant,

the pellet was resuspended in 3ml M9 medium and a single gradient loaded and centrifuged as described above. Again, the upper 65% of the minicell band was recovered and transferred to a sterile SS34 tube, on ice. An equal volume of M9 medium was added and the absorbance measured at 600nm. The minicells were centrifuged at 10,000g for 10min, at 4°C. After discarding the supernatant, the pellets were resuspended in M9 medium containing 30%$^{v}/_{v}$ glycerol to obtain an A_{600} of 2.0 per ml for minicells containing high copy number plasmids and 5.0 per ml for low copy number plasmids. The minicells were stored at -70°C.

2.21.3.3 Labelling the minicells with [^{35}S]-methionine

The frozen stocks of minicells were thawed on ice and 100µl of minicells containing the plasmids of interest transferred to fresh tubes. The remainder of the minicells were refrozen without noticable loss of potency. The minicells were centrifuged at 13000g for 3min, the supernatant discarded and resuspended in 200µl M9 medium and 3µl methionine assay medium. The minicells were preincubated at 37°C for 60min to reduce possible background due to pre-existing mRNA. 1µl (0.555MBq) of [^{35}S]-methionine (Amersham International PLC) was added to each tube of minicells, mixed and incubated for a further 60min. The labelled minicells were centrifuged at 13000g for 3min, the supernatant discarded and the pellets resuspended in 200µl PBS. The minicells were again centrifuged for 3min, resuspended in 100µl PBS and 100µl 2x PAGE sample buffer, and boiled for 5min. 15µl of each minicell extract was loaded onto a 12% polyacrylamide gel and analysed by fluorography.

2.22 Photomicroscopy

Liquid cultures were examined by phase contrast at 1000x magnification, using a Zeiss Axoskop microscope with Leica 35RC camera body attached. Photographs were recorded on 400ASA Ilford HP5 35mm film.

2.23 Preparation of *E. coli* cells for electron microscopy

The preparation of the bacteria for electron microscopy was kindly performed by Mr S. Hyman, Electron Microscopy Laboratories, University of Leicester.

5ml cultures of the bacterial strains to be processed were grown overnight in NB at 30°C. Approximately 4.0 A_{600} units of each culture were centrifuged briefly at 13000g, the cells washed in 1ml PBS, and pelleted by brief centrifugation at 13000g. Fixation of the cells was performed according to the method of Todd (1986). The cells were resuspended in 0.5ml 2.5%$^{v}/_{v}$ glutaraldehyde (Sigma), 1.25%$^{v}/_{v}$ formaldehyde (Sigma),

0.3%$^{w}/_{v}$ $CaCl_2$ and 0.03%$^{w}/_{v}$ picric acid for 60min at room temperature. The cells were washed 3 times by resuspending in 1ml 0.1M sodium cacodylate pH7.4 and incubuating for 10min, followed by centrifuging at 13000g for 60sec. The cells were post-fixed by incubating in 0.5ml 1%$^{w}/_{v}$ OsO_4 buffered in 0.1M sodium cacodylate, pH7.4 for 60min. The cells were washed 3 times by resuspending in 1ml double distilled H_2O, and centrifuging for 10min at 13000g. After the final spin, the cells were embedded in 2%$^{w}/_{v}$ agar and the agar blocks cut into small pieces. The agar pieces were embedded in Spurrs resin using the standard method. Finally, sections were prepared and examined using a Jeol JEM-100CX transmission electron microscope.

Chapter 3
Isolation of a Verapamil Resistant Temperature Sensitive Mutant and the Cloning of the Complementary Genes

3.1 Introduction

A simple approach for the possible isolation of genes encoding voltage operated calcium channels (VOCCs) in *E. coli* is to treat the strain with calcium channel antagonists to which *E. coli* is known to be sensitive. Once the concentration of drug required to inhibit growth (the minimal inhibitory concentration, MIC) has been established, the bacteria can be grown in the presence of the drug at a concentration just in excess of the MIC, and resistant mutants isolated. Since some drug resistant mutants will result from mutations causing non-specific changes *eg* those affecting membrane permeability, a screen to eliminate, as far as possible, such mutants is required. In anticipation that the required mutants would be affected in an essential gene, screening for colonies unable to grow at 42°C in the absence of the drug is also neccessary after isolation of drug resistant mutants at 30°C. Once a temperature sensitive (*ts*) mutant has been isolated, it can be transformed with a plasmid library prepared from a *wt* strain and colonies that grow rapidly at 42°C selected. In principle, the gene(s) isolated in this way should be the wild type copy, complementing the mutant chromosomal gene. However, this should not be assumed, since the cloned gene(s) could be suppressing the mutant phenotype. Additional tests as indicated below, can be carried out to clarify this.

3.2 Selection of a suitable strain for isolating mutants.

Nakamura and Suganuma (1972) described a strain of *E. coli,* N43, that has a mutation, *acr*, encoding AcrA apparently localised in the cell envelope. Recent studies (Ma *et al.*, 1993; 1995) indicate that AcrA is an essential component of a drug detoxification pump traversing the inner and outer membrane. As a consequence of this mutation, the cells are more sensitive to several drugs. However, *acr*$^-$ mutants are also more sensitive to 0.3%($^w/_v$) SDS, thus, resistant bacteria can be tested for their ability to grow in the presence of 0.3%($^w/_v$) SDS, and SDS resistant mutants discarded as *acr*$^+$ revertants.

3.3 Selection of a suitable drug for the isolation of VOCC mutants.

As mentioned in **Chapter 1**, the commonest and most widely distributed type of calcium channel in eukaryotes is the L-type channel. Therefore, when looking for calcium channels in bacteria, it was regarded as most prudent to initially look for L-

type channels. A number of antagonists and agonists are available against L-type VOCCs (see **Table 6** for examples; see also **Chapter 1**).

CLASS	DRUG
Diphenylpiperazines	Cinnizarine
	Flunarizine
Phenylalkylamines	Verapamil
	Gallopamil
Benzothiazepines	Diltiazem
Dihydropyridines	Nifedipine
	Nitrendipine
	BAY K 8644

Table 6. Examples of some common L- type voltage operated calcium channel inhibitors and agonists.

3.4 Determination of MICs to VOCC inhibitors.

In this study, the drugs nifedipine, verapamil and diltiazem were tested for their ability to inhibit the growth of N43, since verapamil and diltiazem in particular have been reported to act upon different sites of classical L-type channels (Rosales and Brown, 1992). 100mM stocks of each drug were prepared, nifedipine in DMSO, verapamil and diltiazem in 50% ethanol/water, filter sterilised and kept in the dark. The drug concentrations used in the MIC assays ranged from 1.6mM to 3.125μM in 2-fold dilution steps, diluted in M9 medium. The MICs were determined after growth overnight at 30°C as described in Materials and Methods. In the case of nifedipine, the drug was found to be insoluble in M9 medium and failed to inhibit growth of N43. Further work with this drug was therefore abandoned. The results obtained indicated an MIC of 0.4mM for verapamil and 0.8mM for diltiazem.

3.5 Isolation of verapamil resistant mutants.

An overnight culture of N43, grown in M9 medium at 37°C was diluted 1:100 in 10ml prewarmed M9 medium and incubated at 37°C until the A_{600} reached 0.05. Verapamil was added to the culture to a final concentration of 0.8mM and the culture incubated for a further 3h at 30°C. 100μl aliquots of this culture were then inoculated onto M9 agar plates containing 0.8mM verapamil and incubated in the dark at 30°C for 3 days.

3.6 Testing of verapamil resistant mutants.

The additionally required properties of verapamil resistant mutants were that they should be SDS and temperature sensitive. Thus, all mutants growing on the verapamil plates were initially inoculated onto NA master plates and incubated overnight at 30°C. The next day, the colonies on the master plates were plated onto an M9 plate lacking verapamil, and a nutrient agar plate containing 0.3%$^{w}/_{v}$ SDS. The M9 plate was incubated at 42°C and the SDS plate at 30°C. Any mutants that failed to grow at 42°C or in the presence of SDS were selected for further analysis after confirming that they still demonstrated the same auxotrophies as N43. One such *ts* mutant, designated *verA1* which formed small colonies at 30°C was retained for further analysis.

3.7 Transformation by the gene library.

Fig. 9 summarises the stages in the isolation of the wild type genes complementing N43*verA1*. The gene library used for transformation into the mutant was constructed by M. Chen, in this laboratory (Chen *et al.*, 1991) and consists of 2-4kb Sau3A partially digested chromosomal DNA derived from the *E. coli* K12 strain MC4100, cloned into the *Bam*HI site within the tetracycline resistance gene of the low copy number vector pLG339 (see **Fig. 10**), also carrying a Kan^R gene (Stoker *et al.*, 1982). 200µl aliquots of frozen bacteria (DH5) containing the library were inoculated onto six 12cm NA + kanamycin plates and incubated overnight at 37°C. The reason for this approach was that in liquid media, there was a possibility that the gene(s) encoded by a complementary plasmid might have slowed the growth of the host strain, resulting in bacteria with non-inhibitory cloned genes out-growing the cells with the complementary gene(s) of interest. The next day, the bacteria were scraped from the plates and plasmid DNA was extracted using a scaled-up version of the small-scale plasmid purification method described in Materials and Methods.

The temperature sensitive mutant, *verA1* was transformed with 1µg of bank DNA per transformation by electroporation, and plated onto M9 + Km. One plate of mutant bacteria transformed with bank DNA was incubated at 30°C, together with all other control plates. The remaining plates of transformations were incubated at 42°C to identify ts^+ colonies.

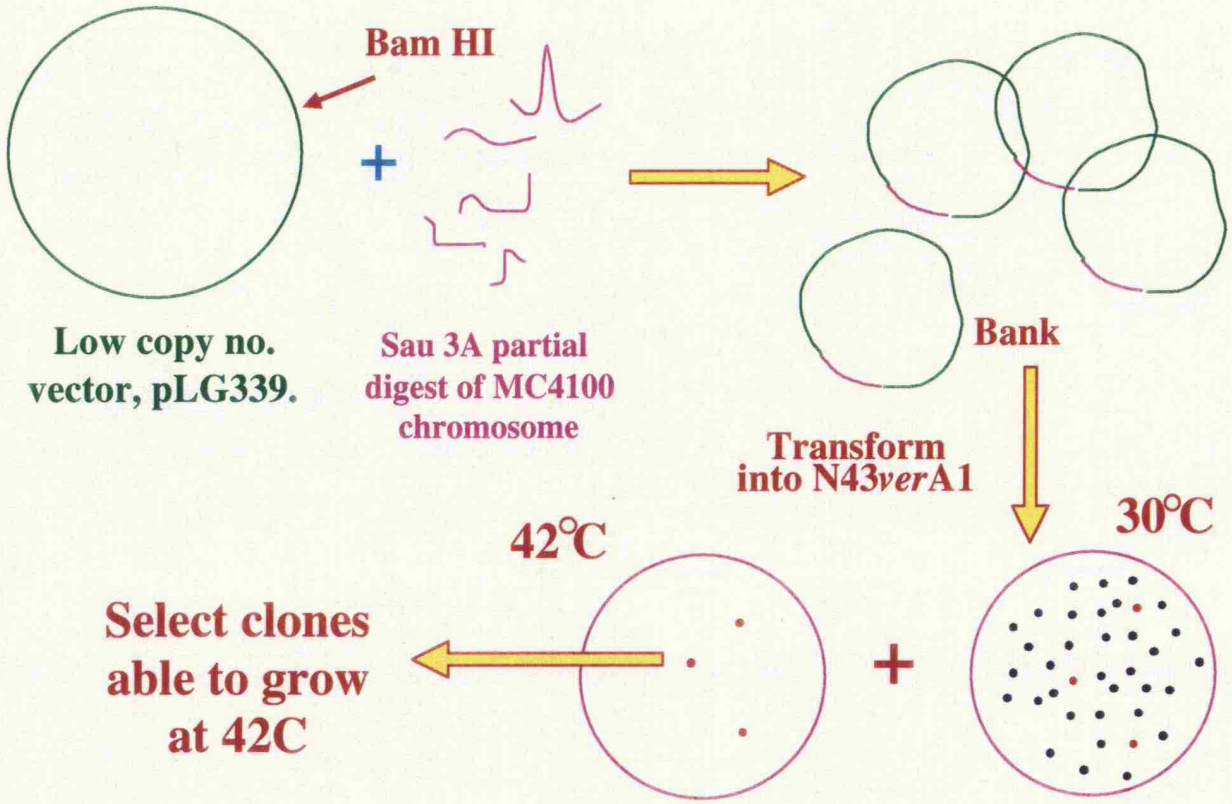


Fig. 9. Screening of the pLG339-based gene library to isolate clones capable of restoring growth of mutant N43*verA1*.

3.8 Isolation of pLG701 (*verA*$^+$) and confirmation of restoration of the wild type phenotype resulting from introduction of this complementary plasmid into the mutant.

Colonies found to be *ts*$^+$ were tested for their ability to grow on M9 plates containing 0.8mM verapamil, at 30°C. A *ts*$^+$ clone was tested, and found to be unable to grow in the presence of verapamil. Thus, the plasmid also restored verapamil sensitivity. Additionally, in order to confirm that the *ts*$^+$, verapamil^S phenotype was due to complementation and not to recombination or reversion, the recombinant plasmid was isolated from several transformants, retransformed into the *verA1* mutant at 30°C, and the phenotype of these transformants again tested. The results demonstrated once again that the plasmid restored both wild type characters. The complementing plasmid was designated pLG701. In a parallel experiment, plasmids from 5 **rapidly growing** clones of the mutant transformed with the gene bank and incubated at 30°C were isolated. In this case, isolation of the complementary clones was obtained without forced selection at 42°C. These plasmids were also shown to restore growth of N43*verA1* at 42°C, and were designated pLG701/2-6. In subsequent analyses, a probe derived from pLG701 was shown to hybridize with all complementing clones (see later).

3.9 Restriction enzyme analysis of the complementary plasmid, pLG701, that restores the *wt* phenotype in the verapamil resistant mutant.

pLG701 consists of pLG339 with a 5kb insert cloned into the *Bam*HI site within the Tc^r gene. Prior to sequencing of the insert, and to establish the nature of the complementing gene(s), restriction analysis of the plasmid was performed and a restriction map compiled. Single and double restriction enzyme digests of CsCl purified plasmid were performed as described in Materials and Methods. The results are shown in **Fig. 11.**

3.10 Subcloning of pLG701 to reduce the size of the cloned DNA fragment needed for complementation of the mutants.

In view of the relatively large size of the insert in pLG701, attempts were made to isolate a smaller subclone, still capable of complementing the *verA* mutation. Therefore, fragments of pLG701 were isolated by the use of specific restriction enzymes, subcloned into pLG339 and tested for their abilities to complement the mutation. As shown in **Fig. 12**, three constructs were prepared using pLG339 as the cloning vector: 1) the 4.2kb *Eco*RI-*Eco*RI fragment from pLG701 into the *Eco*RI site of pLG339 to form pLG718, 2) the 3.1kb *Hpa*I-*Hpa*I fragment into the blunted *Eco*RI

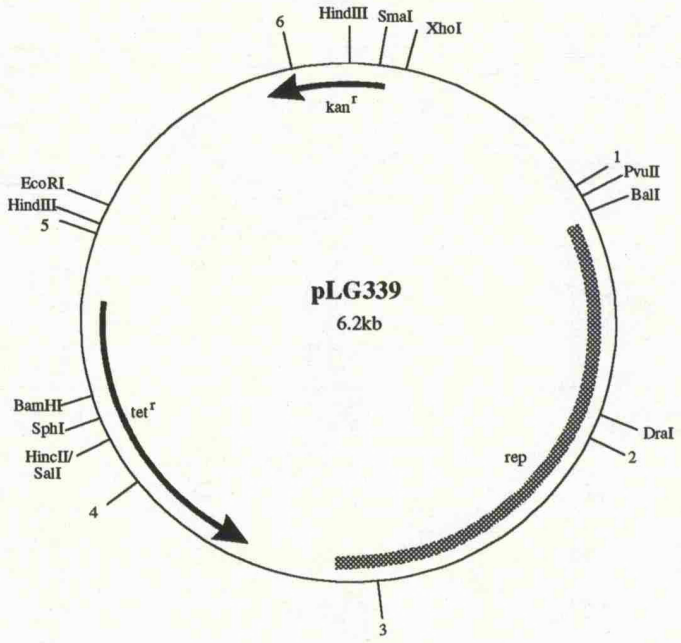


Fig 10. Plasmid pLG339, a low copy number vector (Stoker *et al.*, 1982), used for construction of the plasmid library (Chen *et al.*, 1991).

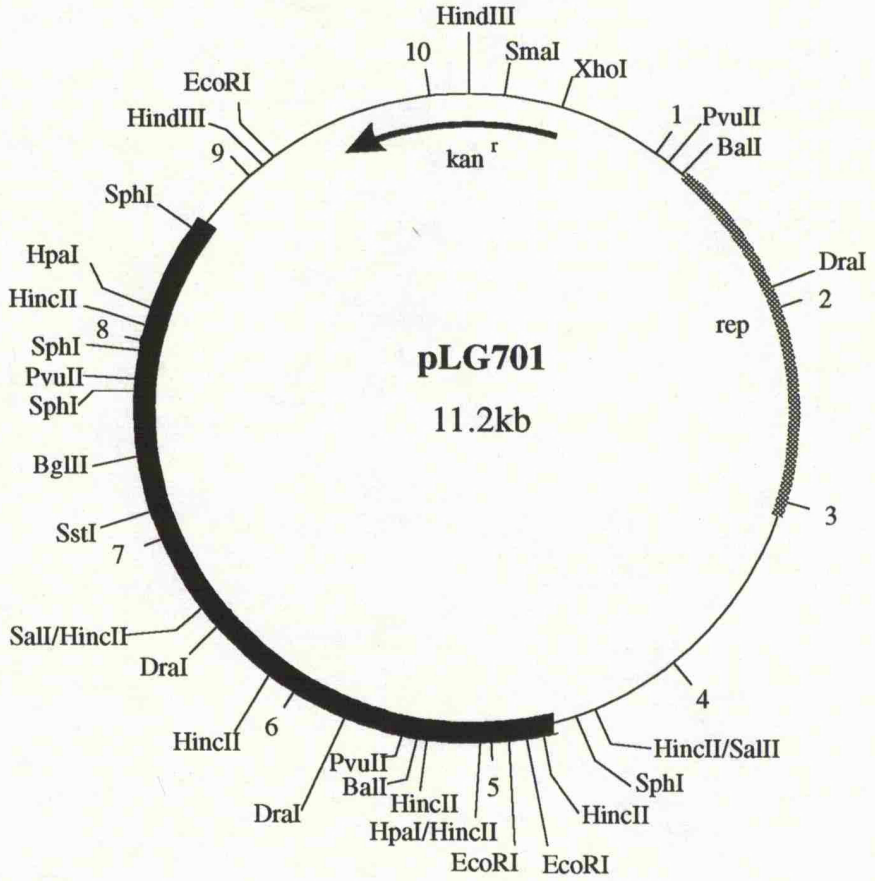


Fig 11. Plasmid pLG701, that complements the *verA* phenotype.

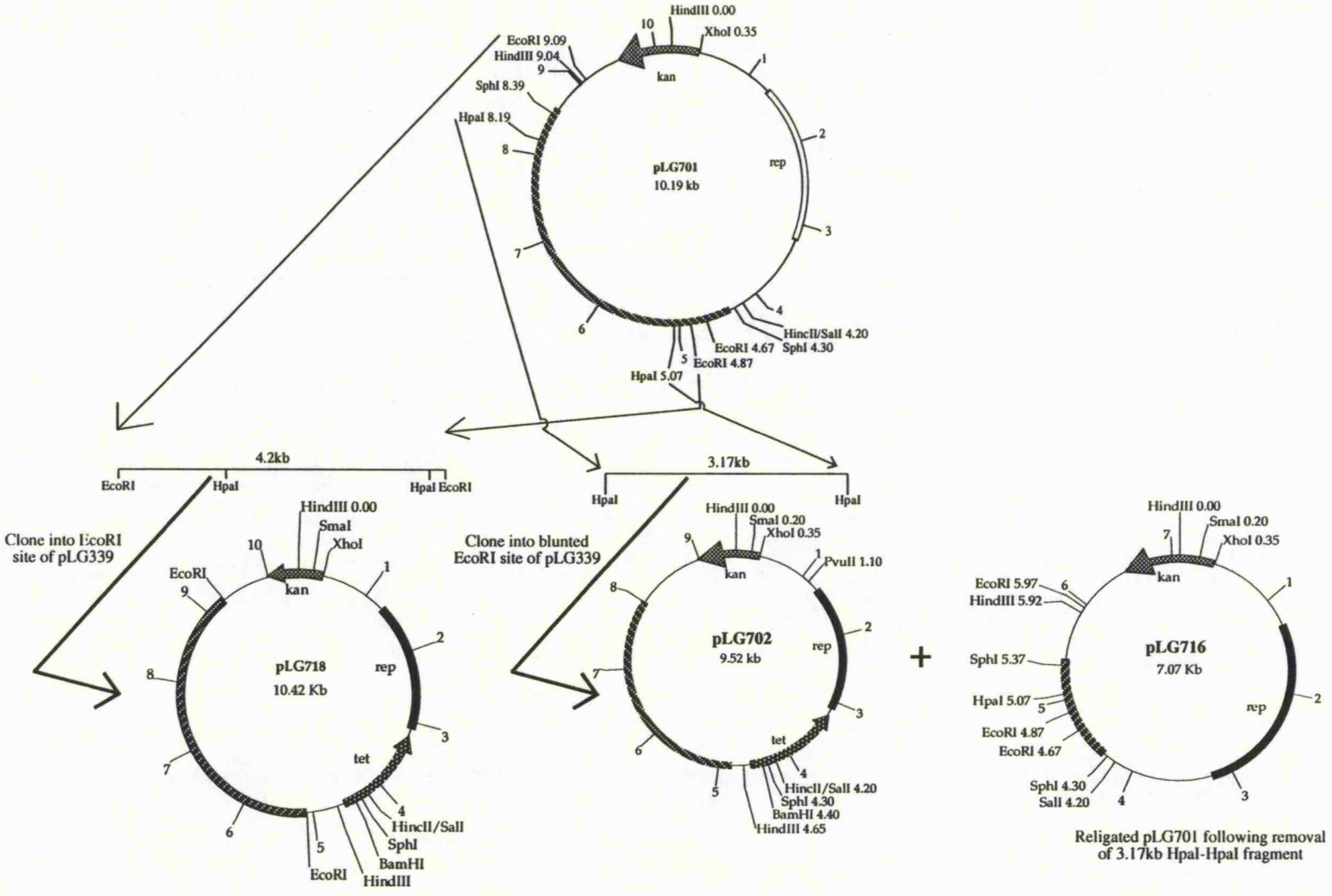


Fig 12. Subcloning of pLG701 to derive a smaller subclone that complements the temperature sensitive phenotype.

site of pLG339 to create pLG702, and 3) religation of the remaining plasmid resulting from the removal of the 3.1kb *Hpa*I fragment from pLG701, yielding plasmid pLG716.

3.10.1 Subcloning of the 4kb *Eco*RI-*Eco*RI fragment from pLG701

5μg of pLG701 was digested with *Eco*RI (Gibco-BRL) to yield restriction fragments of 5.8, 4 and 0.2kb. The digested DNA fragments were separated by agarose gel electrophoresis, the 4kb fragment excised from the gel and recovered by the Qiaex™ technique (see **Chapter 2**, Materials and Methods). 5μg of pLG339 was digested with *Eco*RI and dephosphorylated using calf intestinal alkaline phosphatase (CIP) again, as described in Materials and Methods. Vector and insert DNA were ligated together according to the method described in **Chapter 2**, generating pLG718, and transformed into the *verA1* mutant by electroporation. The transformed cells were plated onto two M9+Km plates and tested for growth at 30°C and 42°C (see below).

3.10.2 Construction of a deletion of pLG701 and subcloning of the 3.1kb *Hpa*I-*Hpa*I fragment from pLG701

5μg of pLG701 was digested with *Hpa*I (Gibco-BRL) and the two fragments of 3.1 and 7.1kb separated by agarose gel electrophoresis. The two bands were excised from the gel and purified by the Qiaex™ method. The 7.1kb DNA fragment which consists of pLG701 with the *Hpa*I fragment deleted, was religated to itself to form pLG716. The 3.17kb *Hpa*I fragment was cloned into the blunted *Eco*RI site of pLG339, resulting in pLG702. These constructs were transformed into the *verA1* mutant and tested for their ability to restore growth at 42°C. Both pLG718 and pLG702 restored growth at 42°C, whereas pLG716 failed to complement the *ts* phenotype. It was therefore concluded that the gene(s) complementing the mutation must be located within the 3.17kb *Hpa*I-*Hpa*I fragment of pLG701. However, an interesting observation was made regarding the degree of complementation conferred by pLG702, such that restoration of growth of the *verA* mutant at 42°C was not as effective when compared with pLG701 or pLG718. At this stage, complementation analysis of verapamil resistance and reduced growth rate of the *verA1* mutant was not determined but this will be described in detail in **Chapter 7.3**.

3.11 Mapping of the cloned region of pLG701 on the *E. coli* chromosome by Southern blot analysis.

A simple method for mapping gene(s) of interest onto the *E. coli* chromosome makes use of the restriction map and corresponding λ bacteriophage library constructed by Kohara *et al* (1987), combined with Southern blot analysis to identify

the size of the restriction fragments that hybridize with a probe derived from the gene(s) of interest.

3.11.1 Southern blot analysis

The procedure employed for performing the Southern blot analysis involved the use of a non-radioactive system (Boehringer-Mannheim) as described in Materials and Methods. The probe used for the mapping experiment was the 2.4kb *Pvu*II-*Pvu*II fragment from the central part of the insert of pLG701 (see **Fig. 11**) and labelled with digoxygenin-11-dUTP, as described in Materials and Methods. The labelled probe was purified and used at a final concentration of 0.05μg/ml.

The chromosomal DNA for the blot was prepared from *E. coli* MC4100 according to the procedure in Materials and Methods. Aliquots of 5μg of chromosomal DNA were digested with eight restriction endonucleases and 2μg of pLG701 digested with *Pvu*II. The digestions were conducted for 2h to ensure that the DNA was completely digested. The eight chromosomal digests, pLG701 digested with *Pvu*II, and 1μg λ*Hind*III molecular weight markers were loaded onto a 0.8%$^{w}/_{v}$ agarose gel and electrophoresed in the absence of ethidium bromide. Following electrophoresis, the gel was stained in 1x TAE buffer containing 10μg/ml ethidium bromide. The DNA was then denatured and neutralised, followed by overnight transfer onto Hybond N^{TM} nylon membrane (Amersham International PLC). Detection of the probe was performed as described in Materials and Methods. By calculating the M_r of the DNA fragments detected by the probe (see **Fig. 13**), and comparing these fragments with the Kohara restriction map (not shown), two possible loci were identified, corresponding to map locations 8.8' and 27.25' on the *E. coli* linkage map. λ bacteriophages from the Kohara miniset library corresponding to these two chromosomal locations were identified as follows: clone 141 (6E2) from location 8.8', and 250 (4D8) and 251 (3D5) from location 27.25'.

3.12 Confirmation of the chromosomal map locus of pLG701 by hybridization of the 2.4kb *Pvu*II-*Pvu*II probe to the Kohara λ phages

DNA from the above λ bacteriophages and λ DNA from an unrelated Kohara clone, λ41, as a negative control, were prepared from the Kohara library as described in Materials and Methods. In addition, five other plasmids, pLG701/2-6 that were isolated during the screening of the pLG339 chromosomal library for fast growth at 30°C and shown to complement the *verA* phenotype (see section **3.8** above) were tested for homology. As a positive control, pLG701 was used. The probe was found to hybridize with the λ clones 250 and 251, and all the plasmids that complement the slow growth mutant phenotype of N43verA1, pLG701/1-6. This therefore confirms the

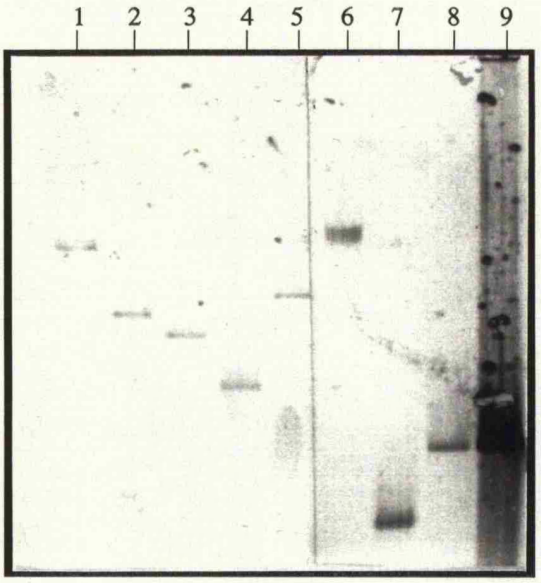


Fig 13. Southern blot of chromosomal DNA extracted from *E. coli* MC4100. Chromosomal DNA from *E. coli* MC4100 was digested with the following enzymes: *Bam*HI, lane 1; *Bgl*I, lane 2; *Eco*RI, lane 3; *Eco*RV, lane 4; *Hind*III, lane 5; *Kpn*I, lane 6; *Pst*I, lane 7; *Pvu*II, lane 8; and pLG701 digested with *Pvu*II, lane 9. The probe consisted of the 2.4kb *Pvu*II-*Pvu*II fragment derived from pLG701 and was labelled with digoxygenin as described in Materials and Methods.

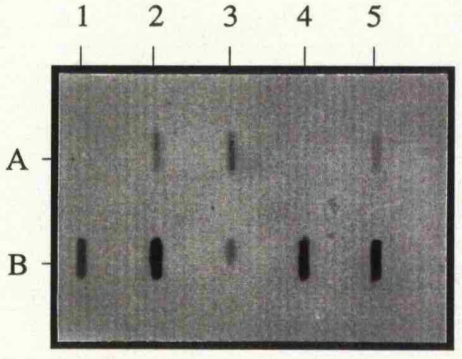


Fig 14. Slot blot hybridization of Kohara λ clones and complementing plasmid clones pLG701/1-6. Sample order is: λ141, A1; λ250, A2; λ251, A3; λ41, A4; pLG701/1, A5; pLG701/2, B1; pLG701/3, B2; pLG701/4, B3; pLG701/5, B4; and pLG701/6, B5. The probe used was the 2.4kb *Pvu*II-*Pvu*II probe, also used in **Fig. 13**. The clone containing pLG701/1 was isolated by initial selection at 42°C, whilst clones carrying pLG701/2-6 were initially selected as rapid-growing colonies, growing at 30°C.

position of the *verA* locus as being at 27.25' on the *E. coli* chromosome, and that a specific gene or set of genes is required for complementation.

3.13 Confirmation of the position of the *verA* locus on the *E. coli* chromosome by P1 transduction.

Having confirmed the position of the complementing fragment carried by pLG701 on the physical map of the *E. coli* chromosome, it was neccessary to determine using genetic analysis, whether the region also corresponded to the location of the *verA* mutation itself. Carol Gross has constructed a library of *E. coli* strains with transposon Tn10 linked to antibiotic resistance markers, located at numerous positions on the chromosome. Growing bacteriophage P1 on *wt* strains containing Tn10 close to the position on the physical map identified in the previous section, followed by transduction into the *verA* mutant, should allow the demonstration of linkage if indeed *verA* is present at this locus. Following selection for antibiotic resistant transductants, and scoring for the proportion of antibiotic resistant transductants that have become ts^+, it is possible to estimate the genetic distance between the transposon and the mutation. Similarly, in a reciprocal test, P1 phage subsequently grown on Tn10-containing *ts* mutants, when transduced into the *wt* strain, N43, should yield exactly the same proportion of drug resistant transductants that become temperature sensitive as a result of co-transducing the mutation. Methodology for the preparation of $P1_{vir}$ phage and production of the transducing phages is described in Silhavy *et al* (1984) and in Materials and Methods. The source strains supplied by Carol Gross were *E. coli* 12016, which contains a Tn10 transposon at 26.75' on the chromosome, and 12169 which has a Tn10 transposon at 27.25'. P1 phage grown on these strains was used to transduce the mutant. Tetracycline resistant colonies were tested for their ability to grow on M9 agar at 42°C. In a reciprocal experiment, a culture of the mutant that had acquired tetracycline resistance (Tn10 at 26.75') but was still unable to grow at 42°C (*ie* was still *ts*), was infected with $P1_{vir}$ and the progeny phage, $P1_{verA1}$ used to infect N43. These tetracycline resistant transductants were tested for their ability to grow at 42°C.

In both experiments, the proportion of tetracycline resistant ts^+ colonies, as a proportion of the total number of tetracycline resistant colonies, was determined. The results shown in Table 7 indicate that by both procedures, $verA^{ts}$ was closely linked to the 27 min region of the chromosome. In fact, co-transduction of *verA1*, *ts* with Tn10 placed at 27.25 min was 100% (38 out of 38). Moreover, when all the transductants were tested for verapamil resistance, this was found to be 100% linked to the ts^- character (data not shown). These results clearly indicate that temperature sensitivity and verapamil resistance are due to a single mutation. In addition, the apparent

coincidence of the genetic location of the *verA* mutation and the physical location of the DNA carried by pLG701 strongly suggest that the plasmid carries the wild type *verA* locus rather than a suppressor.

P1 phage source strain	Phenotype of N43*verA1* transductants		
	Tet^r *ts*$^+$	**Tet^r *ts***	**Total transductants (n)**
P1$_{12016}$ *verA*+	30 (61%)	19 (39%)	49
P1$_{12169}$ *verA*+	38 (100%)	0 (0%)	38
	Phenotype of N43 transductants		
P1$_{verA1}$ Tn10	7 (37%)	12 (63%)	19

Table 7. Summary of the results from the P1 transduction experiments, confirming the location of the *verA* locus on the *E. coli* chromosome.

Bacteriophage P1 was initially grown on *verA*$^+$ strains containing the transposon, Tn10 at 26.75 min (strain 12016) and 27.25 min (strain 12169) on the *E. coli* chromosome. The resulting phage progeny were then used to infect N43*verA1* and the percentage of *ts*$^+$ tetracycline resistant transductants determined. In a reciprocal experiment, P1 was grown on N43*verA1* containing Tn10 at 26.75' on the chromosome, that had not acquired the *wt verA*, allele following P1 transduction, and these progeny phage were then used to infect N43. The percentage of tetracycline resistant, *ts* N43 colonies resulting from this transduction was again determined.

Chapter 4
Sequencing and Sequence Analysis of the *verA* Locus

4.1 Introduction

In the previous chapter, plasmid pLG701, isolated from a plasmid library, and its smaller subclone, pLG702 (see **Fig. 12**) were shown to restore growth of N43*verA1* on M9 minimal medium plates at 42°C. It was however noted that growth at 42°C with N43*verA1* was significantly less well restored in the presence of pLG702, than when compared with the larger plasmid, pLG701. However, it was decided that useful and important information could be obtained by sequencing the insert contained within pLG702. The *Hpa*I-*Hpa*I region from pLG701 that was cloned into pLG339 to obtain pLG702, was therefore cloned into M13mp19, in both orientations. The integrity of the insert contained within M13 was checked prior to the construction of a series of nested deletions. Sequencing of the insert corresponding to pLG702 (see section **3.10.2**) revealed a 3117bp fragment of DNA encoding 2 open reading frames (ORFs) and potentially the 3'-end of a third. At the time of sequencing, no identity was available from the databases, regarding the two complete and the one incomplete ORF. Therefore, by looking for distinctive features within the DNA and predicted peptide sequences, hypotheses were raised in order to postulate a role for the genes constituting the *verA* locus.

4.2 Subcloning of the *Hpa*I-*Hpa*I region from pLG701 into bacteriophage M13.

Plasmid pLG701 was digested with *Hpa*I as described in section **3.10.2**, and the 3.1kb fragment recovered by Qiaex™ treatment of the solubilized agarose block containing the DNA (see Materials and Methods). Bacteriophage M13mp19 (Messing, 1981), was digested with SmaI and ligated with the *Hpa*I-*Hpa*I fragment in both orientations. The ligations were transformed into *E. coli* XL-1 Blue by electroporation. Recombinant clones containing the 3.1kb insert were screened by using blue/white plaque selection (see Materials and Methods). It was noted that the white plaques formed two discrete populations of small and large plaques. A selection of each type was picked, the DNA extracted and purified, and checked by restriction enzyme analysis. It was found that the large plaques contained plasmids with inserts considerably less than 3.1kb whereas the small plaques gave rise to plasmids containing inserts of the correct size. This indicated that one or more of the genes constituting *verA* might be toxic in high copy number. M13mp19 clones containing the 3.1kb insert in both orientations were obtained, and designated pLG712f and pLG712r.

4.3 Confirmation of the integrity of the inserts cloned into M13.

It is recognised that cloning into M13 can occasionally result in partial deletions or rearrangements of the insert. In order to check that no changes had occurred in this case, the entire inserts from both the pLG712f and pLG712r constructs were recovered by digesting the plasmids with EcoRI and BamHI, and cloning back into pLG339 that had also been digested with these enzymes. The resulting plasmids were then transformed by electroporation into N43*verA1* and tested for their ability to restore growth at 42°C. Complementation was clearly demonstrated and the M13 clones were therefore considered to be intact.

4.4 Preparation of nested deletions of the M13 clones.

The method of Henikoff (1984) was used to prepare the nested deletions of the pLG712f and pLG712r containing the *verA* locus in both orientations. The Nested Deletion kit manufactured by Pharmacia was used to generate the nested deletions required to sequence the entire 3.1kb insert. Details of the method are described in Materials and Methods. **Fig. 15** summarises the procedure used and **Fig. 16** shows the result of this procedure. The ligated DNA from selected time-points was transformed into *E. coli* XL-1 Blue by the calcium chloride method (see Materials and Methods). The pLG712 nested deletions from a given time-point contained inserts of widely differing sizes. Thus, a small number of plaques from each transformation were selected at random, inoculated into cultures of *E. coli* XL-1 Blue, small-scale preparations of DNA obtained, and the sizes of the inserts determined by restriction enzyme digestion and agarose gel electrophoresis. A series of pLG712f and pLG712r clones were identified where the insert was found to be progressively deleted in approximately 300bp steps.

4.5 Sequencing of *verA*.

M13 single stranded DNA from each of the selected clones indicated above, was prepared as described in Materials and Methods, and labelled by means of the dideoxy nucleoside chain-terminating method (Sanger *et al.*, 1977), using the M13 forward sequencing primer (see Materials and Methods). The DNA, labelled with α-[^{35}S]-dATP (15TBq/mmol, Amersham International PLC), was separated by electrophoresis using 6% polyacrylamide wedge extension gels, buffered with 0.5x concentration of TBE using the BRL-Gibco S2 electrophoresis apparatus (see Materials and Methods). Typically, 320-350bp of sequence was reliably obtained from individual clones. Sequence analysis was performed and processed using the Genetics Computer Group (1991)

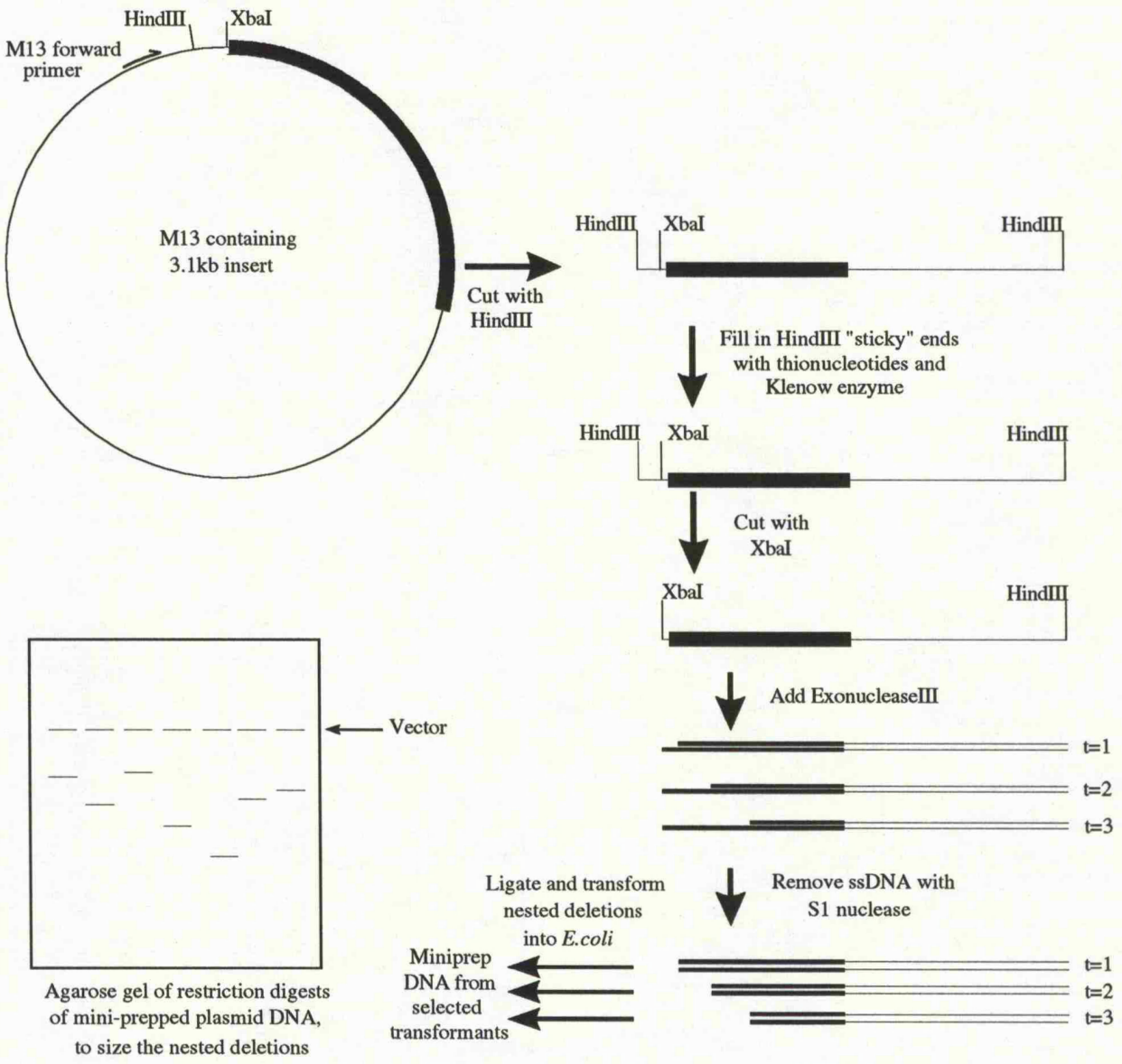


Fig 15. Diagram illustrating the stages involved in the preparation of nested deletions (Henikoff, 1984) for the sequencing of the genes constituting the *verA* locus.

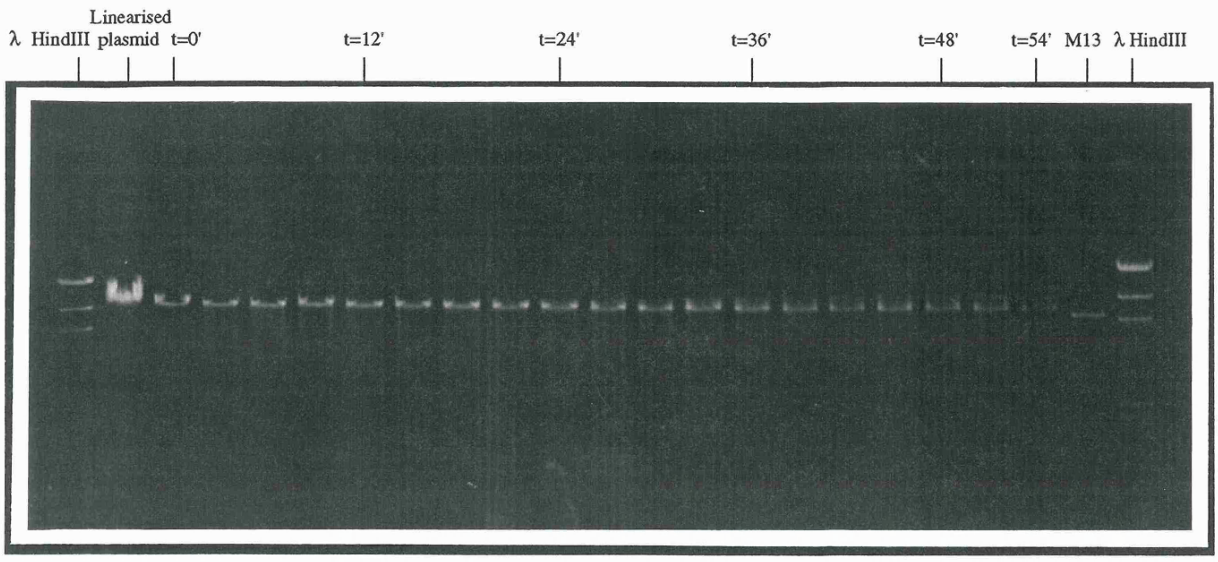


Fig 16. Nested deletions of M13mp18 containing the 3.1kb *Hpa*I-*Hpa*I insert subcloned from pLG701. The plasmid was first digested with *Hind*III and the ends filled in and protected with thionucleotides. The linearized DNA was then digested with *Xba*I and treated with Exonuclease III (*Exo*III). Samples of the *Exo*III-treated DNA were removed at 3 minute intervals and, following removal of single stranded DNA by S1 nuclease, some of each sample was loaded onto an 0.8% agarose gel to verify that the reaction had taken place successfully. Lane 1: λ*Hind*III molecular weight markers; lane 2: linearized, untreated DNA control; lane 3: commencement of the nested deletion procedure (time = 0); lanes 4-21: samples of the nested deletion reaction taken at 3 minute intervals; lane 22: linearized M13, digested with *Xba*I and lane 23: λ*Hind*III molecular weight markers. The λ*Hind*III molecular weight markers visible in this figure were 23.13kb, 9.416kb, 6.557kb, 4.361kb, 2.322kb and 2.027kb.

version 7, suite of molecular biology programs. Sequencing of both the M13mp18 and mp19 clones enabled both strands of the insert to be reliably sequenced. The DNA and predicted peptide sequences obtained are shown below. The data presented also includes additional sequence (bp 1-768) obtained subsequently from the EMBL database, for completeness. Distinctive features have been indicated such as promoters (-10 and -35), ribosome binding sites (RBS), a potential **Fnr** (Eighmeier *et al.*, 1989) binding site upstream of ORF 3, a putative *rho*-independent transcription terminator between ORFs 2 and 3, and a potential nucleoside binding site in ORF 2, identified using the Motifs program supplied with the GCG molecular biology program. This motif has since been corroborated (Ueguchi and Ito, 1992).

```
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       T  S  K  G  A  N  M  A  S  Y  Y  F  Y  L  Q  A  M
                                                    <--tdk

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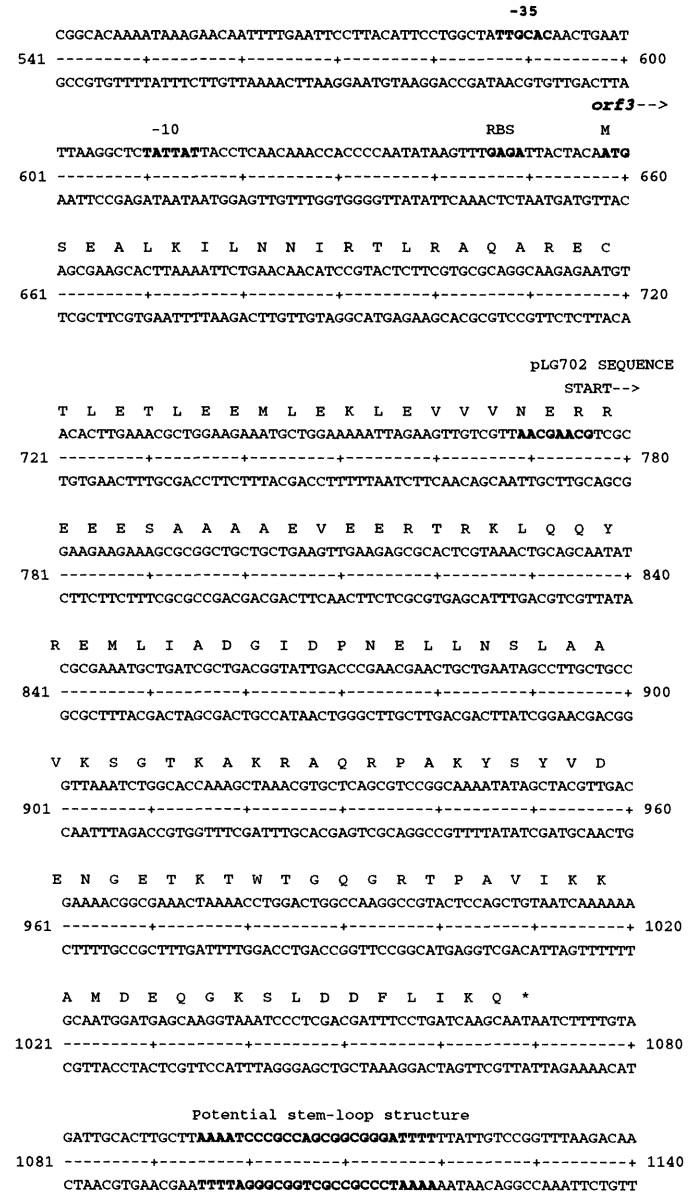
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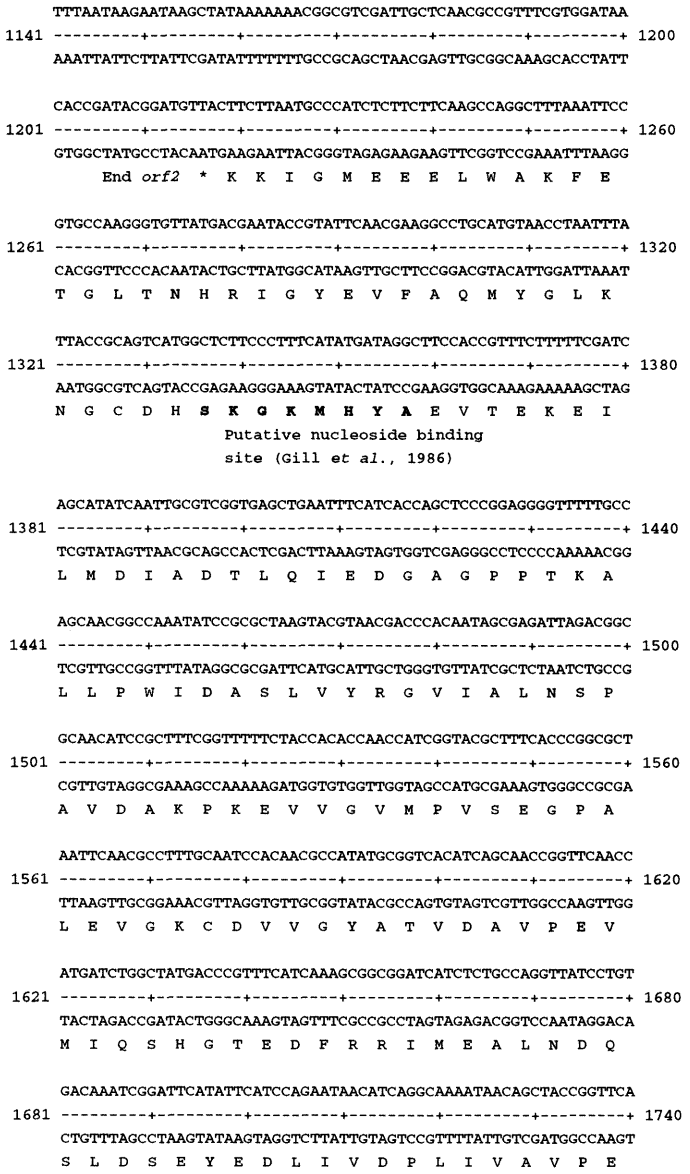
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Putative nucleoside binding
site (Gill et al., 1986)
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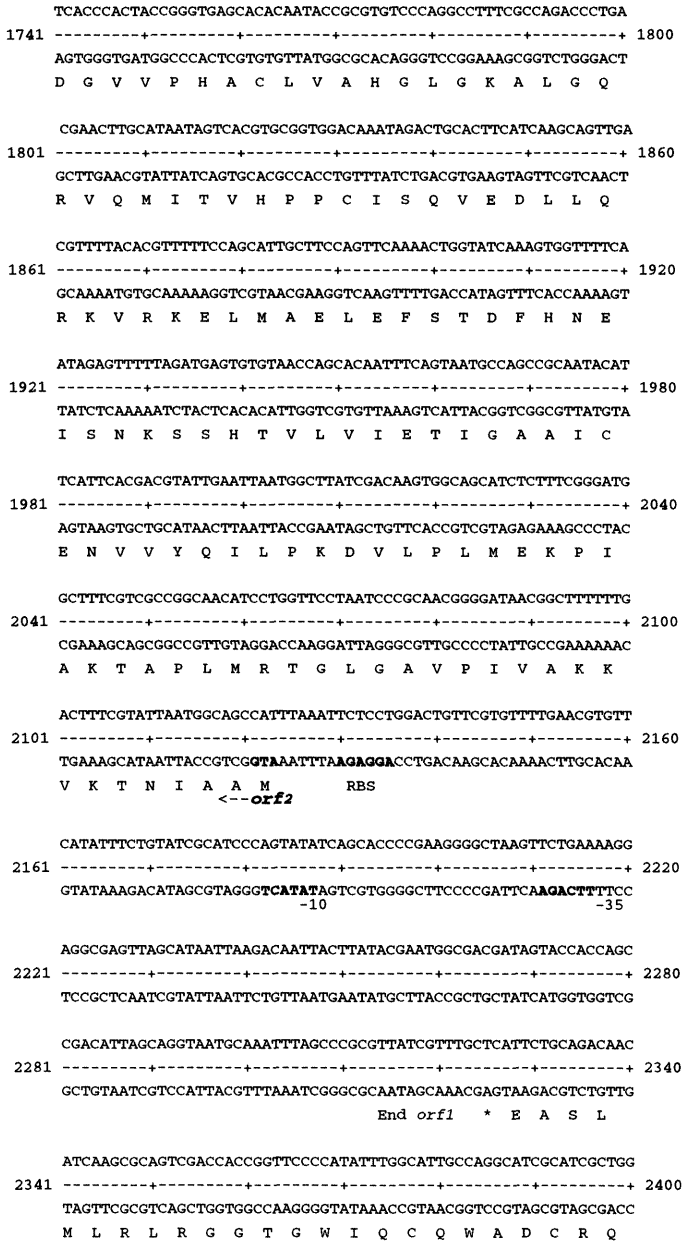
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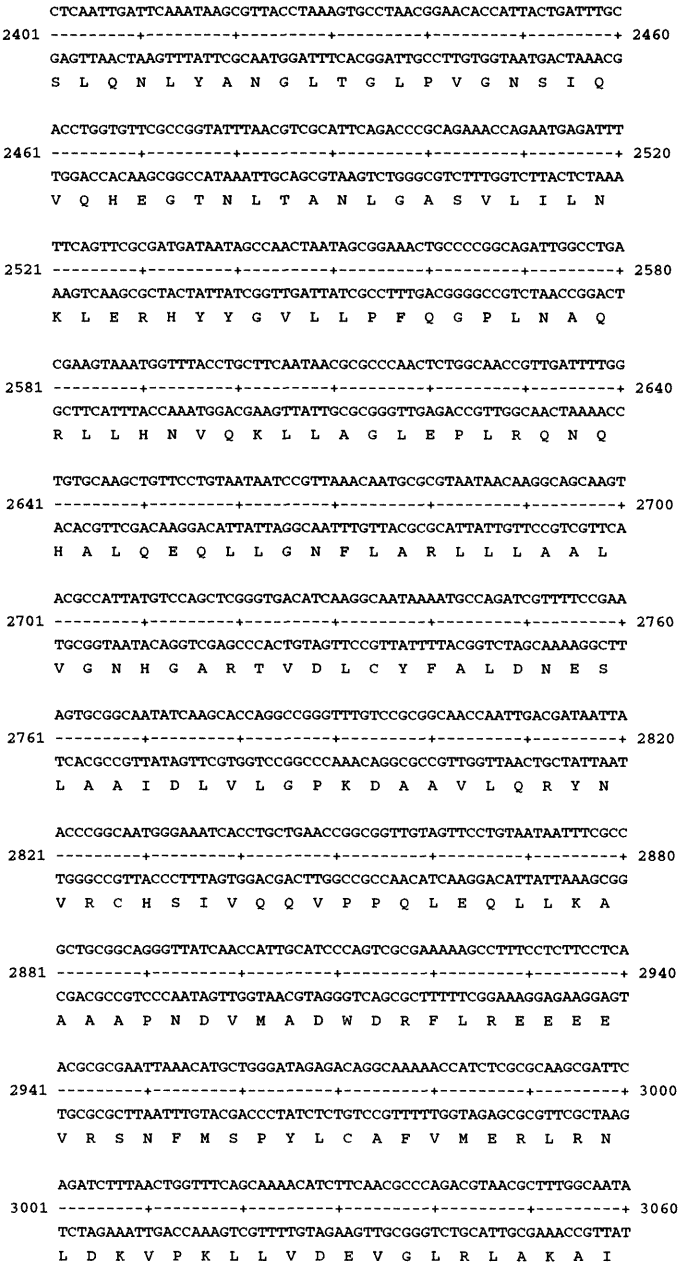
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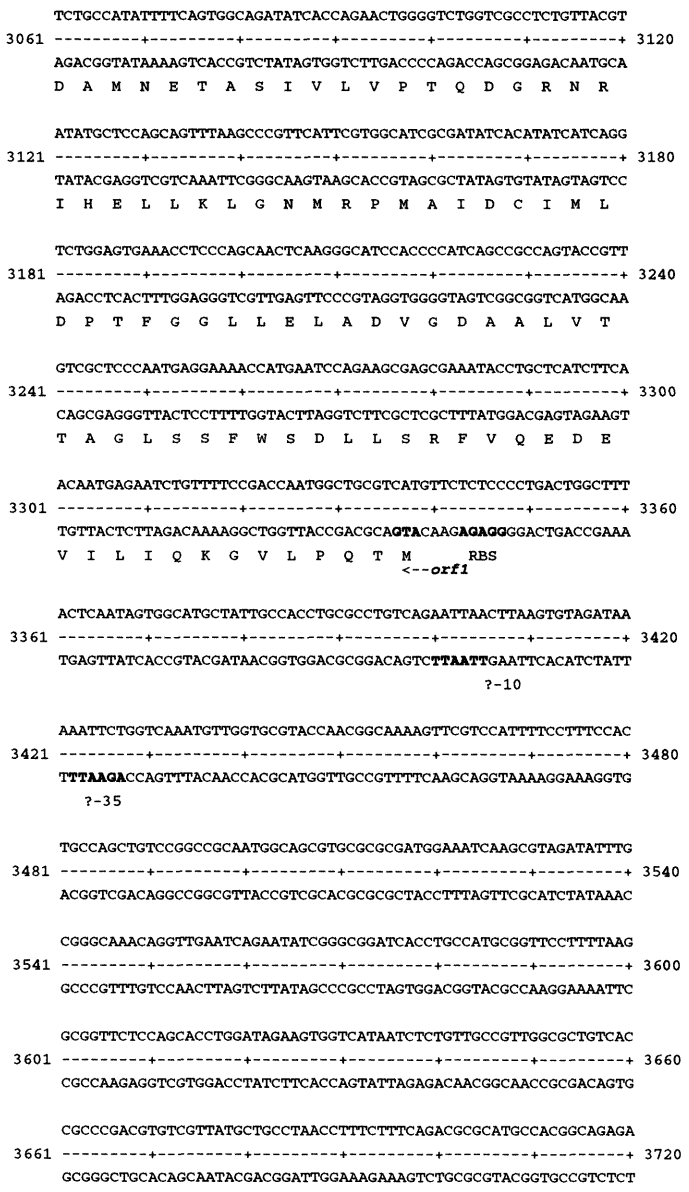
TCTGCCATATTTTCAGTGGCAGATATCACCAGAACTGGGGTCTGGTCGCCTCTGTTACGT
3061 ---------+---------+---------+---------+---------+---------+ 3120
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D A M N E T A S I V L V P T Q D G R N R
ATATGCTCCAGCAGTTTAAGCCCGTTCATTCGTGGCATCGCGATATCACATATCATCAGG
3121 ---------+---------+---------+---------+---------+---------+ 3180
TATACGAGGTCGTCAAATTCGGGCAAGTAAGCACCGTAGCGCTATAGTGTATAGTAGTCC
I H E L L K L G N M R P M A I D C I M L
TCTGGAGTGAAACCTCCCAGCAACTCAAGGGCATCCACCCCATCAGCCGCCAGTACCGTT
3181 ---------+---------+---------+---------+---------+---------+ 3240
AGACCTCACTTTGGAGGGTCGTTGAGTTCCCGTAGGTGGGGTAGTCGGCGGTCATGGCAA
D P T F G G L L E L A D V G D A A L V T
GTCGCTCCCAATGAGGAAAACCATGAATCCAGAAGCGAGCGAAATACCTGCTCATCTTCA
3241 ---------+---------+---------+---------+---------+---------+ 3300
CAGCGAGGGTTACTCCTTTTGGTACTTAGGTCTTCGCTCGCTTTATGGACGAGTAGAAGT
T A G L S S F W S D L L S R F V Q E D E
ACAATGAGAATCTGTTTTCCGACCAATGGCTGCGTCATGTTCTCTCCCCTGACTGGCTTT
3301 ---------+---------+---------+---------+---------+---------+ 3360
TGTTACTCTTAGACAAAAGGCTGGTTACCGACGCAGTACAAGAGAGGGGACTGACCGAAA
V I L I Q K G V L P Q T M RBS
<--orf1
ACTCAATAGTGGCATGCTATTGCCACCTGCGCCTGTCAGAATTAACTTAAGTGTAGATAA
3361 ---------+---------+---------+---------+---------+---------+ 3420
TGAGTTATCACCGTACGATAACGGTGGACGCGGACAGTCTTAATTGAATTCACATCTATT
?-10
AAATTCTGGTCAAATGTTGGTGCGTACCAACGGCAAAAGTTCGTCCATTTTCCTTTCCAC
3421 ---------+---------+---------+---------+---------+---------+ 3480
TTTAAGACCAGTTTACAACCACGCATGGTTGCCGTTTTCAAGCAGGTAAAAGGAAAGGTG
?-35
TGCCAGCTGTCCGGCCGCAATGGCAGCGTGCGCGCGATGGAAATCAAGCGTAGATATTTG
3481 ---------+---------+---------+---------+---------+---------+ 3540
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3541 ---------+---------+---------+---------+---------+---------+ 3600
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3601 ---------+---------+---------+---------+---------+---------+ 3660
CGCCAAGAGGTCGTGGACCTATCTTCACCAGTATTAGAGACAACGGCAACCGCGACAGTG
CGCCCGACGTGTCGTTATGCTGCCTAACCTTTCTTTCAGACGCGCATGCCACGGCAGAGA
3661 ---------+---------+---------+---------+---------+---------+ 3720
GCGGGCTGCACAGCAATACGACGGATTGGAAAGAAAGTCTGCGCGTACGGTGCCGTCTCT

```
     ATCACCATTCTCGCTATTTTCTTCACTGACATTAAAGGAGAGCAAATCTTGTTGCATCAA
3721 ---------+---------+---------+---------+---------+---------+ 3780
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     ATGAGCATCGTGCTGCAGGTCAACCGCTATCACAATATCAGCCCCCAATGCACGCGTGAG
3781 ---------+---------+---------+---------+---------+---------+ 3840
     TACTCGTAGCACGACGTCCAGTTGGCGATAGTGTTATAGTCGGGGGTTACGTGCGCACTC

     GGAAATAGGATTGGGTGGGTACGAGCTCGATTCGTATCATGGTC
3841 ---------+---------+---------+---------+---- 3884
     CCTTTATCCTAACCCACCCATGCTCGAGCTAAGCATAGTACCAG
                                                  <-----pLG702 SEQUENCE FINISH
```

4.6 Comments and conclusions based upon the sequence data.

The information obtained from sequencing the insert contained within pLG702 indicates that there are 2 complete ORFs, *orf*1 and *orf*2, encoding polypeptides with predicted molecular weights of 38 and 33 kDa respectively, and the C-terminus of a third. Since, at the time of sequencing, no information was available in the databases to indicate the nature of the genes, the sequence was studied for distinguishing features and motifs. In addition, to test whether any of the ORFs encoded membrane or secreted proteins, hydropathy plots were obtained to look for signal sequences and hydrophobic domains.

Orf1 does not appear to contain any distinctive motifs that give any useful clues to its function. An examination of its hydropathy profile (see **Fig. 17**) indicates two hydrophobic peaks, immediately followed by hydrophilic regions. Orf2 contains a putative nucleoside binding site (Gill *et al.*, 1986) which suggests that it could either require ATP (or another nucleoside) for activity or that it is a kinase. Its hydropathy profile (see **Fig. 18**) contains a hydrophobic N-terminus that could be a signal sequence suggesting that it is a membrane protein, although an examination of the predicted amino acid sequence does not support this hypothesis. In addition, there are two reasonably large hydrophobic domains which are however, probably not sufficiently large to be transmembrane domains (minimum of 21 amino acids usually needed). Since there is no obvious sequence that could act as a transcriptional terminator between *orf1* and *orf2*, there is a possibility that they may form part of an operon. *orf*3 again does not contain any disinctive features, although an examination of its predicted secondary structure (see **Fig. 19**) indicates two distinct domains, the N-terminus (approximately 6 kDa) consisting of two strong α-helices, followed by β-pleated sheet throughout the rest of the molecule. An, examination of the distal promoter region of *orf3* obtained subsequently, reveals a putative **Fnr** binding site 200bp upstream of the start codon (based upon the consensus sequence AXXXTTGACXXXXATCA, (Eighmeier *et al.*, 1989). This suggests that expression of

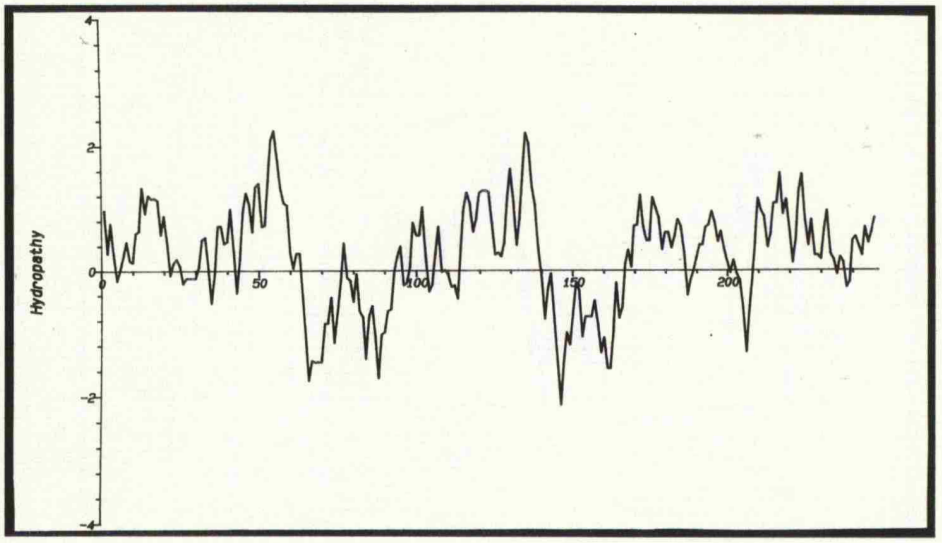


Fig 17. Hydropathy plot of the peptide sequence corresponding to Orf1

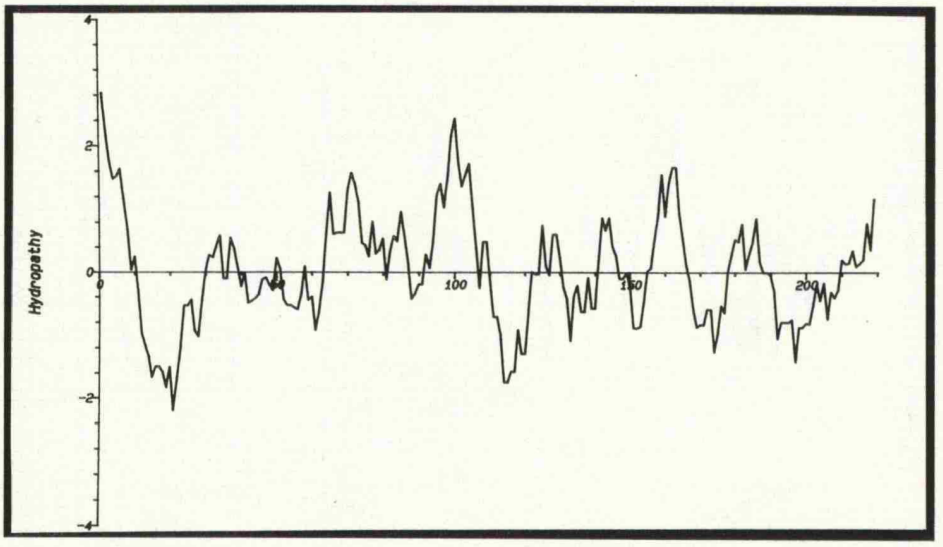


Fig 18. Hydropathy plot of the peptide sequence corresponding to Orf2.

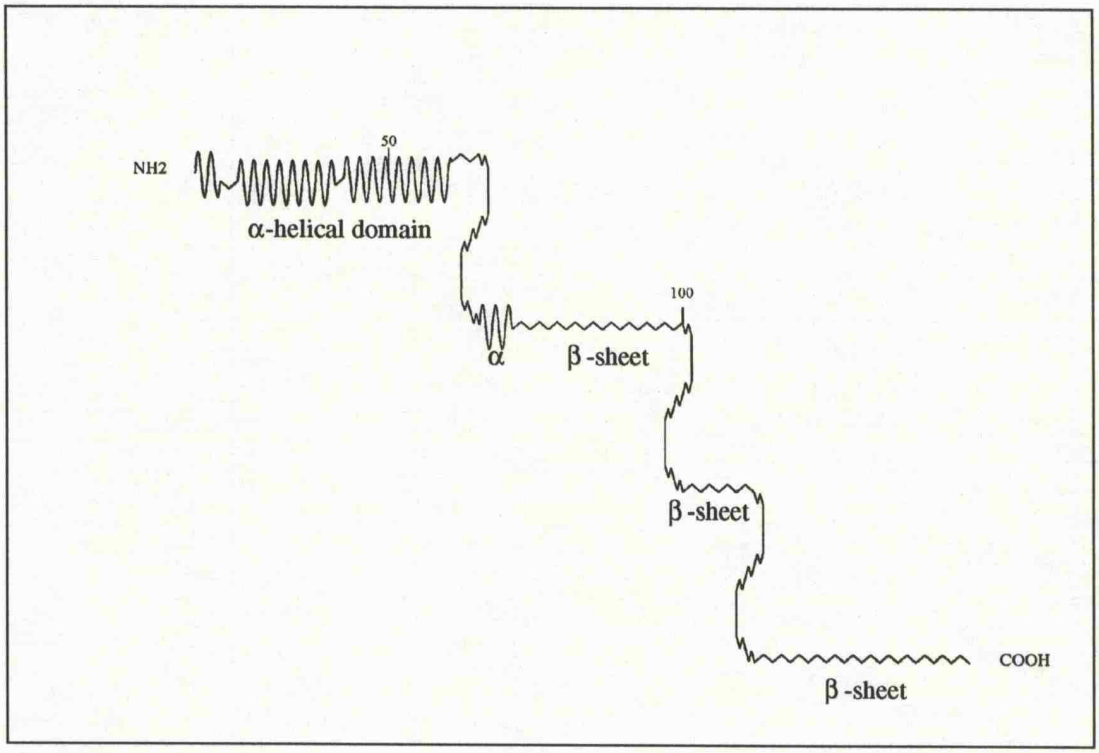


Fig. 19. Structural predictions of the protein encoded by *orf3*.

orf3 may be regulated by changes in oxygen availability. Orf3, according to the structural prediction plot of Chou and Fassman (see **Fig. 19**) consists of two distinct domains, the N-terminus is composed of an α-helical domain whereas the structure of the C-terminus is much more open.

The results did not indicate that any of the genes encoded by pLG701 or pLG702 were likely to form ion channels, and there was no indication of particular features to suggest their interaction with the cytoplasmic membrane or translocation through it. Nevertheless, some specific *in vitro* experiments were designed to test this more directly (see **Chapter 5**). In the next section however, the protein products of Orf1, 2 and 3 were specifically identified.

Chapter 5
Expression and Confirmation of the Gene Products Encoded by *verA*

5.1 Introduction

Having established that pLG701 restores wild-type levels of growth and pLG702 partially complements the *ts* phenotype of N43*verA1*, it appears from the sequence data of pLG702 that at least two genes are involved. A second approach was considered useful to confirm the expression and molecular weights of the three proteins predicted to be encoded by pLG701. Plasmids pLG701 and pLG702 were expressed in *in vitro* coupled transcription - translation reactions (Zubay) in order to facilitate this. Transposon mutagenesis of pLG702 was undertaken to confirm which peptide is encoded by which ORF, and to try, if possible, to establish which gene on this plasmid is required for restoration of growth of N43*verA1* at 42°C. In addition, a minicell experiment was conducted in order to examine the levels of expression of the proteins encoded by *verA* under semi *in vivo* conditions. The results for the first time raised the possibilities of post translational processing of one of the proteins. A further Zubay experiment was undertaken to investigate this possibility, as described below.

5.2 *In vitro* expression of the genes encoded by pLG339, pLG701 and pLG702

Zubay (1973; 1980) first described a method for preparing extracts from *E. coli* which, when incubated with a plasmid containing genes with their own promoters and ribosome binding sites, and a radio-labelled amino acid such as methionine, radio-labelled proteins can be synthesised, corresponding to the plasmid encoded genes. These can be separated by polyacrylamide gel electrophoresis and visualised by exposing the gel to X-ray film. In this project, Zubay experiments were carried out with a commercially prepared *E. coli* S30 *in vitro* coupled transcription translation system supplied by Promega, which requires the addition of [^{35}S-] labelled methionine for labelling the proteins.

5.2.1 Confirmation of predicted peptide molecular weights encoded by pLG701.

In Chapter 4, pLG701 and pLG702 were predicted to encode 3 and 2 peptides respectively, provisionally designated ORF1, 2 and 3. ORF1 is predicted to have a molecular weight of 38kDa; ORF2 33kDa and ORF3 15.5kDa. 1μg of pLG339, pLG701 and pLG702 were incubated in separate reactions, using the Promega Zubay system referred to above. The procedure for using the system was as instructed by the manufacturer, except that all recommended quantities of reagents were reduced by 75%, and the entire reaction mixture was precipitated with 4 vols acetone, rather than the small amount of reaction mixture indicated in the protocol (see also Materials and

Methods). The reaction mixtures were incubated at 37°C for 60min in the presence of [^{35}S-] methionine, followed by acetone precipitation of the proteins and separation on a 15% polyacrylamide gel. The gel, following electrophoresis, was fixed, washed and treated with sodium salicylate as described in Materials and Methods. The labelled proteins were visualised by exposing the dried gel to Fuji RX X-ray film overnight (see **Fig. 20**).

From **Fig. 20**, it is possible to clearly discern one of the three 3 peptides encoded by the vector, pLG339 (Stoker *et al.*, 1982), corresponding to the kanamycin resistance protein, Kan. The tetracycline resistance and copy-number regulating proteins, Tet and Rep respectively, are normally expressed at very low levels, and cannot be seen in the pLG339 samples on this figure. However, a candidate for Rep is visible below the 38kDa protein from pLG701 and pLG702. In pLG701, 2 new peptides are now visible, with molecular weights of 16 and 38kDa. In pLG702, the 16kDa peptide is absent, presumably disrupted by the subcloning procedure used to construct this plasmid, and in addition, a band of 33kDa is now visible, together with the 38kDa peptide. This result is curious, since pLG701 is predicted to encode three proteins within the cloned fragment, whereas pLG702 is only expected to encode two, plus the Tet protein (not visible), since the *tet* gene is intact in this construct. Two possible explanations can account for these observations: 1) there is an error in the DNA sequence, and there are in fact only two genes encoded by pLG701, and during construction of pLG702, an artificial open reading frame has been created, which results in a peptide of 33kDa being formed. 2) The 16kDa protein in pLG701 which is predicted to be inactive in pLG702 due to loss of the N-terminus, is somehow suppressing expression of the 33kDa protein in pLG701 and, because this gene is absent in pLG702, repression no longer occurs. Since the DNA sequence in fact predicts that pLG702 should encode two peptides of 33 and 38kDa, and proteins of these sizes are visible, the second hypothesis is more likely to be correct. This can be simply tested by repeating the experiment, and in addition, plasmids pLG701 and pLG702 can be incubated together, to test whether the 16kDa protein can act in trans to repress the expression of the 33kDa protein.

5.2.2 Does the 16kDa protein repress expression of the 33kDa protein?

In this experiment, the aim was to determine whether the 16kDa protein is indeed regulating the expression of the 33kDa protein. This was tested by repeating the experiment described in **5.2.1** but two additional reactions were also included. Plasmids pLG701 and pLG702 were added together in the same reaction. If the hypothesis is correct, the 33kDa protein should be expressed in reactions

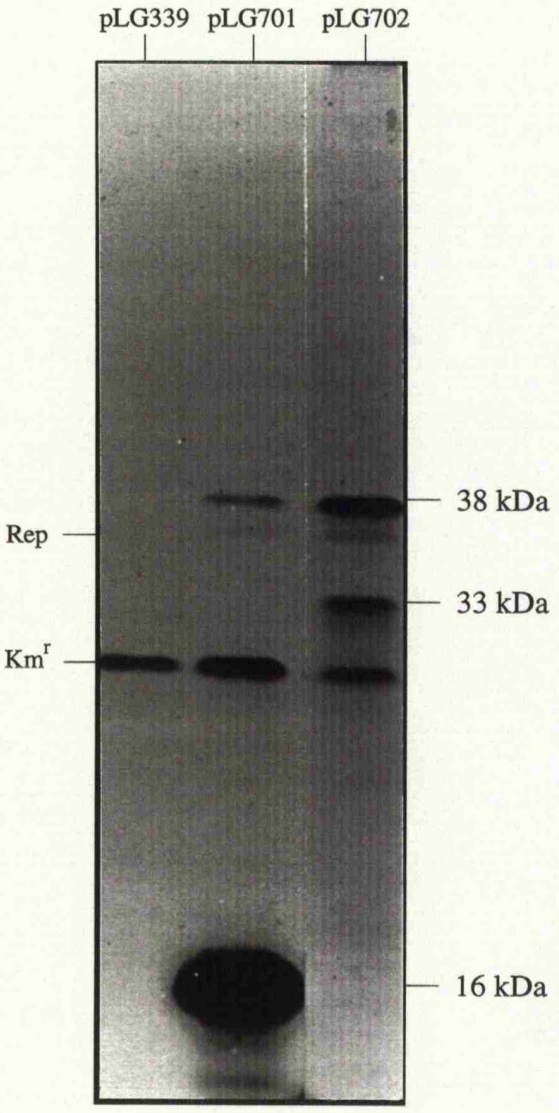


Fig 20. *In vitro* expression of the genes encoded by plasmids pLG339, pLG701 and pLG702. Plasmids pLG339, pLG701 and pLG702 were incubated in coupled transcription-translation reactions (Zubay), and the proteins encoded by the plasmids labelled with [^{35}S-] methionine, resolved by SDS-PAGE and visualised by autoradiography.

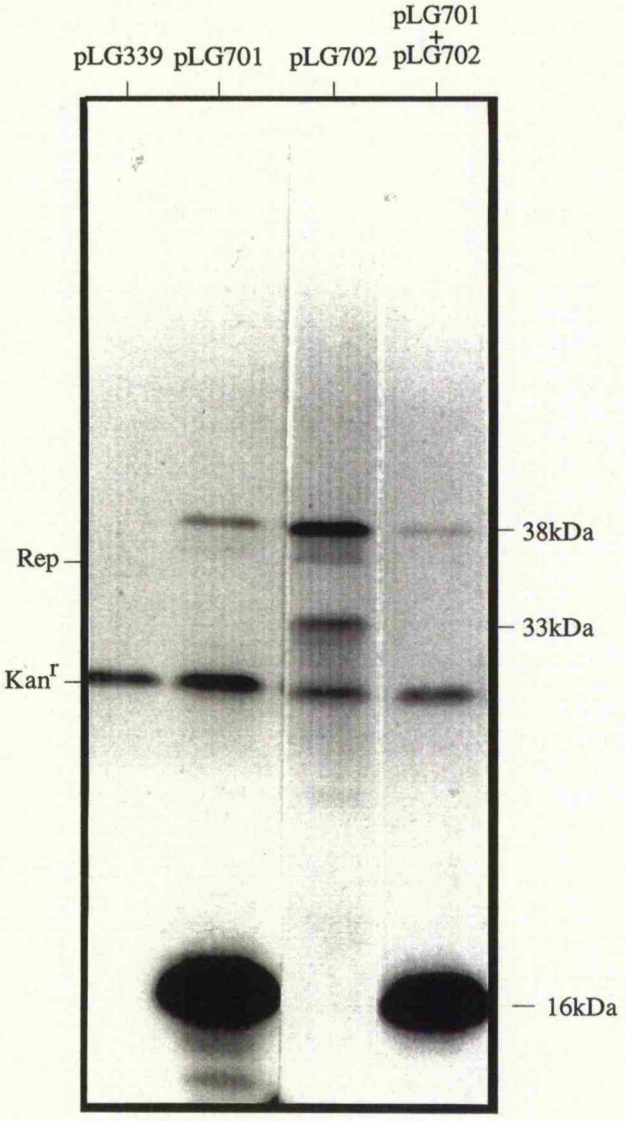


Fig 21. Zubay experiment to investigate whether expression of the 38kDa protein is repressed by the 16kDa protein encoded by *orf3*.

The proteins encoded by plasmids pLG339, pLG701 and pLG702 were labelled with [^{35}S-] methionine in Zubay reactions as already described in **Fig. 20**. In addition to incubating each plasmid in separate reactions, pLG701 and pLG702 were also incubated together.

containing only pLG702 but not when pLG701, which contains the gene encoding the 16kDa protein, is included. **Fig. 21** shows the result of this experiment. In the reactions containing pLG339, 3 bands are visible, corresponding to Kan, Tet, and presumably Rep. From pLG701, 4 bands are visible, corresponding to the 16kDa protein, Kan, the, Rep (very weakly) and the 38kDa proteins. pLG702 only expresses the Kan, 33kDa, Rep and 38kDa proteins. When pLG701 and pLG702 are added together, the 33kDa band is absent. As a comparison, the band intensities of the other proteins are virtually identical when pLG702 is incubated either singly or in combination with pLG701. These results therefore strongly suggest that the 16kDa protein encoded by *orf3* on plasmid pLG701 is a regulator of transcription or translation and appears to affect the expression of the 33kDa protein, and also (less markedly) the 38kDa protein, under these conditions.

5.3 Confirmation of the assignment of ORFs identified from the sequence data with the proteins visualised in the *in vitro* coupled transcription-translation experiments.

From pLG701, three ORFs have been identified, which are predicted to encode proteins of 38, 33 and 16kDa, corresponding to *orf1*, *2* and *3* respectively (see Chapter 4). *orf3* has been shown to encode the 16kDa protein. However, the hydropathy profile from *orf2* (see **Fig. 18**, Chapter 4) contains a number of hydrophobic domains which could affect its mobility through a polyacrylamide gel. A quick method to resolve these questions is to perform transposon mutagenesis of plasmid pLG702. Tn1000 (formerly transposon γδ) is a convenient transposon for such purposes. Tn1000 is located between 4.2F-9.9F map units on the F plasmid (Willets and Skurray, 1987) and is reported to be fairly random in its target sites for transposition (Guyer, 1978). Mutagenesis of a gene(s) of interest can be accomplished simply, by transforming a non-mobilisable plasmid (in this case pLG702) containing the gene(s) of interest, into a strain harbouring the F plasmid. Tn1000 transposes at a high frequency, and will readily transpose into the non-mobilizable plasmid. **Fig. 22** summarises the method and principle behind Tn1000 mutagenesis, details of the procedure are described in Materials and Methods.

An elegant feature of this system is that it enriches for transpositions within the insert of the plasmid, since integration of the transposon into the vector, is likely to inactivate an essential function such as plasmid replication or antibiotic resistance genes. Conversely however, transpositions into the insert of the plasmid should have no adverse effect upon plasmid maintenance or the selective markers. The strain used to perform the Tn1000 mutagenesis, RB308, is sensitive to streptomycin, whereas N43*verA1* and its parent strain are resistant to this antibiotic. Therefore, following

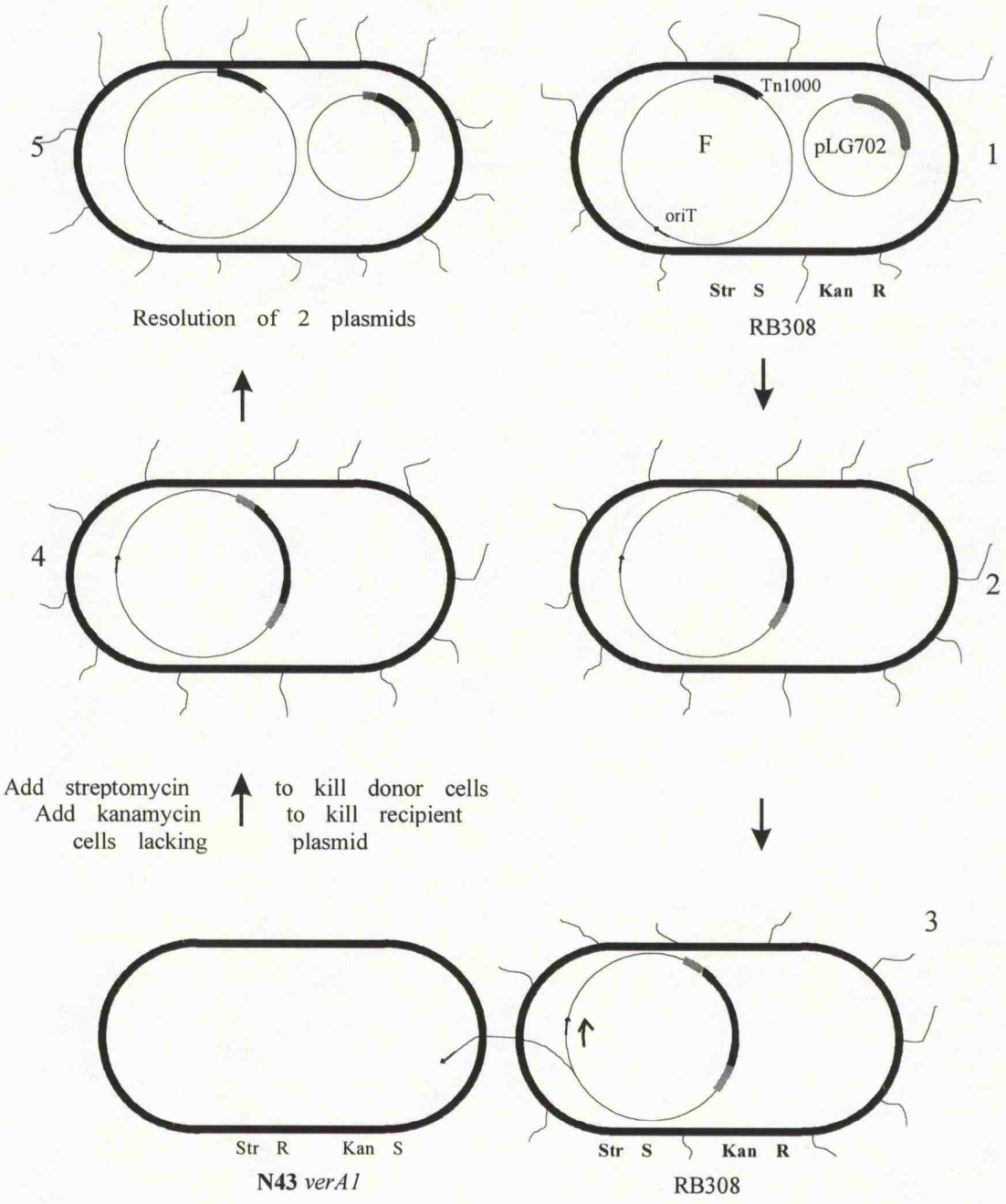


Fig 22. Tn1000 mutagenesis of pLG702

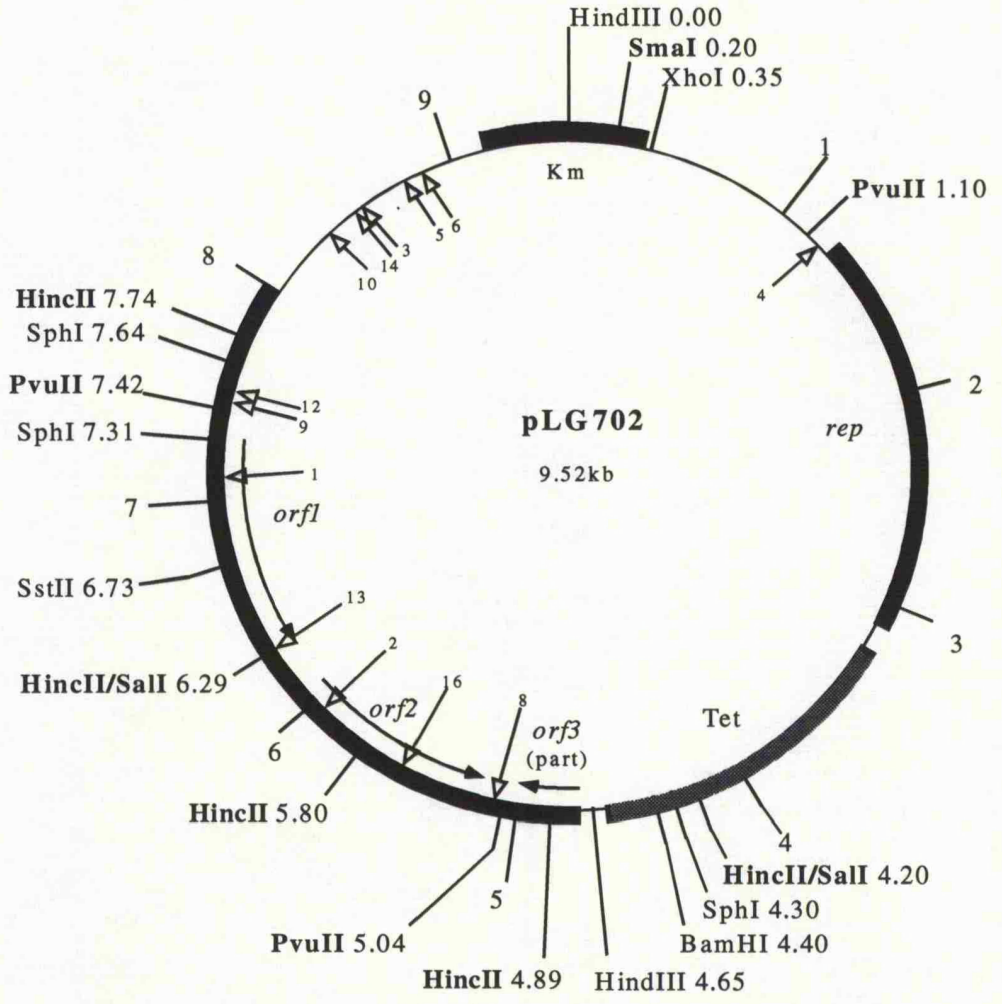
transfer of the F - pLG702 hybrid plasmid into N43*verA1*, the donor strain can be selected against, by inoculating the mating mixture onto NA plates containing kanamycin, tetracycline and streptomycin. The antibiotic plates inoculated with the mating mixtures were incubated overnight at 30°C. 15 colonies were selected at random from the plates, and tested for their ability to grow at 42°C (intact pLG702 enables N43*verA1* to grow at 42°C), by inoculating onto M9 agar containing kanamycin and tetracycline, and incubating overnight at 42°C. In addition, plasmid DNA was extracted from these strains and the position of transposons mapped by restriction enzyme analysis. **Fig. 23** summarises the results of the restriction enzyme mapping of the Tn1000 mutants, including a restriction map of Tn1000, and **Table 8** summarises the ts^+/ts^- phenotype of N43*verA1* containing the Tn1000 mutated pLG702 plasmids. However, the results failed to establish a simple correlation between loss of restoration of ts^+ and the interuption of a specific gene by Tn1000.

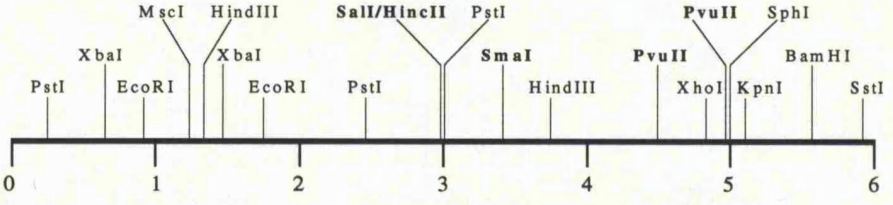
Mutant no.	1	2	3	4	5	6	8
Insertion site in pLG702	ORF1	ORF2	Vector	Vector	Vector	Vector	Between ORF2/3
Phenotype	*ts*	*ts*	*wt*	*wt*	*wt*	*wt*	*wt*

Mutant no.	9	10	12	13	14	16
Insertion site in pLG702	Upstream ORF1	Vector	Upstream ORF1	3'-end ORF1	Vector	ORF2
Phenotype	*wt*	*wt*	*wt*	*wt*	*wt*	*ts*

Table 8. Summary of the phenotype of N43*verA1* containing the pLG702::Tn1000 plasmids. The *wt* designation indicates growth of N43*verA1* at 42°C (*ie* complementation of the temperature sensitive phenotype), comparable with pLG702 lacking Tn1000. The *ts* designation indicates the loss of the complementing ability by pLG702, due to the insertion of Tn1000.

In three instances, Tn1000 interrupted one of the cloned genes, *orf1* or *orf2*. Plasmid pLG702::Tn1000/1 contains a transposon in *orf1*; pLG702::Tn1000/2 and 16 contain transposons in *orf2*. When tested for the ability of each plasmid to restore growth of N43*verA1* at 42°C, it became clear that interruption of either ORF resulted in a loss of complementing potential (see **Table 8** and **Fig. 23).** This therefore confirms that both genes are minimally required for complementation of the *ts* phenotype. If the transposon inserts elsewhere in the plasmid (excluding sites vital for plasmid function), the plasmid is still able to complement the *ts* phenotype, thus enabling the mutant to





Restriction map of transposon Tn1000 (data obtained from Genbank database).

Fig. 23. Restriction map of pLG702, showing the sites of transposition by Tn1000, and a restriction map of Tn1000. Following transformation of pLG702 into the F plasmid containing "donor" strain RB308, RB308 was mated with the recipient strain, N43*verA1* and transconjugants selected by inoculating the mating mixture onto NA plates containing streptomycin, kanamycin and tetracycline. Plasmid DNA was extracted from the transconjugants and the transposons mapped by restriction enzyme analysis. The restriction enzyme sites used in the mapping of the transposons are indicated in bold type.

grow at 42°C. Plasmid pLG702::Tn1000 #2 was selected for further subsequent experimentation, and was designated pLG707 (see **Chapter 7**). It was found that when pLG707, which contains a Tn1000 transposon in *orf2*, was incubated in a Zubay reaction, the protein that migrates as a polypeptide of 38kDa, as seen with pLG702 (see **Fig. 20**), was absent (see **Fig. 24**). This was surprising since the predicted molecular weight of Orf2, based upon the sequence data, is 33kDa. The initial conclusion from this result was that the Tn1000 transposon had been incorrectly mapped. However, when *orf2* alone was subsequently cloned into pLG339 to form pLG708 (see **Chapter 7**), and expressed in a Zubay reaction (data not shown), a polypeptide with an apparent molecular weight of 38kDa was observed. This result therefore indicates that the proteins, Orf1 and Orf2 migrate aberrantly on SDS-PAGE gels, such that the polypeptide encoded by *orf1*, which has a predicted molecular weight of 38kDa, migrates with an apparent molecular weight of 33kDa. Equally, the *orf2* gene product, which has a predicted molecular weight of 33kDa, migrates with an apparent molecular weight of 38kDa.

5.4 Use of an *in vivo* model to test the hypothesis that the 16kDa protein encoded by *orf 3* regulates the expression of the *orf1* gene product.

Having established that *orf*1 and 2 encode proteins that migrate aberrantly using PAGE under denaturing conditions, such that the apparently smaller protein of 33kDa is in fact encoded by the larger *orf*1 (1.01kb) gene and the protein running as 38kDa is encoded by the smaller 907bp *orf2*. The faster-migrating Orf1 encoded protein will from now on be referred to as having a molecular weight of 38kDa, according to its DNA sequence. Similarly, the slower migrating Orf2 protein, which appears to have a molecular weight of 38kDa will in future be referred to as having a molecular weight of 33kDa, again as indicated by its corresponding DNA sequence. In sections 5.1 and 5.2, the 16kDa polypeptide encoded by pLG701 apparently represses the expression of the *orf*1 gene. To test whether the phenomenon also occurs *in vivo*, expression of these genes was studied using minicells. Minicells have the intrinsic advantage that they possess all the features of an intact cell except that they do not contain chromosomes. Thus, if the minicells contain a plasmid, the genes encoded by the plasmid will be expressed, the corresponding proteins labelled with [^{35}S-] methionine and can be visualised by autoradiography.

The formation of minicells is the consequence of mutations affecting the *minB* operon (de Boer *et al.*, 1992). This operon is required for correct positioning of the division septum and deletion of this operon results in some cell divisions taking place at the cell poles. In order to facilitate high expression of the genes encoded by pLG701 and pLG702 in minicells, construction of high copy-number equivalents of these

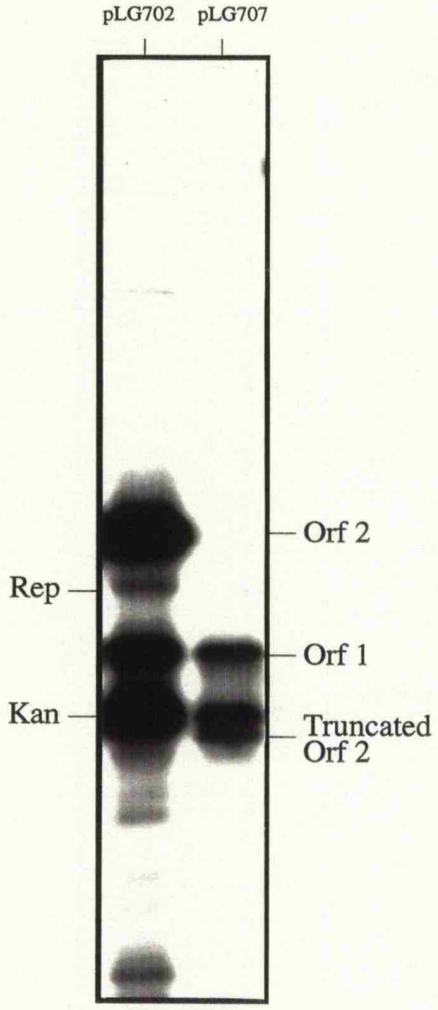


Fig. 24. Demonstration of the aberrant mobility of the polypeptide, encoded by *orf2*. Plasmids pLG702 and pLG707 (pLG702::Tn1000 no. 2, see **Fig. 23**) were incubated in Zubay reactions, and the corresponding proteins encoded by the plasmids labelled with [^{35}S-] methionine.

plasmids was attempted. pLG704, which contains *orf* 1 and 2, was constructed by digesting the M13mp18 plasmid containing the 3.17kb *Hpa*I-*Hpa*I fragment originally used for sequencing the *verA* region, with *Eco*RI and *Xba*I, and subcloned into the *Eco*RI and *Xba*I sites of the high copy-number vector, pUC19. Attempts to clone the 2.8kb fragment containing *orf*1, 2 and 3 from pLG701 into pUC19 by digesting both plasmids with *Eco*RI and *Sph*I failed, and it was therefore concluded that the protein encoded by *orf*3 is toxic when expressed from high copy number plasmids. The minicell producing strain, DS410 was transformed with pUC19, pLG704, pLG339 and pLG701 by the $CaCl_2$ method and minicells prepared containing each of these plasmids, using the method described in Materials and Methods. Since the copy-number of plasmids pLG339 and pLG701 are much lower than the pUC19 based plasmids, the quantity of minicells used containing the low copy-number plasmids was increased by a factor of 5, relative to the minicells containing the pUC19 based plasmids. In addition, plasmid-free minicells were also prepared, to act as a negative control. The plasmid encoded genes in each sample of minicells were expressed and their corresponding proteins labelled with [^{35}S]-methionine as described in Materials and Methods. The labelled minicells were then boiled in SDS-sample buffer and the proteins separated on a 13%$^{v}/_{v}$ polyacrylamide gel. The gel was prepared for autoradiography as described in Materials and Methods, and exposed to Fuji RX X-ray film overnight.

Two interesting results emerge from this experiment (see **Fig. 25**). Firstly, as demonstrated in the Zubay experiments, the 38kDa protein encoded by *orf*1 (which migrates with an apparent molecular weight of 33kDa) is only expressed at very low levels from pLG701 whereas remarkably large amounts are expressed from pLG704. A useful internal control is provided by the other protein products which are of comparable intensities, when expressed from other plasmids. Secondly, below the 16kDa Orf3 protein in the polyacrylamide gel, there is another smaller peptide of approximately 9-10kDa, which is not expressed in the pLG339 vector control or from pLG704. Since this low molecular weight peptide was not visible in the Zubay experiment when pLG701 was expressed *in vitro*, this either indicates that the *in vitro* system fails to transcribe a small ORF, or post-translational processing of one of the proteins takes place in minicells. Subsequent experiments confirmed that the 9-10kDa protein was a cleavage product of Orf 3 (see below).

A further interesting discovery was made following the construction of pLG704. It was noted that the host strain DH5α, used during the construction of the plasmid, and subsequently N43 and N43*verA1*, always grew very poorly when transformed with the high copy-number plasmid, pLG704. Microscopic examination of the cultures revealed two striking features (see **Fig. 26**), which were subsequently

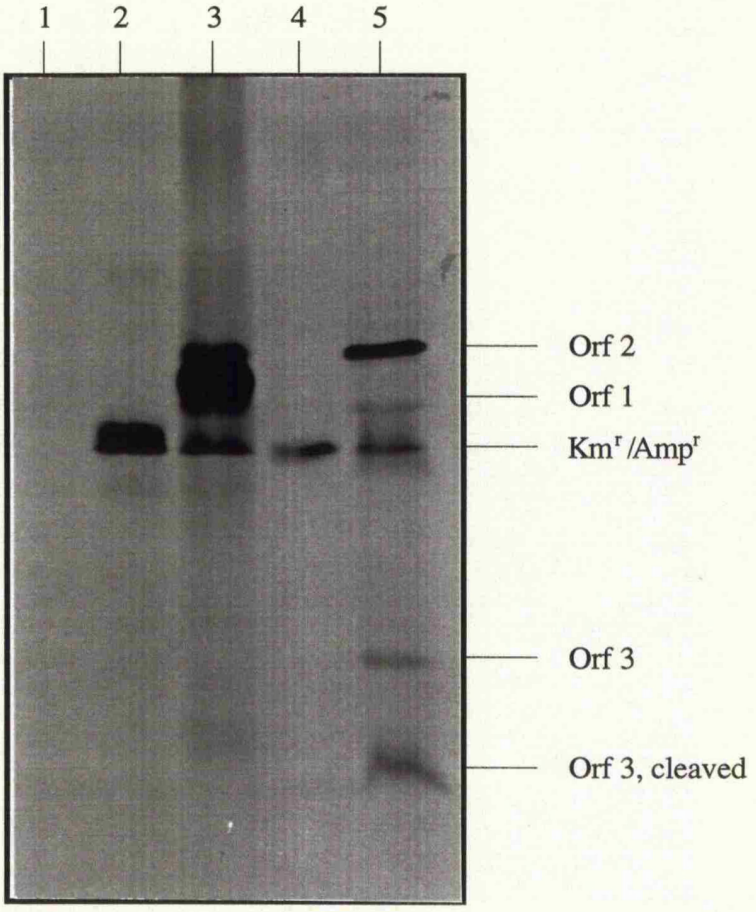


Fig. 25. Expression of plasmids encoding *orf*1, 2 and 3 in minicells.

The genes encoded by plasmids pUC19, pLG704 (pUC19 + *orf1* + *orf2*), pLG339 and pLG701 (pLG339 + *orf1*, *2* and *3*) were expressed in minicells, and their corresponding proteins labelled with [^{35}S-] methionine. The labelled proteins were resolved by SDS-PAGE and visualised by autoradiography.

Minicells containing no plasmid (lane 1); pUC19 (lane2); pLG704 (lane 3); pLG339 (lane 4) and pLG701 (lane 5).

shown specifically to be due to overexpression of *orf2* (see **Appendix 2**). The bacterial cells form very long, fat, irregular filaments ("chains of sausages") and chains of minicells. In addition, normal septation is not taking place, with several cells showing angular rather than vertical cleavage planes. This is in fact a characteristic of some specific FtsZ mutations (Bi and Lutkenhaus, 1990), and is classical of a defect in the division process, providing evidence that at least one of the *verA* genes might be directly connected with the cell cycle. Also noteworthy of this phenomenon is that the filamentation and minicell formation only occurs as the cells enter stationary phase. During exponential phase, the bacteria appeared virtually normal (data not shown). Some further experiments were performed including electron microscopy and protein purification to study this phenomenon further, but since they were not directly related to the main thesis, they have been documented separately in **Appendix 2.** These experiments specifically identified Orf2 as responsible for this phenomenon.

5.5 Are any of the proteins encoded by pLG701 influenced by the addition of everted inner membrane vesicles?

Since verapamil inhibits calcium channels in eukaryotes, it was always one possibility that verapamil resistance might result from the alteration of an inner membrane or periplasmic protein linked to calcium transport. Although the hydropathy plots of proteins encoded by *orf2* and *orf3* (see **Figs. 18** and **19**) did not directly indicate an envelope location for the proteins, it was not specifically ruled out and therefore, it was of interest to analyse the expression of these proteins and of that encoded by *orf3*, *in vitro*, in the presence of everted inner membrane vesicles.

1μg of CsCl purified pLG339, pLG701 or pLG702 was added to duplicate Zubay reaction mixtures, with and without the addition of 1μl of 0.15 A_{280} units/μl everted membrane vesicles (see Materials and Methods). Everted inner membrane vesicles were prepared from *E. coli* MC4100 according to the method described by (Pratt, 1984), except that sonication was used to disrupt the bacterial cells, rather than the French press (see Materials and Methods). Contrary to earlier reports, under certain conditions, the process of sonication does allow the recovery of everted membrane vesicles, resulting in the cytoplasmic surface becoming externalised as demonstrated by Yue (1988). Following labelling of the proteins, the samples were acetone precipitated, the protein pellets resuspended in SDS-PAGE sample buffer, analysed by PAGE and autoradiographed as described in Materials and Methods.

An examination of **Fig. 27** reveals that indeed, one of the proteins encoded by pLG701 is cleaved upon addition of everted membrane vesicles. This protein is in fact the 16kDa protein, encoded by *orf3*, and indicated that the result detailed above with minicells was also due to cleavage of the 16kDa protein. Such is the efficiency of the

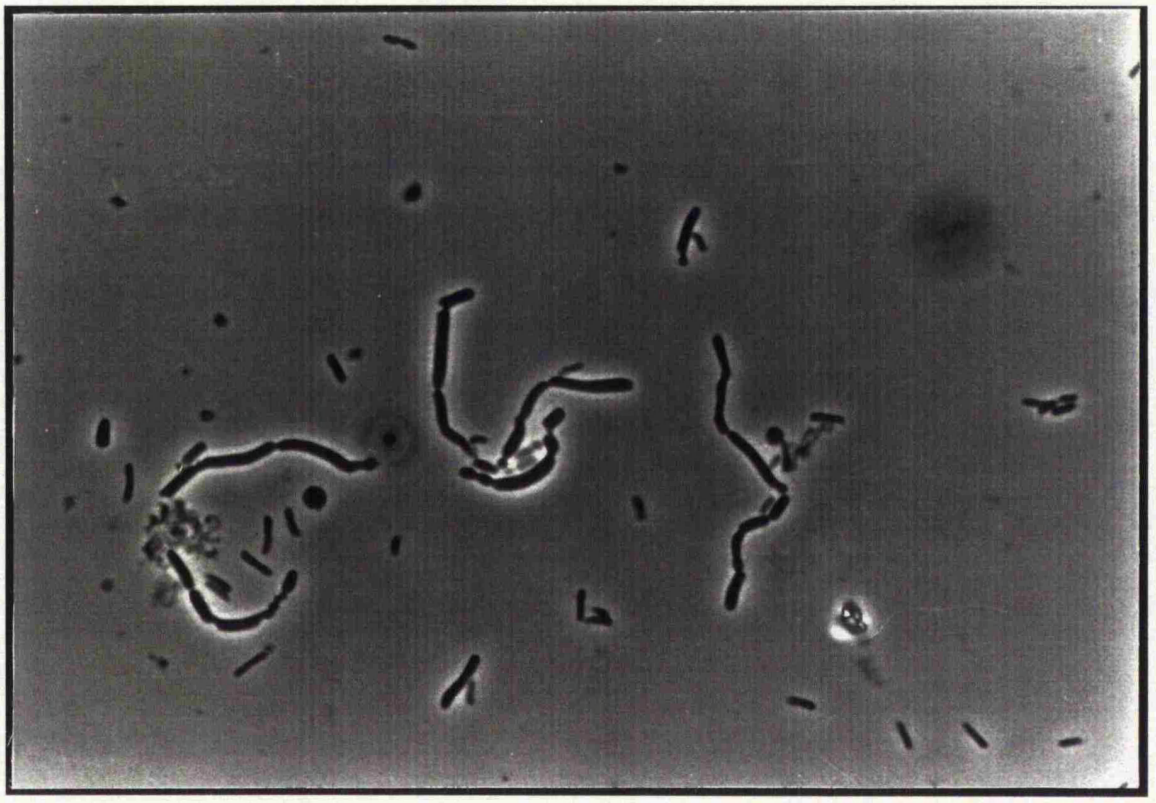


Fig. 26. Photomicrograph of N43 containing plasmid pLG704 (pUC19 containing *orf 1* and *2*).

N43 containing pLG704 was incubated overnight in nutrient broth + ampicillin. A 3µl aliquot of the culture was transferred to a microscope slide and examined by phase contrast microscopy.

Magnification x1000.

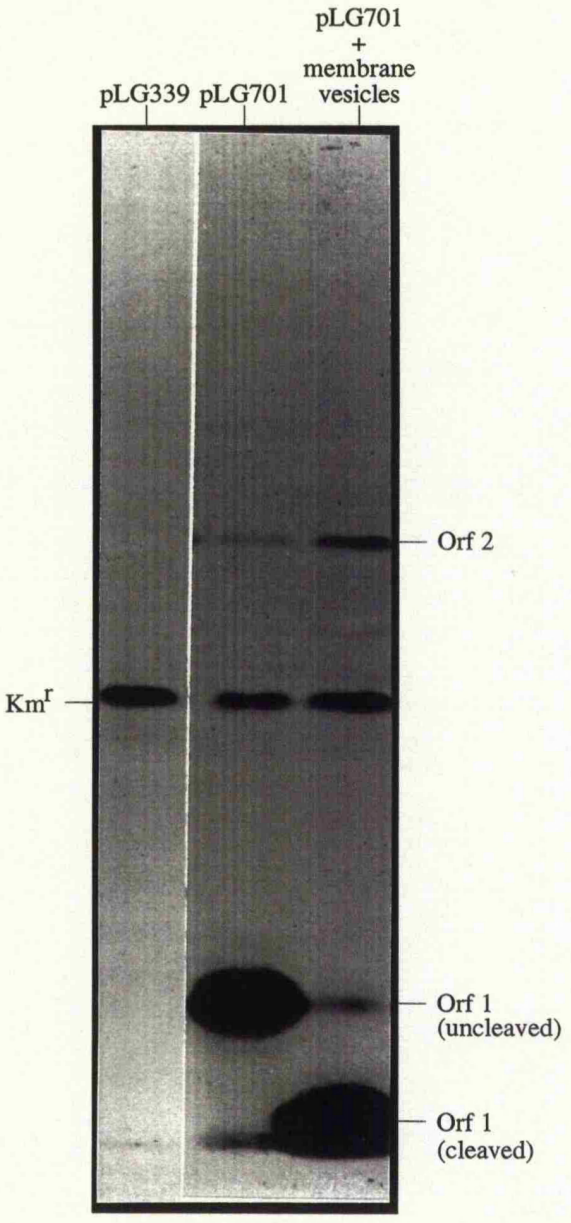


Fig 27. Zubay experiment to investigate possible cleavage of pLG701 encoded proteins by inner membrane peptidases.

Plasmids pLG339 and pLG701 were incubated in Zubay reactions, as previously described. Into one of the reactions containing pLG701, everted inner-membrane vesicles were added. The plasmid-encoded proteins were labelled with [^{35}S-] methionine, resolved by PAGE and visualised by autoradiography.

cleavage mechanism, that virtually all of the 16kDa protein was converted to the cleaved form of approximately 9kDa. The significance of this observation was not clear at the time, since the 16kDa protein does not demonstrate typical features of a membrane protein such as a hydrophobic N-terminal signal sequence. The cleaved form was moreover completely digested by the subsequent addition of 10μg of subtilisin (data not shown), indicating that the cleaved product had not been translocated to the interior of the vesicles, and thus protected. Indeed, the products of *orf1* and *orf2* were also completely digested by the protease, demonstrating that these proteins were not translocated in any detectable way (data not shown).

The cleavage of the Orf3 protein described above was subsequently confirmed by use of the purified 16kDa protein (kindly provided by Professor C.F. Higgins), incubated in the presence of everted inner membrane vesicles. Although the cleavage reaction raised the interesting possibility of post translational regulation of this protein *in vivo*, other experiments (C. Anderson and D. Laoudj, personal communication) demonstrated that cleavage was in fact due to the action of the outer membrane protease, OmpT, and was abolished in OmpT$^-$ mutants. There are now several repeated examples of OmpT cleavage of certain proteins during preparation of cell extracts (see for example, Henderson *et al.*, 1994).

5.6 Summary and conclusions.

Using a combination of *in vitro* and *in vivo* techniques, preliminary sequence data which predicted that plasmid pLG701 encodes 3 genes was confirmed. Two of the genes, *orf1* and *orf2* are orientated in one direction, and encode proteins of 38 and 33kDa respectively. Overexpression of the 33kDa protein results in cell division defects. The third gene *orf3*, is orientated towards *orf* 1 and 2, and encodes a 16kDa protein that appears to be toxic when expressed from high copy number plasmids. This protein demonstrates features of a regulatory protein both *in vitro* and *in vivo*, by apparently repressing the expression of the 38kDa protein encoded by ORF1. If the 16kDa protein is expressed in the membrane vesicle preparations, proteolytic cleavage is observed, which can be attributable to the action of the OmpT protease. The Tn1000 experiment demonstrated a) that no single gene within pLG701 or pLG702 will completely complement the mutant phenotype, and b) permitted the assignment of ORFs derived from the sequence data, with their corresponding peptides as visualised from the Zubay experiments.

Chapter 6
Phenotypic characterisation of N43*verA1*

6.1 Introduction

In Chapter 5, it was established that the two genes *orf1* and *orf2* are minimally required to restore growth of N43*verA1* at 42°C, apparently by complementation, on the basis of the Tn1000 mutagenesis experiments. Nevertheless, complementation is less complete than with pLG701. Since none of the 3 genes required for full restoration of *wt* growth characteristics at 42°C have any known role in calcium uptake / regulation, or homology with known Ca^{2+} binding proteins, the question that clearly needs to be asked is whether the mutant has any demonstrable phenotype which can be linked to calcium regulation or metabolism. MICs were determined to establish the degree of resistance of the parent and mutant strains to verapamil, ethylene glycol - bis (β-aminoethyl ether) N,N,N',N', - tetracetic acid (EGTA) (a chelator of calcium ions), $CaCl_2$, calmodulin inhibitors and a number of unrelated drugs. In addition, both parent and mutant strains were labelled with [^{35}S-] methionine following treatment with verapamil, EGTA and $CaCl_2$, to examine their responses to these treatments. In addition, the mutant and parent were examined microscopically, since our hypothesis states that calcium plays a role in the control of the cell cycle, a perturbation in the cell division process of N43*verA1* might therefore be expected, in particular at the non - permissive temperature.

6.2 Determination of the verapamil MIC of N43*verA1*.

Serial doubling dilutions of verapamil ranging from 3.2mM to 0.05mM were prepared in M9 minimal medium. Each tube in the dilution series was inoculated with 10^5 cfu of an overnight culture of N43 or N43*verA1* and incubated overnight at 30°C in the dark. The MIC for growth was determined as described in Materials and Methods. N43 was again found to have an MIC of 0.4mM and N43*verA1*, an MIC of 0.8mM.

6.3 Determination of the EGTA MIC for N43 and N43*verA1*.

Duplicate series of doubling dilutions of EGTA, ranging from 20mM to 0.16mM were prepared in M9 medium and inoculated with 10^5 cfu of overnight cultures of N43 or N43*verA1*, and incubated overnight at 30°C. N43 was found to grow in all concentrations of EGTA tested. However, N43*verA1* was found to be significantly more sensitive by comparison, with an MIC of 5mM.

6.4 Determination of the Ca^{2+} MIC for N43 and N43*verA1*.

Since N43*verA1* has been found to be much more sensitive to EGTA than the parent, another important question that should be addressed is whether the mutant is also more sensitive to the amount of Ca^{2+} in the medium, compared with the parent strain. The medium TCG (see Materials and Methods), chosen for this experiment was buffered by Tris-HCl pH7.5 rather than phosphate as in M9 medium, because of the risk of formation of an insoluble precipitate of Ca_2PO_4. The concentrations of $CaCl_2$ chosen for this experiment were 0.05mM, 0.1mM, 0.2mM, 0.4mM, 0.8mM, 1.2mM, 1.6mM, 2.0mM, 2.4mM and 2.8mM. As in all the MIC determinations, 10^5 cfu of N43 or N43*verA1* were added to each tube and incubated overnight at 30°C. N43 and N43*verA1* were found reproducibly to have MICs of 1.6 and 0.8mM respectively. This result again suggests a reduced ability of the mutant to regulate its $[Ca^{2+}]_i$. Such a phenotype has been ascribed to *calA* and *calD* mutants (Brey and Rosen, 1979), defective in outward transport of Ca^{2+}.

6.5 Verification that the *verA* mutation does not cause a generalised change in drug resistance.

To check that the *verA* mutation results specifically in an increased resistance to calcium channel inhibitors and not to other drugs acting upon unrelated targets, MICs were determined for N43 and N43*verA1* to a few unrelated drugs, and to another VOCC inhibitor, diltiazem, for comparison (see **Table 9** below). *E. coli* is clearly very sensitive to the calmodulin inhibitor trifluoperazine (TFP), and the fact that both mutant and parent have the same MIC indicates this drug acts quite differently to verapamil. The MIC of the mutant to diltiazem is 2-fold greater than that of the parent (exactly the same margin of difference as for verapamil), and suggests that the target for both drugs could be the same. Both strains are equally sensitive to chloramphenicol, an inhibitor of protein synthesis which inhibits the peptidyl transferase activity of the 50S ribosomal subunit in prokaryotes (Stryer, 1981). Most curious are the MIC results for rifampicin and nalidixic acid. In both cases, the mutant is *more* sensitive than the parent. Rifampicin acts upon the β-subunit of RNA polymerase and inhibits the initiation of RNA synthesis. Nalidixic acid acts by binding to the α-subunit of DNA gyrase (Brown, 1991). The possible significance of these results is discussed later, in **Chapter 10**.

DRUG	TARGET	DRUG MIC	
		N43	N43*verA1*
Trifluoperazine	Calmodulin	17μM	17μM
Chloramphenicol	Ribosomes	1μg/ml	1μg/ml
Rifampicin	RNA polymerase	10μg/ml	<2.5μg/ml
Nalidixic acid	DNA gyrase	4μg/ml	2μg/ml
Diltiazem	L-type VOCCs	0.8mM	1.6mM
Verapamil	L-type VOCCs	0.4mM	0.8mM

Table 9. Summary of MICs of N43 and N43*verA1*, treated with various drugs, and their normal biological targets.

6.6 [^{35}S]-labelling of N43 and N43*verA1* treated with verapamil and EGTA.

As discussed in the introduction, Ca^{2+} is potentially extremely toxic to the cell. The external concentration is typically 10,000 times greater than the intracellular concentration of free Ca^{2+} (Clapham, 1995), and numerous mechanisms, both active and passive, have been evolved by the eukaryotic cell to maintain this gradient, and indeed by bacteria also (Gambel *et al.*, 1992; Ivey *et al.*, 1993). The above experimental results suggest that the mutant may have an impared ability to regulate its $[Ca^{2+}]_i$ compared with the parent. Since calcium acts on a number of targets in the eukaryotic cell, and levels of $[Ca^{2+}]_i$ are very tightly regulated (see **Chapter 1**), it might be expected that if the same is true in *E. coli*, a number of changes might be observed in the protein profiles of the mutant compared with the parent, when challenged with verapamil or EGTA. Calcium binding proteins such as calmodulin are heat stable, consequently when protein extracts containing calmodulin are boiled and centrifuged, calmodulin remains in the supernatant, unlike the majority of proteins that become denatured and precipitate. This method therefore provides a simple means of enriching for heat stable proteins such as those that bind calcium.

The full method for labelling N43 and N43*verA1*, treated with EGTA and verapamil, is described in Materials and Methods. Each culture was grown at 30°C in M9 minimal medium to mid-exponential phase (A_{600}=0.4) and then, 20ml aliquots were transferred to 3 prewarmed flasks. To one flask, nothing was done, to a second flask, 100mM verapamil was added to a final concentration of 0.8mM for the mutant, and 0.4mM for the parent; and to the third flasks, 0.5M EGTA was added to a final concentration of 20mM. Following treatment of the cultures, they were incubated for 5min before the [^{35}S]-methionine was added. The cultures were labelled for a total of

20min before harvesting. Labelling of the cells and preparation of the total-protein and heat-stable protein fractions was as described in Materials and Methods. The proteins were separated on a 12%$^{v}/_{v}$ SDS-polyacrylamide gel and detected by fluorography on Fuji RX X-ray film (see Materials and Methods).

Fig. 28 is the result from this experiment. Many interesting observations can be made. Firstly, even in the protein profiles of the untreated parent and mutant, a number of differences can readily be seen, especially in the heat-stable protein fractions. There are a number of proteins that appear to be constitutively derepressed in the mutant, many of which are heat-stable, and of low molecular weight *ie* 12-21kDa. Moreover, the level of some proteins with similar molecular weights are further induced when the mutant cells are treated with verapamil or particularly EGTA (the results are summarised in **Table 10**, in section **6.7**). Some of these proteins are also induced in N43, but to a lesser extent. Of considerable interest are the low molecular weight heat-stable proteins that are strongly induced in the mutant by the addition of EGTA. An investigation into the nature of some of these proteins is currently under way (Laoudj *et al.*, 1994).

In addition to the heat-stable proteins that are induced by the treatment of these strains with verapamil or EGTA, a number of heat labile proteins are also induced. Most noticeable however, is the marked reduction in expression of a large number of genes following treatment of both strains with verapamil.

This experiment clearly illustrates the complex effects of treating both the parent and mutant with verapamil or EGTA, and additionally demonstrates the complexity of the phenotype of N43*verA1*. Most excitingly however, was the mounting evidence at this stage of the analysis, indicating that an ostensibly single mutation has resulted in a highly pleiotropic phenotype, which is classical of a mutation in a regulator. If the regulator happens to respond to changes in the $[Ca^{2+}]_i$, the *verA* locus could have an important function, controlling the $[Ca^{2+}]_i$.

6.7 What effect does the addition of Ca^{2+} have upon gene expression in N43 and N43*verA1*?

Section **6.6** established that the addition of verapamil or EGTA to the parent, N43, or the mutant, N43*verA1*, can elicit very different responses. The same question must now be addressed as to what genes are induced / repressed upon addition of calcium to these strains. Clearly, it would be important if possible to demonstrate that some of the genes responding to the addition of compounds that interfere with the uptake or storage of Ca^{2+} in eukaryotes, such as verapamil and EGTA respectively, are also involved when toxic concentrations of Ca^{2+} are supplied to the *verA* mutant. A similar experiment to that

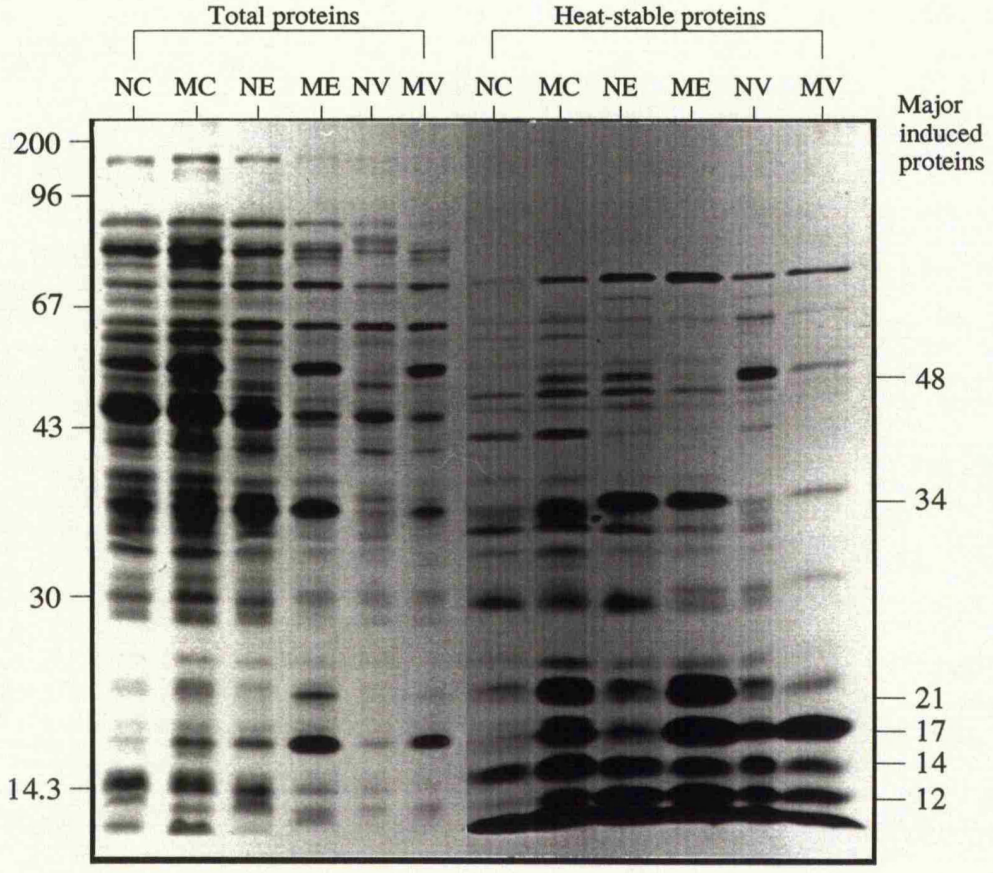


Fig 28. [^{35}S-] labelled total, and heat-stable protein profiles of N43 and N43*verA1*, treated with EGTA and verapamil. N43 and N43*verA1* were grown in M9 minimal medium at 30°C to mid-exponential phase and where appropriate, treated with 20mM EGTA or 0.4 /0.8mM verapamil respectively, as described in the text. After 5min, [^{35}S-] methionine was added to the cultures and incubated for a further 15 min. The samples were processed as described in Materials and Methods, loaded onto an SDS-polyacrylamide gel and the labelled proteins visualised by fluorography. Key: N, N43; M, mutant N43*verA1*; C, untreated control; E, EGTA; and V, verapamil.

described in 6.5 was performed, but this time, the strains N43 and N43*verA1* were grown in TCG medium (see Materials and Methods), to avoid the precipitation of Ca_2PO_4.

All *E. coli* strains grow slowly in TCG, therefore, the 5ml starter cultures were grown for 24-48h at 30°C, and then inoculated into 100ml prewarmed TCG medium. When the A_{600} of the cultures reached 0.4, three 20ml volumes (8.0 A_{600} units) of N43 and N43*verA1* were each transferred to 3 prewarmed 50ml flasks. To the first series of flasks, nothing was added, to the second series, 0.5M EGTA was added to a final concentration of 20mM, and to the third series, 1.6mM and 0.8mM final concentrations of $CaCl_2$ were added to N43 and N43*verA1* respectively. The cultures were incubated at 30° C for a further 5min prior to the addition of 2.22MBq [^{35}S]-methionine (Amersham International PLC). Incubation of the bacteria with the [^{35}S]-methionine was allowed to proceed for 30 min, before transferring the cultures to 50ml centrifuge tubes, and harvesting the labelled cells. The procedures for harvesting the labelled cells, and preparation of the total and heat-stable protein fractions from them, are described in Materials and Methods. The proteins were separated by PAGE using a 12%$^{v}/_{v}$ SDS-polyacrylamide gel, the gel stained with coomassie blue and autoradiographed as described in Materials and Methods. **Figs. 29** and **30** represent the coomassie blue - stained gel and fluorographs of this experiment, respectively.

Both the stained and radiolabelled protein profiles show many differences between the parent and mutant, which again highlight the impact of the mutation upon the overall state of N43*verA1*. In particular, it was again evident with [^{35}S]-methionine labelling that several low M_r heat-stable proteins and amongst total proteins, several species of high M_r, are depressed in the *verA* mutant. However, no differences in the coomassie blue stained protein profiles could be discerned following treatment of the cultures. To investigate changes in the proteins expressed following treatment, the [^{35}S]-labelled gel needs to be examined. In the untreated samples, the protein profiles resemble those seen in section 6.6. Note again, the greatly elevated levels of low molecular weight heat stable proteins in the mutant, even in the untreated control.

It appears that neither added Ca^{2+} or EGTA has much detectable effect upon the **total protein** profiles of N43 or N43*verA1*. A particularly striking observation is the repression of a 12kDa heat-labile protein following treatment with EGTA in N43. In contrast, a protein band corresponding to 21kDa is apparently enhanced by EGTA but repressed by Ca^{2+} in both N43 and N43*verA1*.

Table 10 summarises some of the most prominent changes in gene expression in the parent and mutant following treatment with EGTA, Ca^{2+} and verapamil; there are many other changes that have not been recorded here. Proteins that are differentially

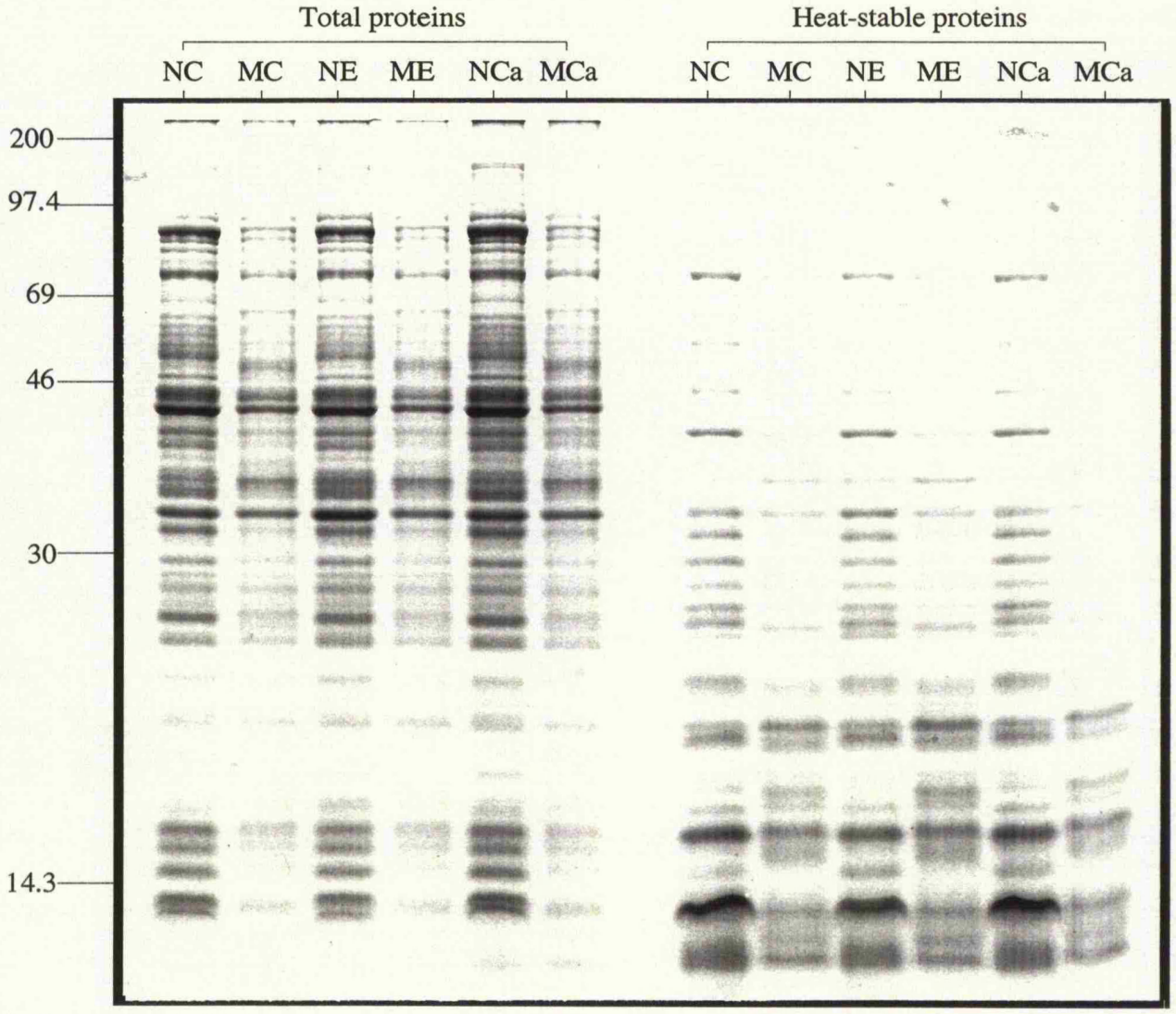


Fig. 29. Coomassie blue stained gel of N43 and N43*verA1* treated with EGTA and $CaCl_2$. Following treatment of N43 and N43*verA1* with EGTA and $CaCl_2$, and labelling with [^{35}S-] methionine, the proteins were processed as described in the text, for the preparation of total and heat-stable protein fractions. After electrophoresis of the proteins, the gel was stained with coomassie blue, photographed, and treated for fluorography (see **Fig. 30**). **Key:** N, N43; M, Mutant N43*verA1*; C, control; E, EGTA; Ca, $CaCl_2$.

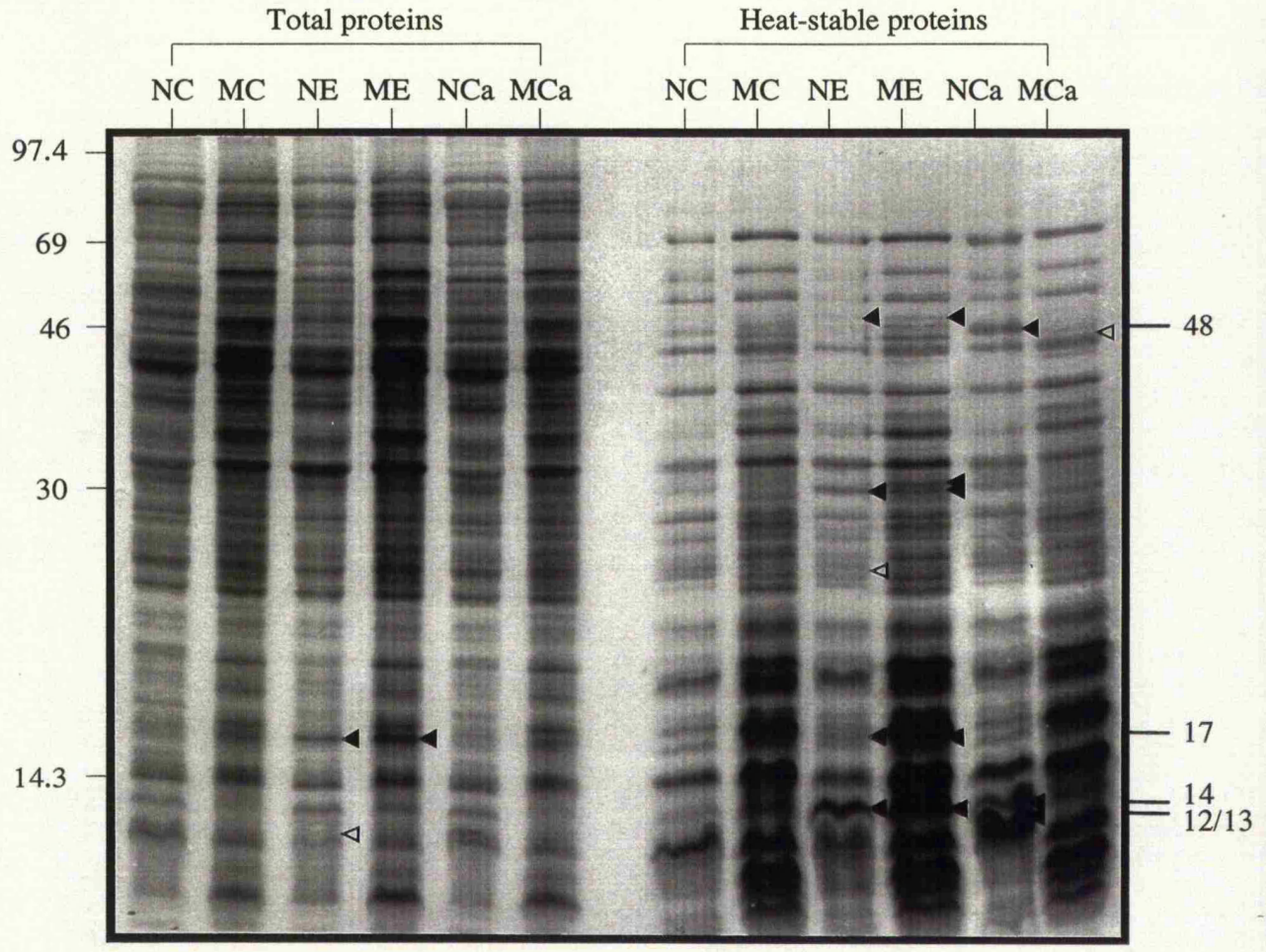


Fig 30. [^{35}S-] labelled total and heat stable protein profiles of N43 and N43*verA1* cells growing exponentially in TCG medium and treated with EGTA and $CaCl_2$.

N43 and N43*verA1* were grown to mid-exponential phase in TCG medium. To one pair of cultures, nothing was added; to a second pair of cultures, 20mM EGTA was added, and to the remaining N43 and N43*verA1* cultures, 1.6 and 0.8mM $CaCl_2$ was added, respectively. [^{35}S-] methionine was added to each culture to label the proteins. Following labelling, the proteins were processed as described in the text, for the preparation of heat-stable and total proteins, separated by SDS-PAGE, and the gel stained with coomassie blue (see **Fig. 29**). The gel was then treated for fluorography and the labelled proteins detected on X-ray film.

Key: N, N43; M, Mutant N43*verA1*; C, control; E, EGTA; Ca, $CaCl_2$.

◀ = proteins induced following treatment.

◁ = proteins repressed following treatment

expressed in the parent and mutant, but are not affected by the treatments described above have been disregarded in order to reduce the complexity, although it is clear that the differences between the parent and mutant are in fact much greater than those induced by the treatments described above. For each treatment, certain genes are being induced or repressed. However, a most exciting observation is that a low molecular weight, heat stable protein of 12-13kDa can apparently be induced by all treatments in both N43 and N43*verA1*. In contrast, a protein of approximately 14kDa is induced by EGTA and Ca^{2+} in N43, but is only induced by EGTA in N43*verA1*. These results are consistent with, but do not prove the hypothesis that there are proteins required for the regulation of the $[Ca^{2+}]_i$ in *E. coli*.

The 48kDa protein induced by verapamil in N43 (see **Fig. 30**, lane 11) is also induced by diltiazem (Cadman, undergraduate project 1994) further demonstrating the common mode of action of these drugs in *E. coli*. Moreover, a protein of the same molecular weight is induced in N43 following treatment with $CaCl_2$. One might imagine that by "starving" the cell of Ca^{2+}, specific proteins would be synthesised to scavenge Ca^{2+}. Similarly, by exposing the cell to toxic concentrations of Ca^{2+}, the cell might express proteins that buffer the excess Ca^{2+}, and lower the $[Ca^{2+}]_i$ *eg* parvalbumin in eukaryotes (Means and Rasmussen, 1988). It is possible therefore that in these experiments, we may be seeing regulatory proteins being induced or repressed, which are concerned with Ca^{2+} homeostasis. However, considerably more detailed studies to identify and characterise the relevant polypeptides will be required to confirm this.

	EGTA treatment		**Ca^{2+} treatment**		**Verapamil treatment**	
Protein M_r (kDa)	N43	N43*verA1*	N43	N43*verA1*	N43	N43*verA1*
65*	†	†	-	-	†	†
56.5	+	+	-	-	-	-
48	+	†	++	-	+++	-
46	-	-	-	†	-	-
40	+	-	-	-	-	-
33	-	+	+	-	-	-
32	+	+	-	-		
21	+	+++	-	-	-	-
17	-	++	-	++	++	+++
14	++	++	++	-	+	†
12/13	+++	+++	+++	-	+++	-

Table 10. Summary of heat stable and labile proteins induced or repressed in N43 and N43*verA1* by the addition of EGTA, Ca^{2+} and verapamil.

All the above results are with reference to the untreated control samples.

Key: * heat labile proteins; - no change in levels compared to untreated control; + increased levels compared to the control; +++ very large increases in expression compared with control; † protein repressed following treatment.

6.8 Microscopic morphology of N43 and N43*verA1*.

A possible consequence of affecting the $[Ca^{2+}]_i$ of the mutant is that cell division or morphology might be affected. This could manifest itself in the formation of filaments, minicells or cells with abnormal girth. 5ml overnight cultures of N43 and N43*verA1* were grown in TCG medium, diluted 1:50 into 10ml fresh medium and grown at 30°C until the A_{600} reached 0.2-0.4. 5ml of each culture was then transferred to 42°C and incubated for a further 4h. 3µl aliquots of the each culture were transferred to microscope slides and examined by phase contrast microscopy, using a Zeiss Axioskop microscope, at 1000X magnification. The cells were photographed using an Leica 35RF camera, and recorded on Ilford HP5 film.

Figs. 31 and **32** represent N43 grown at 30 and 42°C, and **Figs. 33** and **34** represent N43*verA1* grown at 30 and 42°C respectively. A striking difference can be seen in the morphology of the two strains, even at 30°C. At 30°C, the *verA* mutant tends to form long spindly cells, unlike the parent which forms uniformly rod-shaped cells. At 42° C, cells of N43 do not appear significantly different, compared to those grown at 30°C. However, N43*verA1* shows all the classical features of a cell cycle defect. At the non-permissive temperature, the mutant sometimes forms "chains of sausages", whereby the cells appear to be unable to complete septation, resembling the *envA* phenotype (Donachie and Robinson, 1987). Moreover, minicells can also be seen in some instances. These results therefore present the strongest possible evidence for the role of the *verA* locus in the regulation of the cell cycle.

6.9 Conclusions

A large amount of data has been presented to indicate that the mutant, N43*verA1* is affected in a gene(s) that encodes some form of regulatory protein(s) concerned with the regulation of the $[Ca^{2+}]_i$. The mutant responds very differently to the parent following treatment with VOCC antagonists such as verapamil, the Ca^{2+}-chelating agent EGTA and indeed Ca^{2+}. That such a plethora of changes in gene expression occur following treatment of either the mutant or parent with these compounds strongly suggests that Ca^{2+} must have many diverse effects upon the bacterial cell.

An examination of the microscopic appearance of the mutant indicates that normal cell division and morphology is affected, especially at the non-permissive temperature. Coupled with the observations regarding the mutant's apparent responses to treatment with Ca^{2+} or Ca^{2+}-antagonists provides convincing evidence that Ca^{2+} is somehow linked to the *E. coli* cell cycle.

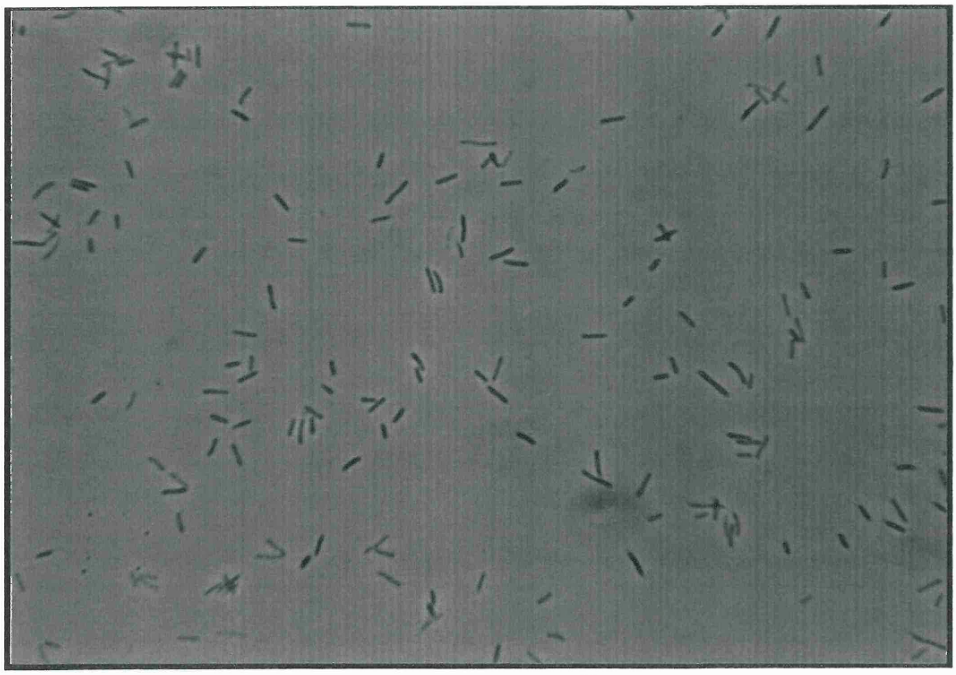


Fig. 31.N43 grown at 30° C

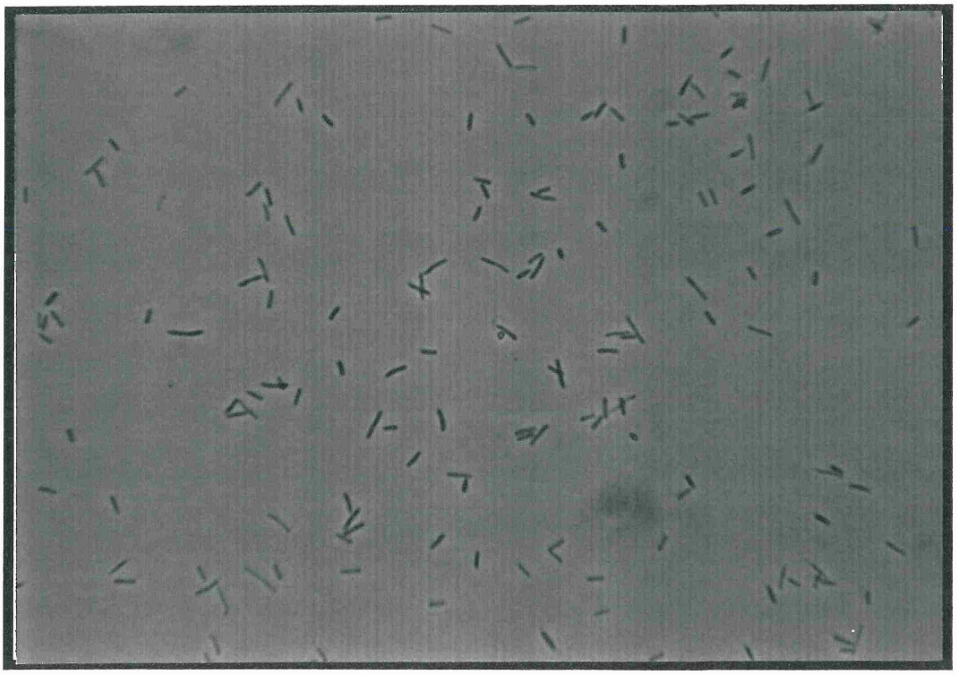


Fig. 32. N43 grown at 42°C

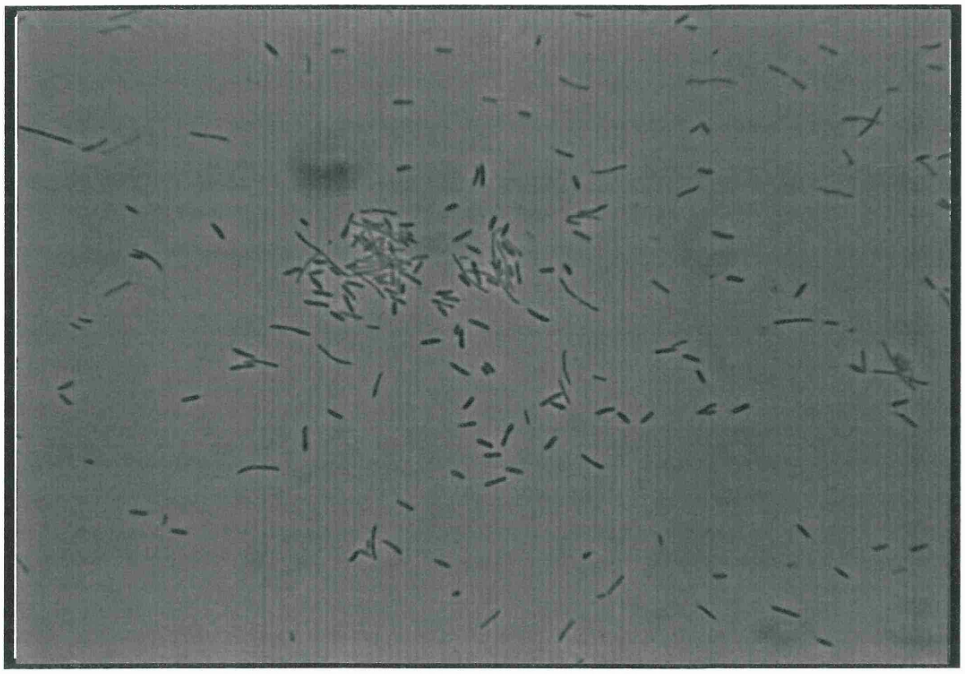


Fig. 33. N43*verA1* grown at 30°C

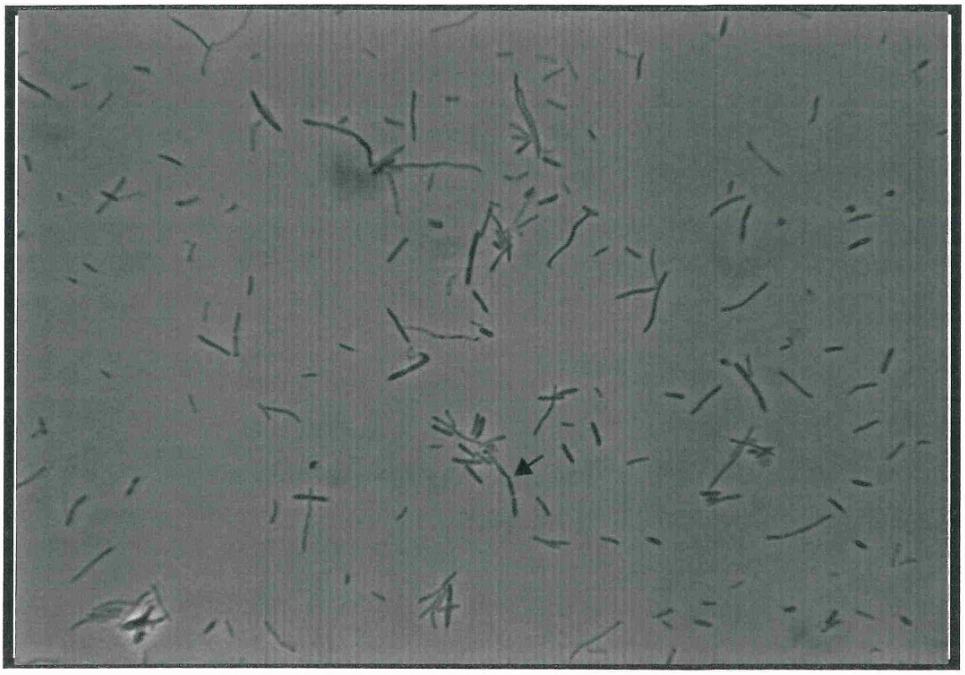


Fig. 34. N43*verA1* grown at 42°C
Note: the arrow indicates a minicell.

Chapter 7
Detailed complementation analysis of N43*verA1*

7.1 Introduction.

Having obtained evidence that the *verA* mutation affects a regulator that is required for the regulation of synthesis of many proteins, and perhaps including some proteins governing the cells' response to changes in $[Ca^{2+}]_i$, more information was required about which gene(s) encoded by plasmid pLG701 is / are needed for normal growth in high Ca^{2+} medium, verapamil sensitivity and growth at 42°C. Consequently, low copy number plasmids containing the different combinations of *orf1*, *2* and *3* were constructed, and their properties determined by studying the growth rates of N43*verA1*, MICs to Ca^{2+} and verapamil, and growth at 42°C. At this stage, as described at the end of this chapter, the identities of the genes corresponding to *orf1*, *2* and *3* were finally obtained due to new information accumulating in the computer database in the later stages of this project. With this information available, further inferences about the roles of the genes that constitute the *verA* locus were formed.

7.2 Subcloning of *orf1*, *2* and *3* into pLG339 to investigate the effects of different combinations of the three genes upon the phenotype of N43*verA1*.

Since the Tn1000 experiments failed to give conclusive results regarding which gene is required for complementation of the *verA* phenotype in **Chapter 5**, this strongly suggested that the genes corresponding to *orf*1, 2 and 3 are perhaps interacting and are all required for full complementation. Different combinations of the three genes were constructed in order to study their effects both singly, and in combination, upon the *verA* phenotypes.

Plasmid pLG703 containing *orf*3 was constructed by digesting pLG701 with *Eco*RI and *Stu*I, and cloning into the *Eco*RI and *Hinc*II sites of pLG339 (see **Figs. 10 and 11**). pLG707 was obtained from the Tn1000 experiments described in the previous chapter and consists of pLG702 with a Tn1000 in *orf2*, thus only *orf1* is functional in this plasmid. pLG708, which only contains *orf2* was constructed by digesting the M13 based plasmid, pLG712f, originally used to sequence the genes corresponding to *orf*1 and 2, with *Sal*I to yield a fragment of 1600bp, which was cloned into the *Sal*I site of pLG339. *orf2* and *3* were cloned into pLG339 by digesting both pLG339 and pLG701 with *Eco*RI and *Sal*I, and subcloning the 1.87kb fragment from pLG701 into pLG339 to form pLG709. Finally, pLG711 was constructed by digesting pLG701 with *Stu*I and *Xmn* I, deleting the 856bp fragment, and religating the plasmid to yield a construct containing *orf 1* and *3*. **Fig. 35** indicates the important restriction sites in pLG701 used in the construction of the subclones described and **Fig. 36** summarises the composition

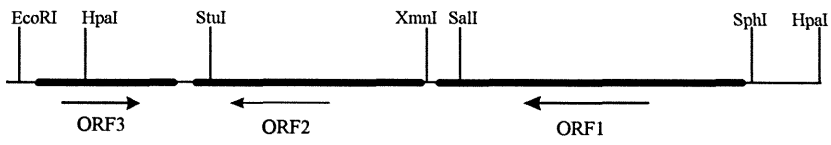


Fig 35. Restriction sites in the *verA* locus used for subcloning.

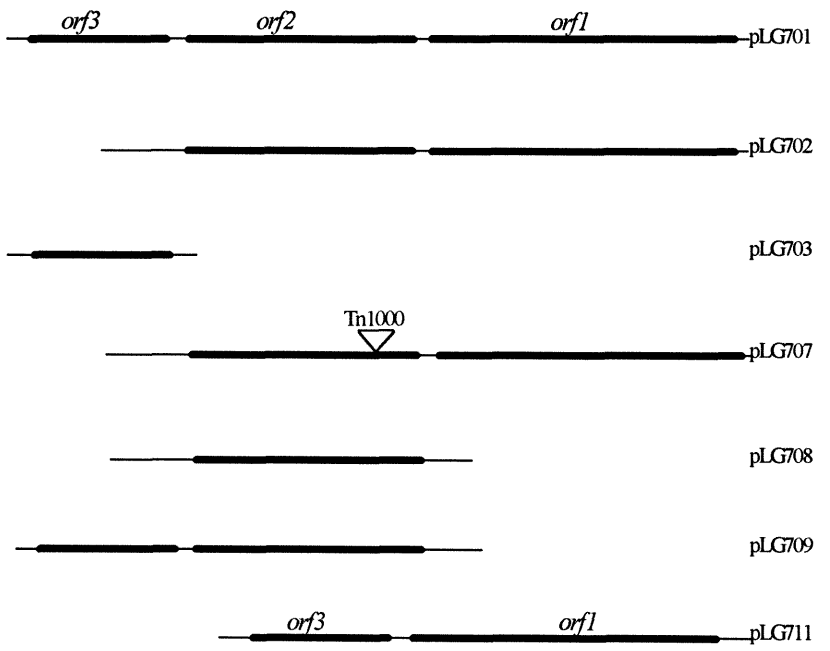


Fig 36. Summary of low copy number plasmid constructs containing the different combinations of genes *orf1*, *2* and *3* (not drawn to scale). The heavy bars correspond to the intact ORFs.

of each plasmid. Each plasmid was transformed into N43*verA1*, and comparisons of the effects of each plasmid construct upon the phenotype of the mutant determined.

7.3 Testing of plasmid constructs for restoration of growth of N43*verA1* at 42°C.

The strains N43, N43*verA1* and N43*verA1* containing either pLG339, pLG701, pLG702, pLG703, pLG707, pLG708, pLG709 or pLG711 were streaked for single colonies onto duplicate M9 plates containing kanamycin, where appropriate. One set of plates was incubated at 30°C and the other set at 42°C, for 24h. The degree of growth was recorded by comparison with N43, on a -, +, ++ or +++ (wild type) scale. The results are summarised below in **Table 12**, and indicate that no single gene will complement the *ts* phenotype. pLG709, which contains *orf2* and *orf3*, appears to restore *wt* levels of growth, and pLG702 (*orf1* and *orf2*) restores growth at 42°C, close to that observed in the wild-type. Interestingly, in an experiment where N43*verA1* and N43 were infected with bacteriophage P1 that previously been grown on the strain GM230 (see **Chapter 8.3** and **Materials and Methods**) containing a Tn10 transposon in *orf3*, both the mutant and parent transductants were found to grow at 42°C as strongly as wild-type N43. Thus indicating that the gene conferring temperature sensitivity is located in either *orf1* or *orf2* (the wild-type allele of these genes would have been introduced during the transduction).

7.4 Comparison of growth rates of N43 and N43*verA1* containing the plasmid constructs.

5ml overnight cultures of the strains indicated in section **7.3** were grown in M9 medium at 30°C, and diluted 1:75 in 20ml of fresh, prewarmed M9 medium and shaken at 30°C. The absorbance of each culture was measured at 600nm at regular intervals with a Pharmacia Ultrospec III spectrophotometer. The absorbance values were recorded on 2-cycle semi-logarithmic graph paper, and the mean generation times (τ) calculated. The τ values which are recorded below in **Table 12**, permit a comparison of the effects of the different gene combinations, upon restoration of the growth rate of N43*verA1* to *wt* values. The results indicate that *orf3* is necessary and almost sufficient for restoration of the *wt* growth rate at 30°C. However, pLG702 (*orf1* and *orf2*) also greatly improves the generation time.

7.5 Comparison of the effects of the different plasmid constructs upon the sensitivity of the mutant to verapamil.

MIC determinations for verapamil were carried out to establish the effects of the different genes upon verapamil sensitivity in the mutant containing the plasmids

described above, and in N43. The MIC procedures were carried out as described in **Chapter 6**, section **6.2** and Materials and Methods, using 2-fold step increases in concentration of verapamil ranging from 0.1 to 6.4mM. The results are summarized in **Table 12**, below and indicate that the level of verapamil resistance is determined by more than one gene, and in fact, all three genes, *orf1*, *orf2* and *orf3* are needed to restore normal levels of verapamil sensitivity. Surprisingly, *orf2* alone or with *orf1* results in significant **hyper-resistance** to verapamil. Equally surprisingly, cloned *orf3* alone actually renders the mutant **hyper-sensitive** to verapamil.

7.6 Determination of the effects of the different plasmid constructs upon the sensitivity of N43*verA1* to Ca^{2+}.

Ten sets of tubes containing 5ml TCG medium supplemented with kanamycin where appropriate, and $CaCl_2$ added exogenously in the following concentrations, were set up: 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4 and 2.8mM. To each tube, 10^5 cfu of an overnight culture of the appropriate strain was added, and all the tubes incubated by shaking at 30°C for 48h. The calcium concentration of the medium without the addition of calcium was determined using a Varian Techtron atomic absorption spectrophotometer model AA-6, and found to have a calcium concentration of less than 2μM. As usual, the MIC was determined as the lowest concentration of $CaCl_2$ to

Strain	ORFs	$CaCl_2$ concentrations (mM)										MIC (mM)
		.05	0.1	0.2	0.4	0.8	1.2	1.6	2.0	2.4	2.8	
N43	1, 2, 3	+	+	+	+	+	+	-	-	-	-	1.6
N43*verA1*		+	+	+	+	-	-	-	-	-	-	0.8
N43*verA1* pLG339		+	+	+	+	-	-	-	-	-	-	0.8
N43*verA1* pLG701	1, 2, 3	+	+	+	+	+	+	-	-	-	-	1.6
N43*verA1* pLG702	1, 2	+	+	+	+	+	-	-	-	-	-	1.2
N43*verA1* pLG703	3	+	+	+	+	+	-	-	-	-	-	1.2
N43*verA1* pLG707	1	+	+	+	+	-	-	-	-	-	-	0.8
N43*verA1* pLG708	2	+	+	+	+	+	+	+	+	+	+	>2.8
N43*verA1* pLG709	2, 3	+	+	+	+	+	+	+	+	+	+	>2.8
N43*verA1* pLG711	1, 3	+	+	+	+	+	-	-	-	-	-	1.2

Table 11. [Ca^{2+}] MIC values for N43 and N43*verA1* containing low copy-number plasmids with different combinations of the *verA* genes. ([Ca^{2+}] of TCG medium without added calcium <2μM). Key: + = growth; - = no growth.

inhibit growth. The results are recorded in **Table 11**, and summarised in **Table 12**, and indicate that the Ca^{2+} resistance levels identical to those of the *wt* parent N43, were only restored when all three ORFs were present, and intact. Interestingly, *orf2* alone again causes hyper-resistance, this time to calcium, but requires the presence of *orf1* to down-regulate this effect, instead of *orf3*, neccessary to down-regulate hyper-resistance to verapamil conferred by *orf2*.

In an unrelated experiment, when N43*verA1* was added to M9 medium containing 1mM $CaCl_2$, the cells lysed (unpublished data). This lysis must involve a metabolically active process, since it is possible to transform N43*verA1*, using the $CaCl_2$ method (see Materials and Methods, **Chapter 2**), which uses 100 mM $CaCl_2$. However, during transformation, the cells were maintained at 0°C, and no apparent loss of viability was noticed.

7.7 Identification of the genes provisionally designated *orf1, 2* and *3*.

At every opportunity, the GenBank database was consulted to find out whether the genes that have been referred to as *orf1*, *orf2* and *orf3* had been identified by other research groups, and any function assigned. However, sequence analysis indicated that *orf1* encoded a novel response regulator component of the super family of 2-component response regulators (see Stock *et al* (1989) for a review). No publications have been produced to date, regarding the function of this gene, which was initially named *hnrG* (Conteras, 1992). Since no function has yet been ascribed to this gene, or its protein product, I propose renaming the gene Response Regulator X (*rrx*). From this point, *orf1* will therefore be regarded as *rrx*. The second gene was identified from the database as *galU*, and encodes the enzyme α-D-glucose-1-phosphate uridylyl transferase (UDPGP) (Fukasawa *et al.*, 1962; Ueguchi and Ito, 1992; Weissborn *et al.*, 1994). *orf3* was found to encode the histone-like DNA binding protein H-NS, the gene being *hns* (Varshavsky *et al.*, 1977; Spassky *et al.*, 1984; Pon *et al.*, 1988; La Teana *et al.*, 1989; Hulton *et al.*, 1990). **Fig. 37** shows a detailed restriction map of pLG701 with the three genes *rrx*, *galU* and *hns* included.

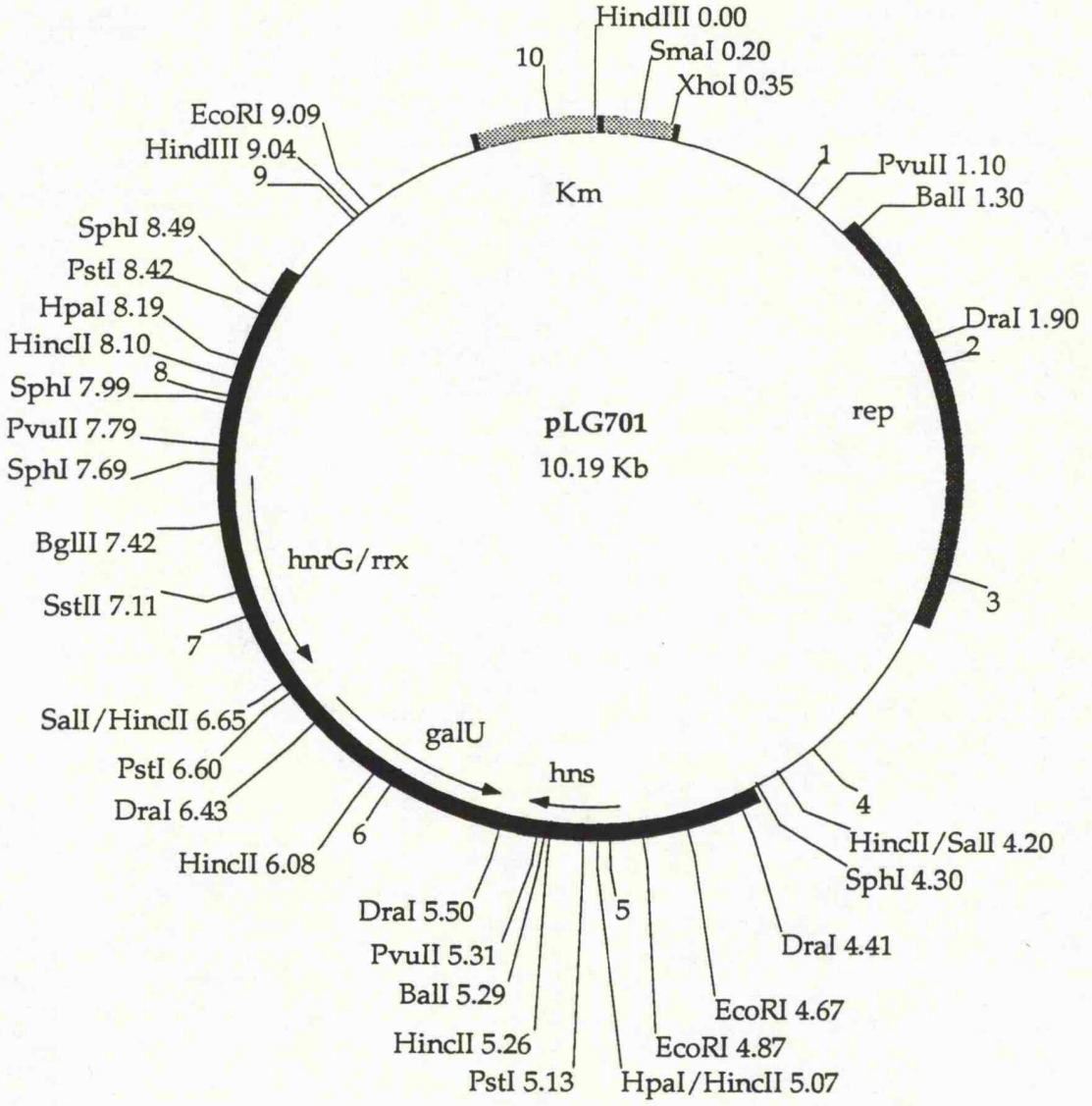


Fig 37. Detailed restriction map of pLG701 indicating the genes *rrx*, *galU* and *hns*.

7.8 Summary of effects of *hns*, *galU* and *rrx* upon the phenotype of N43*verA1*.

Strain (+plasmid)	*hns* (*orf3*)	*galU* (*orf2*)	*rrx* (*orf1*)	Verapamil MIC (mM)	[Ca^{2+}] MIC(mM)	Growth at 42°C	Mean Gen. Time (τ, mins)
N43				0.4	1.6	+++	70
N43*verA1*				0.8	0.8	-	180
N43*verA1*pLG701	+	+	+	0.4	1.6	+++	72
N43*verA1*pLG702	-	+	+	3.3	1.2	++	108
N43*verA1*pLG703	+	-	-	0.05	1.2	-	80
N43*verA1*pLG707	-	-	+	0.8	0.8	-	168
N43*verA1*pLG708	-	+	-	3.3	>2.8	+	170
N43*verA1*pLG709	+	+	-	0.2	>2.8	+++	70
N43*verA1*pLG711	+	-	+	0.2	1.2	-	72

Table 12. Summary of MICs for calcium and verapamil, growth rates (measured at 30°C) and growth at 42°C in N43*verA1* containing low copy-number plasmids with different combinations of the 3 genes *rrx*, *galU* and *hns*. In the column representing growth at 42°C, the symbols -, +, ++, +++ represent no growth, weak growth, intermediate growth and wild type growth respectively, as compared with N43 under identical conditions. The columns labelled *hns*, *galU* and *rrx* are included to indicate the genes encoded by the plasmid.

7.9 Complex interactions between *hns*, *galU* and *rrx*

From the summarised data, it is apparent that some form of complex interaction is taking place between the three genes. An examination of the verapamil MIC results (determined at 30°C) reveals that if the plasmid contains *galU*, but not *hns*, the mutant becomes substantially more resistant to verapamil. The same effect is also observed, using the *galU* plasmid in the parent strain, N43 (data not shown). On the other hand, *hns* alone renders the mutant more sensitive to verapamil than the parent. *hns* and *galU* together also appear to make the mutant hypersensitive to verapamil. This therefore tends to suggest that the mutation giving rise to drug resistance affects both *hns* and *galU*. However, when one considers the restoration of growth at 42°C, a different and much more complicated story emerges. No single gene will restore growth at 42°C. *galU* restores only very weak growth at 42°C, *galU* and *rrx* together restore intermediate levels of growth, whereas *galU* and *hns* restore *wt* levels of growth.

The Ca^{2+} MIC results provide even further indications of interactions. Only N43*verA1* containing pLG701 has an identical MIC to the parent. *galU* again, as for verapamil, makes the mutant much more resistant to the levels of Ca^{2+} in the medium. However, in this instance, *hns* has no effect upon *galU*, in reducing the MIC. Only *rrx* affects the elevated MIC caused by *galU* (see pLG702), and in fact reduces the MIC to

a value below that of N43. *hns* raises the Ca^{2+} MIC of the mutant slightly, but clearly cannot fulfill the entire function.

Measurements of growth rate strongly suggest that *hns* is minimally required for restoring the normally very long generation time of the mutant to a value approaching that of the parent. However, pLG702, which contains *rrx* and *galU* also has a strong influence, but the two genes, when transformed independently into N43*verA1* (pLG707 and pLG708 respectively) have no effect upon the growth rate.

The gene *rrx* does not appear to interact directly with *hns* in any of the tests performed. However, as demonstrated in **Chapter 5**, H-NS appears *in vitro* and in minicells to repress the level of expression of *rrx* (*orf1*). It also appears that *galU* is central to all of the observed phenotypes. However, transforming the mutant with a plasmid containing just *wt galU* does not simply restore the *wt* phenotype. On the contrary, the mutant becomes more resistant to both verapamil and Ca^{2+}, and requires the input of *hns* or *rrx* to reduce the respective hyper resistance to verapamil and Ca^{2+}. Restoration of growth at 42°C again requires *galU*, but this too requires the added input of *hns* and / or *rrx*.

It is finally important to note that although N43*verA1* containing pLG703 (*hns*) and pLG711 (*hns* and *rrx*) appears to grow rapidly in liquid culture, on solid media, these strains grow poorly, and, especially with pLG711, the strain dies after a short period of storage at 4°C. Similarly, N43*verA1* containing pLG707 (*rrx* only) which is also a poor growing strain, does not survive prolonged storage at 4°C. Thus, long term survival clearly requires *galU*, at least in this genetic background.

7.10 Conclusions from the complementation data.

As correctly predicted from the results described in Chapters 5 and 6, the *verA* mutation does affect regulatory proteins. Indeed, 2 of the 3 proteins required for restoration of the *wt* phenotype are regulators and evidence is emerging that GalU/UGPGP also has a regulatory role (see later). H-NS (formerly known as H1a and OsmZ) is currently under very intensive investigation by many groups. It is a heat stable, 15.5kDa, non basic DNA-binding protein that exists mainly as homodimers *in vivo* (Falconi *et al.*, 1988). H-NS has been shown to be primarily associated with the bacterial nucleoid (Dürrenberger *et al.*, 1991), and has been shown by many groups to be involved in the regulation of a large number of genes concerned with responding to environmental stimuli, such as osmoregulation (Higgins *et al.*, 1988), temperature regulation (Göransson *et al.*, 1990; Ito *et al.*, 1994), pH regulation (Shi *et al.*, 1993), growth phase control (Dersch *et al.*, 1993), virulence gene regulation (Higgins *et al.*, 1990; Dagberg and Uhlin, 1992; Dorman and Ní Bhriain, 1993) and supercoiling of the chromosome (Higgins *et al.*, 1988; 1990; Hulton *et al.*, 1990; Owen-Hughes *et al.*,

1992). This gene has been shown to be involved in the regulation of many diverse genes, but little is understood about its mode of action. Two models have been developed, one which involves a role for H-NS in modulating changes in the superhelicity of the nucleoid (Hulton *et al.*, 1990), and the other which involves transcriptional silencing, *ie* H-NS binds to the promoters of certain genes, preventing them from being expressed (Göransson *et al.*, 1990). Increasing amounts of data are now emerging to indicate that the second model is probably correct, and that changes in supercoiling are more likely controlled through an indirect mechanism (see **Chapter 10** for discussion). Since H-NS plays such an important role in the regulation of so many physiological processes, it is highly plausible that it also plays a role in the control of the $[Ca^{2+}]_i$.

Although the sequence of the *E. coli galU* gene has been only recently published (Ueguchi and Ito, 1992; Weissborn *et al.*, 1994), its existence has been known since 1962 (Fukasawa *et al.*, 1962), but its genetic location was not accurately mapped. The enzyme encoded by *galU* (UDPGP) catalyses the reaction UTP + glucose --> UDP-glucose. UDP-glucose is incorporated into the lipopolysaccharide (LPS), as part of a four nucleosidediphosphate (NDP)-hexose oligomer (Jiang *et al.*, 1991), and used as a precursor in trehalose synthesis, which is used as an osmoprotectant (Giaever *et al.*, 1988; Strøm and Kaasen, 1993). Flagella formation is reported to be affected in *galU* mutants (Komeda *et al.*, 1977), as is galactose utilisation (Fukasawa *et al.*, 1962). The role of GalU in resistance to Ca^{2+} and verapamil is unclear, however one possibility is that excess UDP-glucose could result in changes in the LPS, that render the cell envelope more impermeable to some external agents. Finally, UDP-glucose or a closely related compound has been shown to negatively regulate *rpoS* (σ^S, the stationary phase sigma factor) (Böhringer *et al.*, 1995).

The third gene, *rrx*, is the least well characterized of the three genes. However, from its sequence, it appears to be a classical response regulator of the 2-component response regulator family, (see **Appendix 1**). These are involved in the physiological maintenance of many bacterial systems, ranging from the regulation of phosphate metabolism, aerobic / anaerobic respiration, virulence genes, osmoregulation, nitrogen metabolism, chemotaxis, sporulation, and transport. In all cases, the first component of these regulators is a sensor protein, which is almost always bound to the cytoplasmic membrane. This histidine protein kinase is capable of transferring the phosphoryl group from ATP to histidine residues within the kinase domain of the protein. The phosphate is then subsequently transferred to an aspartic acid residue in the response regulator. Once the response regulator has been phosphorylated, it can bind to specific DNA promoters, and thus modulate gene activity. There is a certain amount of cross-talk between sensor proteins and response regulators, which permits a degree of fine tuning

of responses to stimuli (Stock *et al.*, 1989). An important feature of all response regulators, is the requirement for a divalent metal ion. Often the ion is magnesium, however, other ions can also bind and confer activity. Indeed, calcium is sometimes involved (Stock, J. Princeton Univ., N.J., personal communication). Moreover, it has been demonstrated that Ca^{2+} ions enhance the phosphorylation of the response regulator OmpR by the membrane-bound histidine kinase EnvZ (Rampersaud *et al.*, 1991). This 2-component response regulator is concerned with regulating expression of the outer membrane proteins OmpC and OmpF in response to changes in the osmolarity of the growth conditions. As developed in the discussion (**Chapter 10**), whilst this thesis was in the late stages of preparation, it became known that *rrx* regulates RpoS activity by a novel mechanism.

From the complementation data, it is very difficult to identify a specific gene within the *verA* locus that has been mutated, and is responsible for the complex phenotypes observed with the *verA1* strain. The most likely genes to be affected by the mutation are *hns* and *galU*, although interestingly, temperature sensitive growth mutations have not been previously associated with either *hns* or *galU*. Bearing in mind that the mutant is *ts*, the mutation should theoretically be subtle. The precise nature of the mutation in N43*verA1* was therefore investigated in detail and is described in the next chapter.

Chapter 8
Identification of the mutation responsible for the *verA* phenotype

8.1 Introduction

The previous chapter highlighted the complexity of the *verA1* mutant. No single gene, when cloned into the low copy-number plasmid, pLG339 and transformed into the mutant, restores the *wt* phenotype. On the contrary, different phenotypes of the mutant appear to require different combinations of the three genes *hns*, *galU* and *rrx*, (but preferably all) to restore the *wt* phenotype. Additionally, transformation of the *wt galU* gene alone causes the mutant to become hyper-resistant to calcium and verapamil. This therefore creates considerable difficulties in determining which gene to investigate for the presence of a mutation. Several approaches were undertaken to test for mutations in *hns*, *galU*, and *rrx*. These approaches included, a) use of the suicide vector pCVD442, to replace the chromosomal copies of i) *rrx*, *galU* and the 3'-terminus of *hns*, and ii) the 5'-terminus of *hns* by conjugation, b) Western blotting to test for the expression of H-NS in the parent and mutant, c) PCR reactions to isolate, clone and sequence the *hns* gene from the mutant, d) Northern blots to test for expression of *hns* and *galU* in the mutant, and finally, e) Southern blots were performed to confirm the identity of the mutation in N43*verA1*.

8.2 Use of the suicide vector, pCVD442 to introduce the wild-type *verA* genes into N43*verA1*

Until now, all complementation analysis has involved the use of the low copy-number plasmid, pLG339 to act as a vector for introducing the *verA* genes into the mutant, and to test the effects of the different plasmids upon the various phenotypes of the mutant. A possibly more accurate method of identifying the site of the mutation is to replace the mutant chromosomal locus with the equivalent wild-type sequence cloned into a suicide vector, since this avoids any problems with copy-number effect. The suicide vector system chosen for these experiments was based upon plasmid pCVD442 (Donnenberg and Kaper, 1991), and uses the *sacB* positive selection system (see **Fig. 38**). Briefly, the plasmid functions as follows: the origin of replication is based upon plasmid R6K, and can only replicate in a background containing the *pir* gene which encodes π protein, required for functioning of the R6K origin. The remainder of the plasmid is derived from pBR322 and includes the β-lactamase gene for selection purposes. Manipulation and cloning into pCVD442 was performed using the λ*pir* lysogen, SY327. In order to mobilise and conjugate the suicide plasmids into N43*verA1*, pCVD442 and its derivatives were first transformed

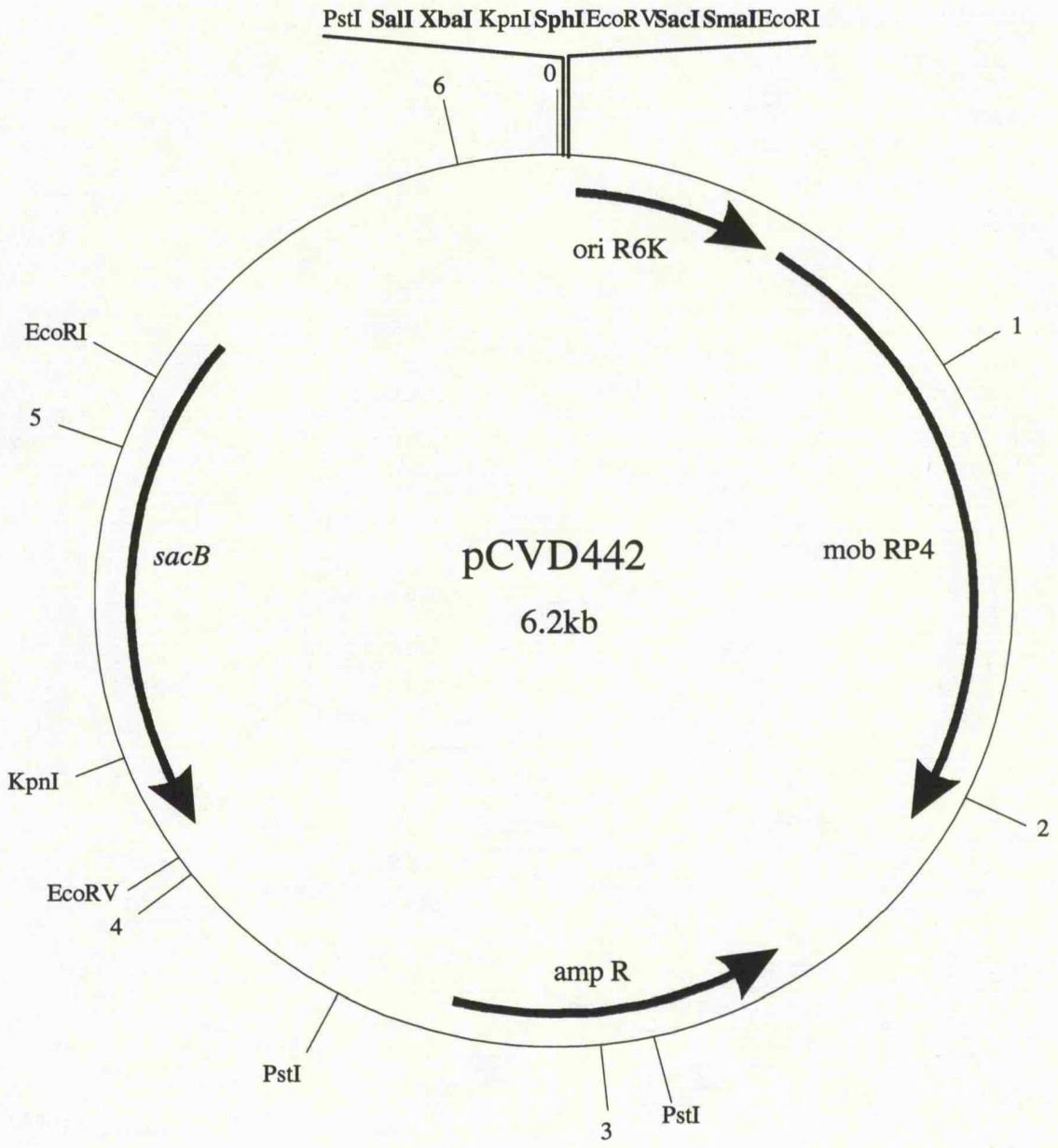


Fig 38. Suicide vector pCVD442 (Donnenberg and Kaper, 1991), used for introducing the wild-type *verA* alleles into the chromosome of N43*verA1*.

The restriction sites in bold represent unique sites.

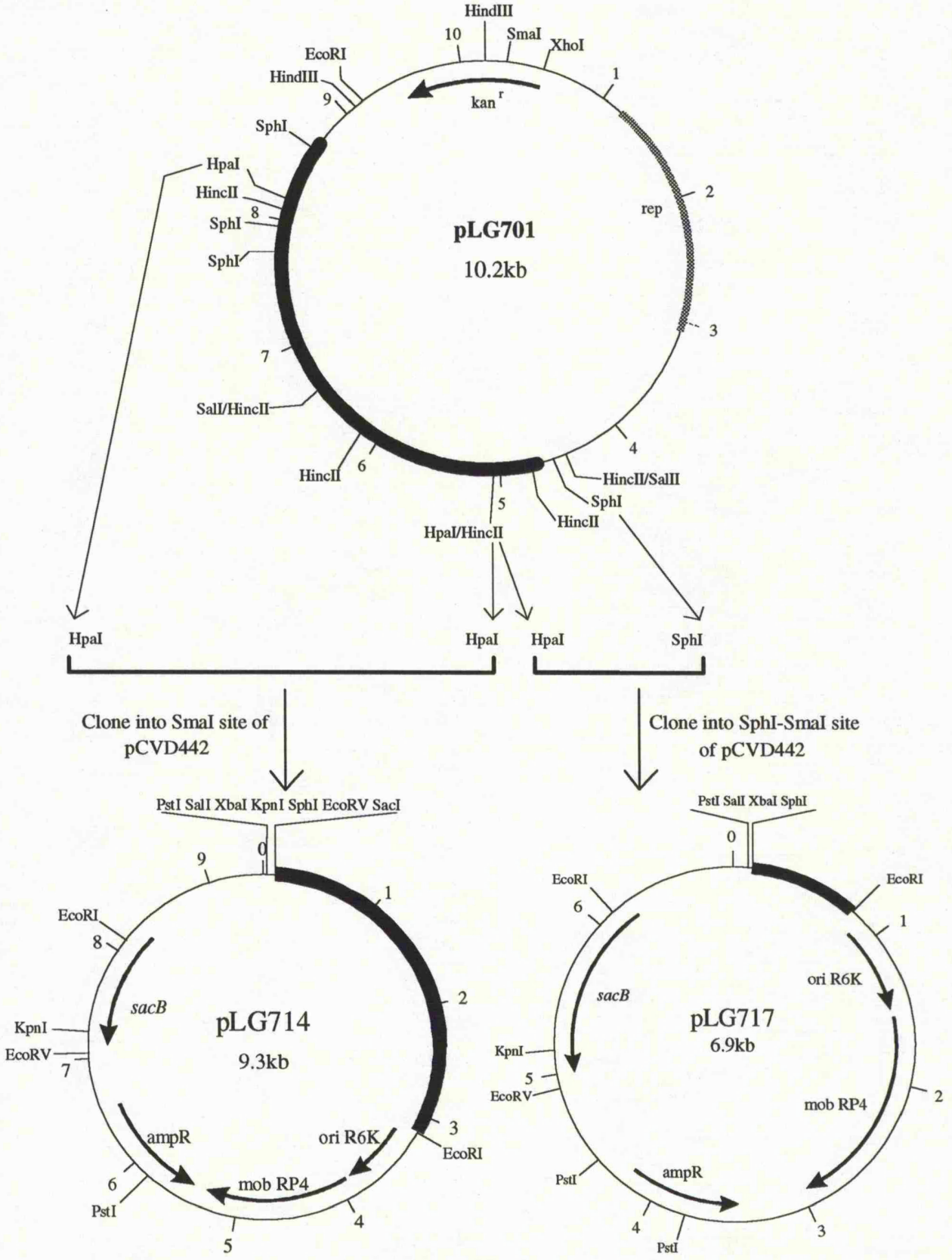


Fig 39. Construction of the pCVD442-based plasmids pLG714 and pLG717, used to introduce the wild-type *verA* alleles into N43*verA1*.

into the strain SM10 λ*pir* which contains the *trans*- acting factors required for mobilisation of pCVD442.

Following conjugation of the suicide plasmids into the λ*pir*⁻ *recA*⁺ streptomycin resistant strain, N43*verA1*, the mating mixture was inoculated onto NA plates containing ampicillin and streptomycin. The streptomycin was added to select against the donor cells in the mating mixture. Since the plasmid cannot replicate within N43*verA1*, it is eventually lost. However, occasionally, a recombination event occurs between the insert cloned into the plasmid and the chromosome, resulting in integration of the plasmid into the chromosome and the formation of an ampicillin resistant stable merodiploid. Replacement of the original mutant copy of the gene(s) of interest requires a second recombination event in which the chromosomally integrated plasmid excises from the chromosome, accompanied either by the wild-type genes originally cloned into the plasmid, or the mutant gene(s) to be replaced. These plasmids are subsequently lost, through their inability to replicate in the *pir*⁻ background. Within plasmid pCVD442 is the gene *sacB* which confers sucrose sensitivity. Thus, any cells containing the chromosomally integrated plasmid will be killed when inoculated onto LA plates containing 6%$^{w}/_{v}$ sucrose. The resulting colonies growing on the 6% sucrose plates should either be *verA* or *verA*⁺, depending upon the fate of the second recombination event. These were tested for their ability to grow on M9 minimal medium plates, at 42°C.

Because of the potential toxicity of cloning the intact *hns* gene into a medium copy-number vector such as pCVD442, two constructs were prepared (see **Fig. 39**): plasmid pLG714, consisting of pCVD442 with the genes *rrx*, *galU* and the 3'-terminus of *hns*, was constructed by cloning the 3.17kb *Hpa*I-*Hpa*I fragment originally cloned into pLG702, into the *Sma*I restriction site of pCVD442. The promoter and 5'-terminus of *hns* was cloned into pCVD442 by digesting pCVD442 with *Sma*I and *Sph*I, and pLG701 with *Sph*I (cuts in the vector) and *Hpa*I, and cloning the 770bp fragment to form pLG717. Having successfully constructed both plasmids using the strain SY327 λ*pir*, the plasmids were then transformed into SM10 λ*pir* and conjugated into N43*verA1* as described in Materials and Methods. **Table 13** below summarises the results of the conjugation stage of the experiment:

Conjugation (Donor-->Recipient)	No. of exconjugants (per 10^8 recipients)	Frequency of integration of plasmid into chromosome.
SM10 pLG714->N43*verA1*	65	6.4×10^{-7}
SM10 pLG717->N43*verA1*	110	1.3×10^{-6}
SM10 pCVD442->N43*verA1*	126	1.25×10^{-6}

Table 13. Summary of results from the conjugation of pLG714, pLG717 and pCVD442 into N43*verA1*.

50 merodiploids from each conjugation were selected at random, inoculated onto NA + ampicillin and M9 minimal medium plates, and incubated at 37 and 42°C. This step was used to check that the merodiploids had potentially inherited a *wt* allele that was capable of restoring growth of the mutant at 42°C. Of the 50 merodiploids that acquired a copy of pLG714, 17 grew well at 42°C, the remaining 33 either failed to grow, or grew very poorly. None of the merodiploids receiving pLG717 or pCVD442 became ts^+. One ts^+ merodiploid containing pLG714, and two merodiploids containing pLG717 and pCVD442 were inoculated into 5ml LB + ampicillin, and incubated overnight at 30°C. 1ml of each culture was centrifuged briefly, and the pellets resuspended in 1ml PBS buffer. In addition, 1/10 dilutions of each suspension were prepared. 100µl of each neat and diluted suspension were inoculated onto LA plates containing 6%$^{w}/_{v}$ sucrose, to select for those cells that had undergone the second recombination event, and lost the suicide plasmid. These plates were again incubated overnight at 30°C. Approximately 800 colonies grew on the plates inoculated with the 1/10 diluted bacterial suspensions. 50 colonies from each sucrose plate were selected at random, inoculated onto M9 plates, and tested for their ability to grow at 42°C. Of the 50 colonies that were derived from the merodiploids inheriting pLG714, 19 grew at 42°C, although not as strongly as the N43 control that had also been included. None of the colonies derived from the merodiploids inheriting pLG717 or pCVD442 grew at 42°C. In conclusion, it appears that the mutation is located within either *rrx*, *galU* or the 3'-terminus of *hns*. However, since pLG702, which contains the same insert as pLG714, enables N43*verA1* to grow to the same degree at 42°C, the most likely conclusion is that the mutation is situated in either of the two intact genes, *galU* or *rrx*.

It is noteworthy that in these experiments, there were a large numbers of exconjugants resulting from the mating of SM10 pCVD442 with N43*verA1*. This observation was also remarked upon by R. Haigh, (this laboratory, personal communication) and Donnenberg and Kaper (1991), who claim that random integration of pCVD442 into the chromosome can occur, and recommend the performance of Southern blots of merodiploids to confirm correct integration of the plasmid into the chromosome. In this instance, because many of the merodiploids resulting from the SM10 pLG714 / N43*verA1* mating became ts^+, it was considered unneccesary to perform Southern blot analysis at this stage.

8.3 Western blot analysis to test for expression of H-NS in N43 and N43*verA1*

Having established that the locus conferring temperature sensitivity in N43*verA1* is located either in *galU* or *rrx*, it was still regarded as important to check that H-NS was being correctly expressed in the mutant, since much of the

complementation data in the previous chapter suggested a possible involvement of H-NS in the mutant phenotypes. A simple technique to test for expression of H-NS is by Western blotting. Rabbit-derived anti-H-NS antibody was kindly donated by A. Spassky, Insitut Pasteur, Paris, France. The method used was based upon the procedure of (Towbin and Gordon, 1984), and is described in detail in Materials and Methods. The strains used in this experiment are listed in **Table 14** below:

STRAIN *	SOURCE / REFERENCE
GM37 (*hns*$^+$)	CF Higgins. ICRF laboratories, University of Oxford, UK (May *et al.*, 1986)
GM230 *hns*::Tn10	CF Higgins. ICRF laboratories, University of Oxford, UK (May *et al.*, 1986)
MC4100 (*hns*$^+$)	(Casadaban, 1976)
MC4100 *hns*::Tn10	F Moreno, Unidad de Genética, Hospital Ramon y Cajal, MADRID, SPAIN
N43 (*hns*$^+$)	(Nakamura and Suganuma, 1972)
N43 *hns*::Tn10	P1 transductant, derived from P1 grown on GM230
N43*verA1*	
N43*verA1 hns*::Tn10	P1 transductant, derived from P1 grown on GM230

Table 14. Strains used to test for the expression of H-NS by Western blotting.
* Full strain details are listed in Materials and Methods

The strains N43*hns*::Tn10 and N43*verA1 hns*::Tn10 were prepared by infecting with bacteriophage P1 that had been previously grown on the strain GM230, using the method described in **Chapter 3**, and in Materials and Methods. Tetracycline resistant colonies resulting from these transductions were isolated, and used for the preparation of protein extracts to provide isogenic negative controls in the Western blot.

The protein samples were prepared from overnight cultures of each strain, grown at 30°C. 1.0 A_{600} unit of each culture was centrifuged, and the bacteria processed for the preparation of heat-stable protein fractions as described in Materials and Methods. Heat-stable protein fractions were used since H-NS has been reported (Spassky and Buc, 1977) and confirmed (data not shown), to remain in solution following boiling and centrifugation, unlike 90% of *E. coli* proteins which become denatured, precipitate and form a pellet during centrifugation (Chen, 1990; Laoudj *et al.*, 1994). Thus, this method was considered useful for reducing potential "background" in the Western blot. A 13%$^{v}/_{v}$ SDS-polyacrylamide mini-gel (BioRad Mini Protean II system) was loaded with 30µl of each boiled protein sample and 5µl of molecular weight standards (Sigma high molecular weight standards, range: 29-116kDa). The proteins were electroblotted onto 0.45µm pore-size nitrocellulose (Schleicher and Schuell) using the conditions described in Materials and Methods.

Successful transfer of the proteins was verified by staining the filter with Ponceau S (Sigma Chemical Corp.). The H-NS protein bound to the filter was probed and detected using the protocol described in Materials and Methods. The anti-H-NS antibody was used at a dilution of 1:500, and the goat-derived anti-rabbit alkaline phosphatase conjugate (DakoPats) was used at a dilution of 1:1000. **Fig. 40** shows the result of the Western blot, and reveals that H-NS is not being detectably expressed in N43*verA1*. H-NS is clearly visible in all the parental strains tested, but absent in their isogenic strains containing Tn10 transposons within the *hns* gene. Note also, that no H-NS cleavage product is detectable on the Western blot, in contrast to the Zubay and minicell experiments, using radiolabel described in **Chapter 5**. It is now clear however, that under these blotting conditions, the cleavage products of H-NS are not transferred (D Laoudj, personal communication). Two possible conclusions can be derived from this result: *either* there is a mutation in *hns* that affects its expression *or* one or both of the other genes (*galU* or *rrx*) is / are required for expression of *hns*. A simple method of testing the latter hypothesis was to perform a Western blot on the *ts*$^+$ N43*verA1* clones isolated from the conjugation of pLG714 into the mutant (see section **8.2**), and N43*verA1* containing pLG702, and to look for restoration of H-NS expression.

8.4 Western blot experiment to test whether introduction of the *wt galU* and *rrx* genes restores expression of H-NS in N43*verA1*

In this experiment, stationary phase cultures of the following strains were tested for expression of H-NS: N43, N43*verA1*, N43*verA1* pLG701, N43*verA1* pLG702, N43*verA1* *ts*$^+$ (exconjugant obtained from the conjugation of pLG714, (pCVD442 containing *rrx* and *galU*) into N43*verA1* (see section **8.2**), and N43*ts*. The N43*verA1* *ts*$^+$ strain was considered to have successfully acquired the *wt galU* and *rrx* genes following introduction of the suicide plasmid, pLG714, since the complementation data obtained in the previous chapter (see **Table 12**) indicates that these genes are required for complementation of the *ts* phenotype. The N43*ts* strain was isolated by P1 transduction (see section **3.13**), where bacteriophage P1 originally grown on *E. coli* 12016 containing a Tn10 transposon at 26.75' on the chromosome (0.5' upstream of *hns* on the *E. coli* linkage map), was used to infect N43*verA1*, and introduce the transposon at 26.75' into this strain. 63% of the tetracycline resistant transductants became *ts*$^+$, the remaining 37% being *ts*. One of these tetracycline resistant, *ts* transductants was selected and used to prepare $P1_{verA1}$, which was then used to infect N43. A high proportion (63%) of the tetracycline resistant N43 transductants became *ts*, and it was one of these clones that was used in this experiment.

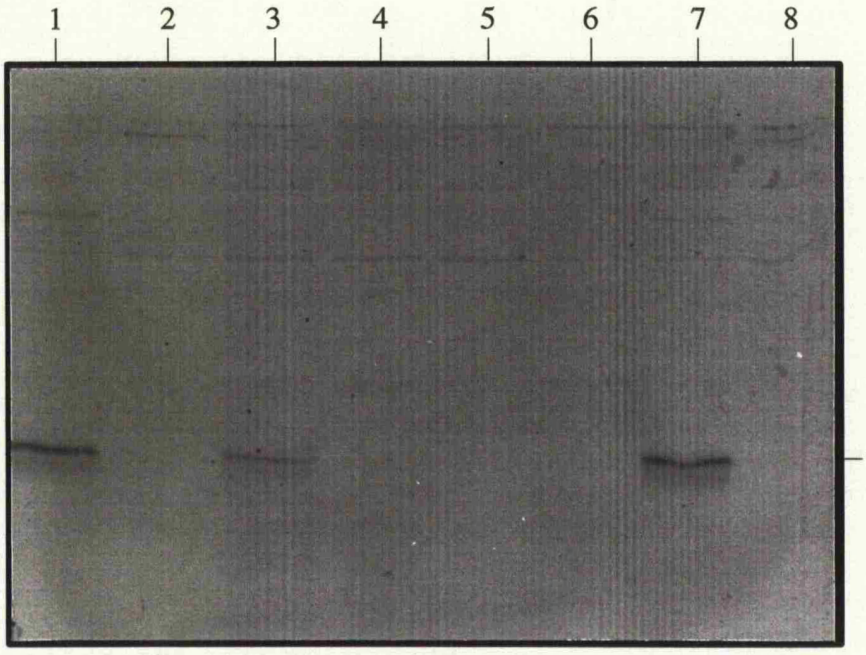


Fig. 40. Western blot testing for the expression of H-NS in N43*verA1*.

Order of samples: lane 1, MC4100; lane 2, MC4100 *hns*::Tn10; lane 3, N43; lane 4, N43 *hns*::Tn10; lane 5, N43*verA1*; lane 6, N43*verA1 hns*::Tn10; lane 7, GM37; lane 8, GM230 *hns*::Tn10.

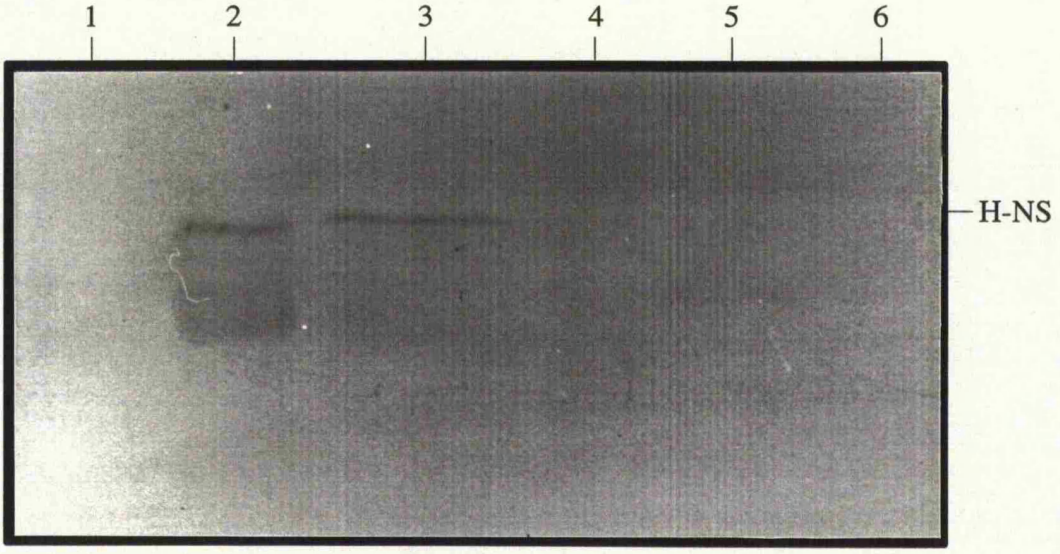


Fig. 41. Western blot to test for restoration of H-NS expression in N43*verA1*, following introduction of the wild-type *galU* and *rrx* genes.

Sample order: lane1, N43*verA1*; lane 2, N43; lane 3, N43*verA1* pLG701; lane 4, N43*verA1* pLG702; lane 5, N43*ts*; lane 6, N43*verA1* ts^+. The N43*ts* strain carrying Tn10 closely linked to *hns* was obtained by P1 transduction, using bacteriophage P1 previously grown on N43*verA1* (see text). The N43*verA1* ts^+ strain was a transconjugant isolated following the conjugation of suicide plasmid, pLG714 containing the *wt* alleles of *rrx* and *galU*, into N43*verA1* (see text).

The protein samples were prepared as described above, and separated on a 13%$^{v}/_{v}$ SDS-polyacrylamide minigel. Probing of the blot, and detection were also performed as described above. **Fig. 41** is the result of this experiment, and clearly demonstrates that the introduction of the wild-type *galU* and *rrx* genes fails to restore expression of H-NS. Also noteworthy is the confirmation that N43*ts* fails to express H-NS. This provides useful confirmation that the temperature sensitive phenotype and failure to express H-NS are at the very least very closely linked, but are both probably consequences of a single mutation. In conclusion, it appears that although the apparent introduction of wild type *galU* and *rrx* genes into N43*verA1* by P1 restores close to wild-type levels of growth at 42°C, expression of H-NS is still affected, and this therefore suggests that the mutation must affect *hns* only, or in addition to the *galU* and *rrx* genes. In order to test for mutations in *hns*, an attempt was made to isolate the gene by PCR, and to clone and sequence it.

8.5 PCR of *hns* from N43 and N43*verA1*

In order to maximise the chances of succesfully amplifying the *hns* gene from N43*verA1*, oligonucleotide primers were designed to anneal within the *tdk* gene upstream of *hns* and in the C-terminus of *galU*, named *hns7* and *hns2* respectively. The sequences of these primers were:

hns7 5'-T A C G C G A A C T G A C T T T C C C G G C A C C -3'

hns2 5'-A T G C A G G C C T T C G T T G A A T A -3'

PCR reactions were set up using the reagents described in Materials and Methods. Chromosomal DNA from N43 and N43*verA1* was prepared as described in Materials and Methods, and used as templates in the reactions. The PCR conditions used are indicated in **Table 15** below:

	1 CYCLE		30 CYCLES		1 CYCLE	
	Time (mins)	Temp (°C)	Time (mins)	Temp (°C)	Time (mins)	Temp (°C)
Denature	1	96	1	94	1	94
Anneal	1	60	1	60	1	60
Extension	3	72	3	72	10	72

Table 15. PCR conditions used to amplify the 1.4 kb fragment encoding *hns* from N43 and N43*verA1*.

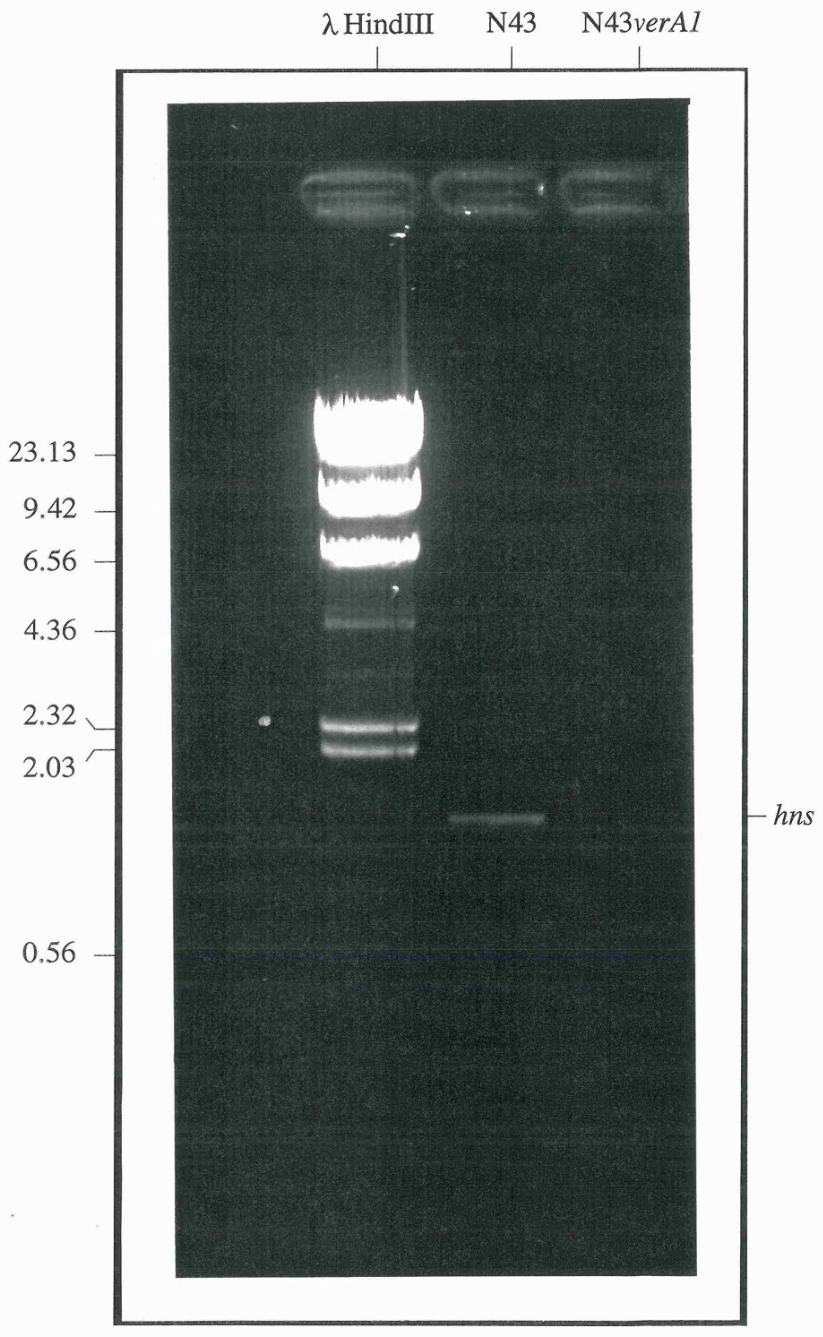


Fig. 42. PCR reaction to amplify and sequence the *hns* gene from N43 and N43*verA1*.

From the published sequence, a predicted PCR product of 1.4kb was expected. The result of the PCR reaction is shown in **Fig. 42**, and shows that a 1.4kb PCR product was indeed produced in the reaction containing the N43 chromosomal DNA. In complete contrast and curiously, no 1.4kb product was produced from the *verA1* mutant. A Southern blot of these PCR products confirmed that the 1.4kb band is *hns* (data not shown). A useful internal control in this experiment was the formation of a small number of much lower molecular weight, non-specific PCR products, that are common to both reactions, thus demonstrating that the PCR reactions worked satisfactorily. This result, combined with the antibody analysis therefore indicates a major lesion in the *hns* gene, resulting in loss of H-NS expression.

As described in Chapter **7.3**, when N43*hns*::Tn10 and N43*verA1 hns*::Tn10 (prepared by transduction using P1 grown on GM230 (*hns*::Tn10)) were tested for temperature sensitivity, they were found to be ts^+. From this observation, it was concluded that a) the lesion affecting *hns* might also affect *galU*, and b) *hns* is not essential for growth at 42°C. To test whether expression of *hns*, *galU* and *rrx* are affected by the *verA1* mutation, a Northern blot was prepared.

8.6 Northern blot to test for expression of *hns*, *galU* and *rrx* in N43*verA1*

In view of the results above with *hns* and the complex phenotypes of the *verA* mutants, it was considered essential to test directly the expression of all three genes at the *verA* locus. RNA was prepared from mid-exponential phase cultures of N43, N43*hns*::Tn10, N43*verA1 hns*::Tn10 and N43*verA1* containing plasmids pLG339, pLG701, pLG702, pLG703, pLG707, pLG708, pLG709 and pLG711, using the procedure described in Materials and Methods (the strains N43*hns*::Tn10 and N43*verA1 hns*::Tn10 were prepared by infecting N43 and N43*verA1* with bacteriophage P1 previously grown on strain GM230*hns*::Tn10 (see **section 8.3**)). The probe for detecting expression of these genes was prepared by digesting pLG701 with *Eco*RI and *Sph*I (see **Chapter 7, Fig. 37**) to yield a 3kb fragment containing *hns*, *galU* and *rrx* and purified from agarose by centrifuging through polyallomer wool (see Materials and Methods). The probe was labelled with fluorescein-dUTP, using the Gene Images™ kit manufactured by Amersham International PLC, following the procedure supplied with the kit (see Materials and Methods).

Preparation of the formaldehyde based 2.1%$^{w}/_{v}$ agarose denaturing gel and the RNA samples loaded on the gel, are described in Materials and Methods. Following electrophoresis of the RNA, the RNA was blotted onto Hybond-N nylon membrane (Amersham International PLC) using the conditions described in Materials and Methods. After cross-linking the RNA to the filter, efficient transfer was confirmed by staining the membrane with methylene blue (see Materials and

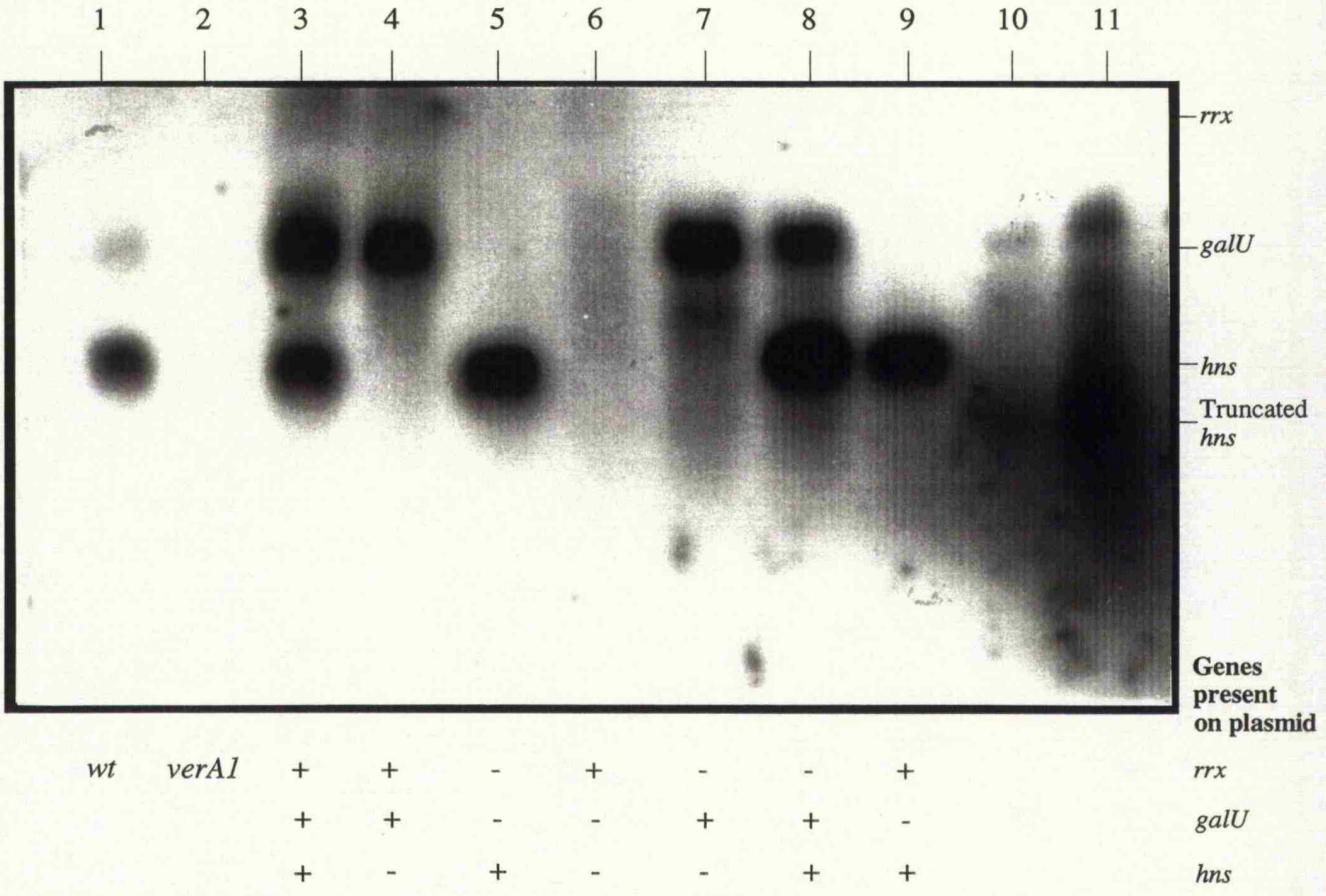


Fig. 43. Northern blot to detect expression of *hns*, *galU*, and *rrx* in N43*verA1*.

Lane 1, N43; lane 2, N43*verA1* pLG339; lane3, N43*verA1* pLG701; lane4, N43*verA1* pLG702; lane 5, N43*verA1* pLG703; lane6, N43*verA1* pLG707; lane7, N43*verA1* pLG708; lane 8, N43*verA1* pLG709; lane 9, N43*verA1* pLG711; lane10, N43 *hns*::Tn10; lane 11, N43*verA1 hns*::Tn10.

Methods). The part of the filter containing the stained RNA markers was cut from the filter and preserved for later comparison. The filter was prehybridized and hybridized using the fluorescein labelled probe described above, and the conditions. Following hybridization, the filter was washed once in 1x SSC, 0.1%$^{w}/_{v}$ SDS at 65°C and twice in 0.3x SSC, 0.1%$^{w}/_{v}$ SDS at 65°C. Detection of the bound probe was performed as instructed in the Gene Images™ protocol (see Materials and Methods).

Fig. 43 represents the result obtained from the Northern blot and clearly shows that *hns* and *galU* are expressed in N43. Unfortunately, *rrx* appears to be expressed at much lower levels, and cannot be seen in N43 under these conditions, and is only just discernable in lanes 3, 4 and 6 from strains containing *rrx* on a plasmid. In order to visualise this transcript, it is probably neccessary to probe the blot for *rrx* specifically. Most interestingly, no signal is detected from N43*verA1* for any of the three genes. This confirms the earlier suspicions alluding to the presence of a major lesion in this locus, that is affecting more than one gene. It appears that a deletion may have occurred, affecting at least *hns* and *galU*. In N43*verA1* containing the different plasmid constructs, transcripts are clearly visible corresponding to *hns* and *galU*, and very weak signals corresponding to *rrx*. In both N43 *hns*::Tn10 and N43*verA1 hns*::Tn10, *galU* is clearly visible (see tracks 10 and 11, respectively) and the *hns* transcript is truncated due to the Tn10 transposon, as expected. The fact that N43*verA1 hns*::Tn10 transductant is ts^{+}, presumably as a result of having *galU* and *rrx* restored during the P1 transduction (although the *rrx* transcript is not visible from these strains), but *hns* has been deliberately disrupted, again strongly implicates that at least one of the two genes *rrx* or *galU* is essential for growth at 42°C on M9 minimal medium plates.

8.7 Confirmation of the nature of the *verA1* mutation by Southern blot analysis

Chromosomal DNA from N43 and N43*verA1* was digested with *Sph*I, *Hinc*II and *Hpa*I, and loaded onto a 0.8%$^{w}/_{v}$ agarose gel. Following completion of the run, the gel was stained with ethidium bromide and photographed. The gel was then denatured and neutralised as described in Materials and Methods. The DNA was blotted overnight onto Hybond N nylon membrane (Amersham International PLC) as described above, and crosslinked with UV. The probe used was the fluorescein-labelled 3kb probe used in **Chapter 8.6** for the Northern blot, containing the genes *hns*, *galU* and *rrx*. Prehybridization, hybridization and detection of the probe utilised the materials and protocol supplied in the Gene Images™ kit manufactured by Amersham International PLC, as described above, and in Materials and Methods.

From **Fig. 44**, it is clear that in N43, all three genes must be present. However, in none of the restriction enzyme digests of N43*verA1*, can any signal be detected. This result therefore confirms that the basis for the *verA* phenotype involves not only a

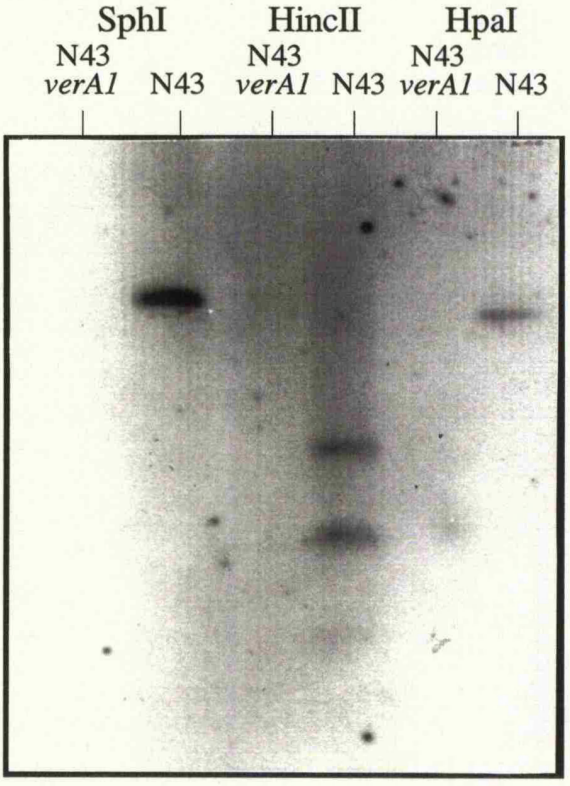


Fig 44. Southern blot analysis of chromosomal DNA from N43 and N43*verA1*, digested with *Sph*I, *Hinc*II and *Hpa*I, and probed for the genes *hns*, *galU* and *rrx*.

Chapter 9
Towards an understanding of the mechanisms resulting in resistance to calcium channel inhibitors in *E. coli*

9.1 Introduction

In the previous chapter, it was demonstrated that N43*verA1*, a spontaneous *ts* mutant strain of *E. coli*, resistant to the calcium channel inhibitors verapamil and diltiazem (see Chapter 6) is the result of a deletion of the three genes *hns*, *galU* and *rrx*. The nature of the *verA* mutation appears to be somewhat extreme, and it is important to consider whether this mutation is simply a chance occurrence, or if there is an absolute requirement for the deletion / inactivation of the *verA* locus to generate temperature sensitive mutants resistant to VOCC inhibitors. To extend the analysis to other verapamil resistant mutants, Southern blot analysis of the diltiazem resistant mutants (also verapamil resistant, data not shown) was performed, to determine whether any genetic rearrangements had occured in the *verA* locus of these strains. It is well known that *hns* regulates many cellular processes, therefore in addition, experiments were undertaken, using *hns* promoter - *lacZ* fusion constructs, to test whether *hns* expression is affected by the addition of EGTA and verapamil in an hns^+ and hns^- background.

9.2 Southern blot analysis of four diltiazem resistant *ts* mutants derived from N43

Four diltiazem resistant *ts* mutants (N43*dilA1-4*) were isolated from N43 by Hayley Sweetman in this laboratory. These mutants all share the same phenotypic characteristics as N43*verA1*, including the absence of H-NS as determined by Western blot analysis (data not shown). Similarly, all are also fully complemented by pLG701. Chromosomal DNA was extracted from each of the strains by the method described in Materials and Methods, and digested with the restriction endonucleases *Hpa*I and *Hinc*II. In addition, chromosomal DNA from N43*verA1* and N43 was digested with *Hpa*I and *Hinc*II, and included for comparison. The digested DNA was electrophoresed in a 0.8%$^{w}/_{v}$ TAE-agarose gel and blotted, using the procedure described in Materials and Methods. The probe used in this experiment was the 3kb fluorescein-labelled probe, used in the Northern and Southern blot analyses in **Chapter 8**. The filter containing the genomic digests was prehybridised and hybridised overnight at 65°C, using a final probe concentration of 5ng/ml, and washed for 15min in 1x SSC, 0.1%$^{w}/_{v}$ SDS at 65°C followed by two 15min washes in 0.1x SSC, 0.1%$^{w}/_{v}$ SDS at 65 °C. Detection of the hybridised probe was performed as directed in the Gene Images™ protocol (Amersham International PLC) (see Materials and Methods), and exposed overnight to Fuji RX X-ray film.

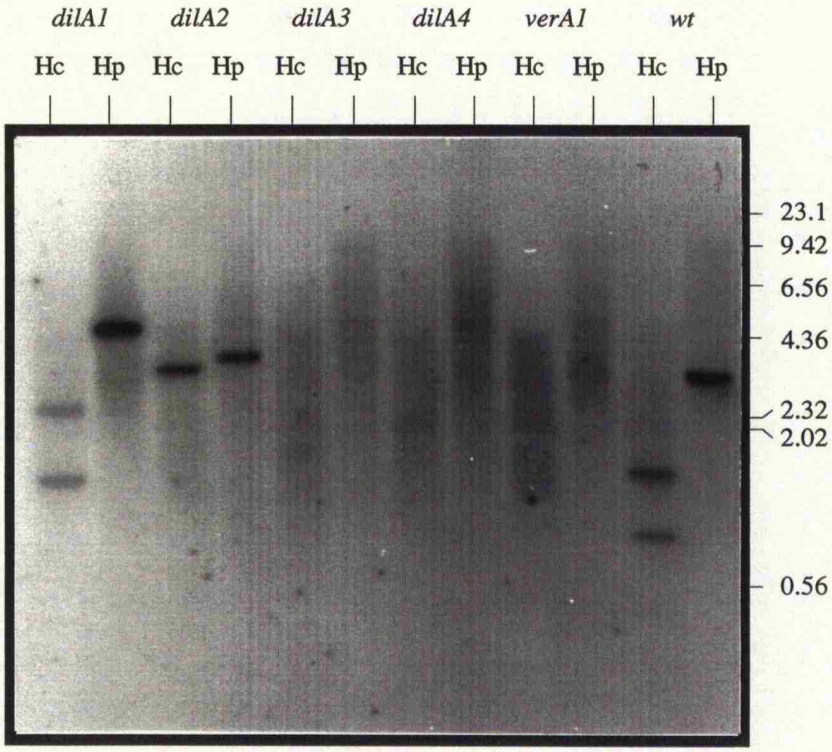


Fig 45. Southern blot of chromosomal DNA from the diltiazem and verapamil resistant mutants N43*dilA1-4* and N43*verA1* and the parent strain N43, to test for rearrangements or deletions of the *verA* locus.

Key: Hc, chromosomal DNA digested with *Hinc*II; Hp, chromosomal DNA digested with *Hpa*I.

Fig 45 shows the result of this experiment and indicates that some remarkable events have occurred in all of the mutants. In two of the mutants (N43*dilA3* and N43*dilA4*), no signal is discernible and indicates that a deletion of the *verA* locus has occured in these strains, as in N43*verA1*. Mutants N43*dilA1* and N43*dilA2* show even more curious results, indicative of genetic rearrangements of the *verA* locus, resulting either from insertional inactivation, transpositions or inversions. Indeed, this result graphically illustrates the absolute requirement for inactivation of the genes constituting the *verA* locus, to generate the observed phenotypes. The mechanism by which these deletions and rearrangements occur needs to be investigated.

9.3 Measurement of *hns* expression in N43 and N43*verA1* following treatment with verapamil

It is well recognised that inactivation of *hns* results in an increase in site specific recombination events and deletion formation (Higgins *et al.*, 1988; Lejeune and Danchin, 1990). It is also conceivable that overexpression of H-NS can cause similar problems. Previous studies, and in this study, it has proved impossible to clone *hns* on high copy-number plasmids (Higgins *et al.*, 1990). Overexpression of H-NS is apparently lethal, resulting, in extreme cases, in hyper-compaction of the nucleoid (Spurio *et al.*, 1992). If treatment of *E. coli* with verapamil or diltiazem were to result in elevated levels of H-NS, hyper-compaction of the nucleoid would occur, resulting in gross changes in gene expression that could ultimately be fatal.

Transforming N43*verA1* or N43 with pLG703, the low copy-number plasmid encoding *hns*, renders both strains hypersensitive to verapamil (see **Chapter 7**), with an MIC of 0.05mM, compared to 0.4mM for N43 without the plasmid. Conversely, N43*hns*::Tn10 has a verapamil MIC in excess of 1mM. It therefore seems that H-NS plays a crucial role in sensitivity to verapamil. (To complicate issues, plasmids containing *galU* alone also result in substantial increases in the verapamil MIC of N43 and N43*verA1*). To test whether verapamil does affect expression of *hns*, strains containing the *hns* promoter (p*hns*) fused to *lacZ* were constructed in N43 and N43*verA1* and the β-galactosidase levels measured during growth and following treatment with verapamil or EGTA.

The strain PD73 (see Materials and Methods for strain details) was kindly donated by E. Bremer (Max Planck Institut für Terrestrische Mikrobiologie, Marburg, Germany) (Dersch *et al.*, 1993), and contains a p*hns-lacZ* fusion construct in the λ*att* site of the the chromosome. A culture of PD73 was infected with $P1_{vir}$ (see Materials and Methods), and the resulting progeny bacteriophage used to infect N43 and N43*verA1*. Following infection, the N43 and N43*verA1* cultures were inoculated onto NA plates containing kanamycin. In principle, all the kanamycin clones should have co-

inherited the p*hns-lacZ* constructs (Dersch *et al.*, 1993). A selection of clones from each transduction, N43 and N43*verA1* were inoculated onto LUA plates containing X-gal (see Materials and Methods). All kanamycin resistant P1 transductants formed blue colonies.

To measure the effects of verapamil and EGTA on p*hns* activity in N43ϕ(p*hns-lacZ*) and N43*verA1*ϕ(p*hns-lacZ*), three 50ml flasks containing 35ml M9 medium, prewarmed at 30°C, were inoculated with overnight cultures of either N43ϕ(p*hns-lacZ*) or N43*verA1*ϕ(p*hns-lacZ*) to give a starting optical density (A_{600}) of 0.02 - 0.03. At intervals, a 700µl aliquot of each culture was withdrawn, and the A_{600} measured to determine the culture density, and duplicate 100µl aliquots of each culture were sampled and assayed for β-galactosidase activity (Miller, 1972) (see Materials and Methods). When the A_{600} reached 0.2-0.4 OD units, two 10ml aliquots of each culture were transferred, using a prewarmed pipette, to two prewarmed 50ml flasks containing verapamil and EGTA. In experiments with N43ϕ(p*hns-lacZ*), verapamil and EGTA were added to give final concentrations of 0.35 and 30mM respectively. With the strain N43*verA1*ϕ(p*hns-lacZ*), verapamil and EGTA were added to give final concentrations of 0.75 and 5mM respectively. Samples of the cultures were withdrawn at frequent intervals to measure the optical density and β-galactosidase activity following each treatment. Calculation of the β-galactosidase activity from the samples was determined as described in Materials and Methods.

Following calculation of the β-galactosidase activity for each culture, the mean optical density and enzyme activity values were determined for each group of three cultures, and the standard deviation values derived (data not shown). The ratio of β-galactosidase activity in the treated cultures was divided by the enzyme activity in the corresponding untreated cultures. **Figs. 46** and **47** represent respectively, the growth curves and β-galactosidase ratios of N43ϕ(p*hns-lacZ*), and **Figs. 48** and **49** represent the respective growth curves and β-galactosidase ratios for N43ϕ*verA1*(p*hns-lacZ*).

From the data presented, it is clear that verapamil induces *hns* expression by at least a factor of 2 over the untreated control in both the parent and mutant. By the time the experiment was terminated, the β-galactosidase levels were still increasing in the mutant, but in N43, the levels began to decrease, which could be the consequence of H-NS autoregulation (see Dersch *et al.*, 1993). Since N43*verA1* has lost the *hns* gene, no auto regulation can take place. In other experiments, when higher concentrations of verapamil (1.3mM) were used, the levels of β-galactosidase activity increased to much higher levels in the parent and mutant (data not shown), and were accompanied by cell lysis. The concentrations of EGTA used did not appear to have a significant effect on *hns* expression. Since the levels of H-NS in the cell are critical, a doubling in the

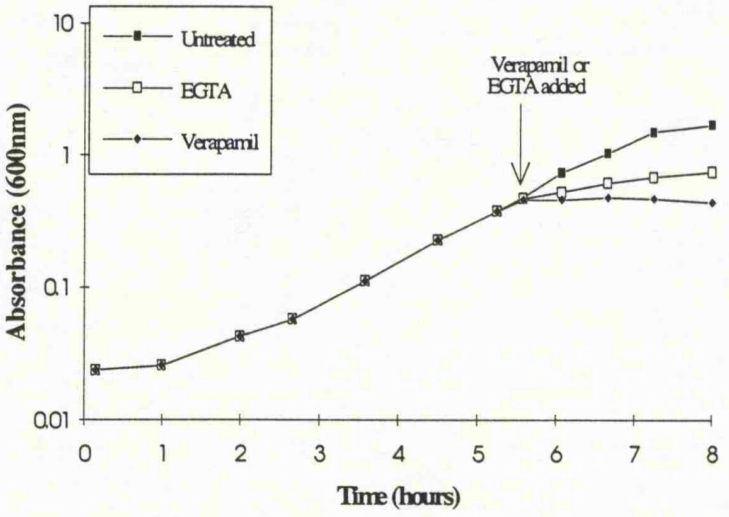


Fig 46. Growth curve of N43 in M9 medium at 30°C, showing the effect of adding 0.35mM verapamil or 30mM EGTA. Triplicate cultures of N43 were grown in M9 medium at 30°C to mid-exponential phase. 3 aliquots of each cuture were then transferred to 3 identical flasks containing a) nothing (control), b) verapamil or c) EGTA. The optical density (**Fig. 46**) and β-galactosidase activity (**Fig. 47**) of each culture was then determined.

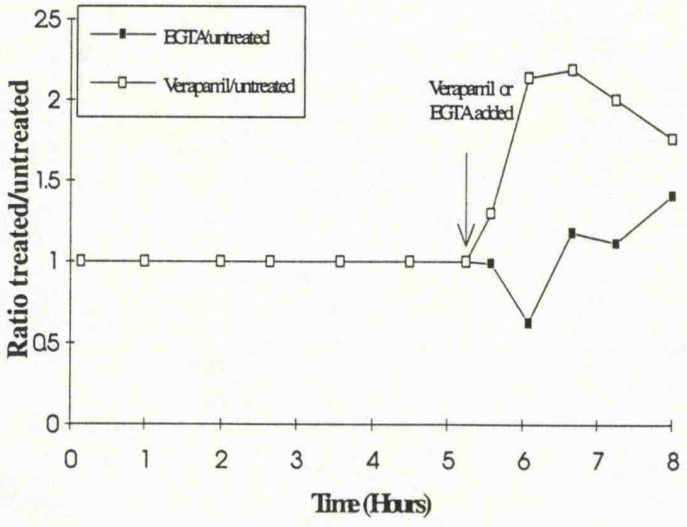


Fig 47. Comparison of β-galactosidase activities in N43 treated with EGTA or verapamil and untreated cultures

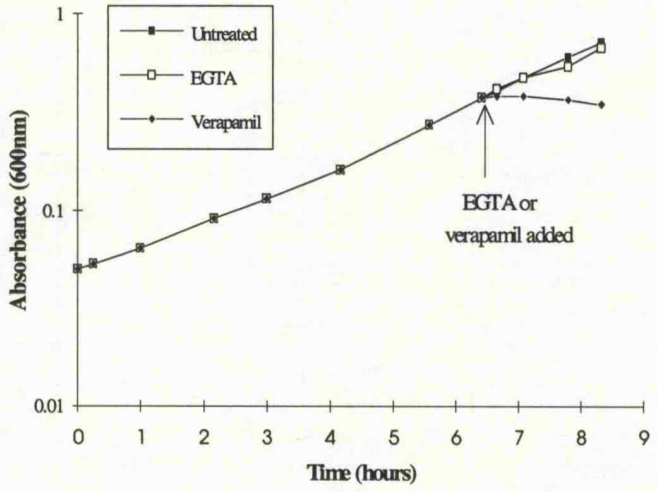


Fig 48. Growth curve of N43*verA1* in M9 medium, at 30°C, showing effect of adding 0.75mM verapamil or 5mM EGTA. Triplicate cultures of N43*verA1* were grown in M9 medium at 30°C to mid-exponential phase. 3 aliquots of each cuture were then transferred to 3 identical flasks containing a) nothing (control), b) verapamil or c) EGTA. The optical density (**Fig. 48**) and β-galactosidase activity (**Fig. 49**) of each culture was then determined.

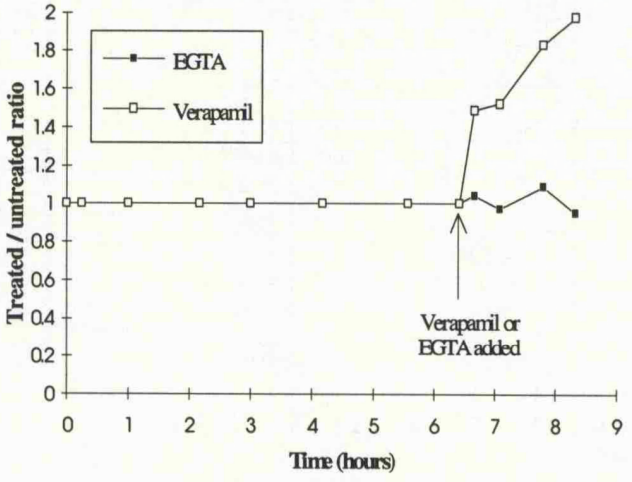


Fig 49. Comparison of β-galactosidase activities in N43*verA1* treated with EGTA or verapamil and untreated cultures

amount of H-NS might be expected to have a significant effect either upon the supercoiling of the chromosome or upon global gene expression. This would certainly affect the expression of many genes, and could be potentially deleterious, since the level of supercoiling is set according to the status of the cell, and the growth conditions. If a sudden change in the levels of H-NS were to take place, resulting in inappropriate changes in global gene expression, the consequences might be toxic to the cell, although whether this is sufficient to explain the observed deletion of *hns* to confer resistance remains debatable (see **Discussion, Chapter 10**). Whether the increases in *hns* expression are due to a direct interaction with verapamil, or verapamil causes a block in the uptake of calcium from the environment, which results in a compensatory change in *hns* expression is not clear.

Chapter 10
Discussion and conclusions

10.1 Discussion

At the outset of this project, the aim was to isolate temperature sensitive (*ts*), verapamil resistant mutants of the *E. coli* strain N43. The hope was that such mutants would be the result of mutations either directly in gene(s) related to voltage operated Ca^{2+} channels (VOCCs), or by more indirectly affecting Ca^{2+} transport. A *ts* phenotype was anticipated because the expected change in channel structure or related protein might affect Ca^{2+} transport, resulting in its inability to regulate the uptake of Ca^{2+} correctly at the non-permissive temperature. Such a scenario was predicted to result either in a generalised internal Ca^{2+}-induced toxicity or starvation, or a specific derangement of normal cell cycle regulation. Indeed, the verapamil resistant *ts* mutant, N43*verA1*, displays very slow growth at 30°C, and is *ts*, at least in minimal medium, where cell division is affected. Moreover, four *ts* mutants were later isolated, resistant to another VOCC inhibitor diltiazem and were named N43*dilA1-4*. Encouragingly, cross-resistance was observed between the verapamil and diltiazem resistant mutants such that N43*verA1* was found to be resistant to diltiazem and *vice versa*. When N43*verA1* was tested for its sensitivity to a range of antibiotics and the calmodulin (CaM) inhibitor trifluoperazine (TFP), the mutant and parent were found to have identical sensitivity profiles to TFP and most of the antibiotics tested, thereby showing that the resistance to the VOCC antagonists was not merely a change in the cells permeability to drugs, and that the resistance to the VOCC antagonists was probably specific. Curiously however, N43*verA1* was found to be hyper-sensitive to the gyrase inhibitor, nalidixic acid and the RNA polymerase inhibitor, rifampicin. This aspect will be discussed later with relation to the genes affected in the mutant.

When a low-copy number genomic library was transformed into N43*verA1*, a number of plasmid clones were isolated that restored verapamil sensitivity, ts^+ and rapid growth. A single clone called pLG701 was selected for further analysis, and was also found to restore the wild-type phenotype in the diltiazem resistant mutants. This therefore gave further evidence for the specificity of the drug resistance. When tested for sensitivity to the Ca^{2+} chelating compound EGTA, the mutant was found to be much more sensitive than the parent, with N43 being at least 4 times more resistant to EGTA than N43*verA1*. A similar situation was found when the mutant and parent were compared for their sensitivities to Ca^{2+}, where N43*verA1* was found to be 2-fold more sensitive to the amount of Ca^{2+} in the medium than N43. Interestingly, when inhibitory concentrations of Ca^{2+} were added to the medium with N43*verA1*, the cells lysed.

In line with the underlying hypothesis that such mutants might be defective in cell cycle controls, a microscopic examination of N43 and N43*verA1* revealed that when grown in TCG minimal medium at 30°C, containing concentrations of Ca^{2+} too low to be measured by the atomic absorption spectrometry equipment available at the time (<2μM), the mutant was found to form cells of pleiotropic morphology, often giving rise to cells that were long, very slender and curved or kinked, whereas the parent formed much shorter, broader rods. Upon shifting to the non permissive temperature, the mutant produced filaments with incomplete septa, occurring at irregular intervals along the cells, and minicells. However, as with the *ts* phenotype, the morphological defects were only observed in minimal medium.

An analysis of the protein profiles of N43 and the *verA1* mutant revealed many differences as seen both by coomassie-blue staining and [^{35}S-] methionine labelling of the proteins. This gave a major clue as to the type or nature of the gene(s) affected, indicating that the role of the protein(s) was connected with regulation of gene expression. Moreover, when the two strains were treated with EGTA, verapamil or $CaCl_2$, and labelled with [^{35}S-] methionine, the two strains responded in very different ways, again showing dramatic changes in the expression of a number of genes both within each strain, but also differences between the two strains. Even more encouraging was the observation in the mutant of several low molecular weight heat-stable proteins that appear to be constitutively expressed, even under unstressed conditions, but are further induced by treatment with EGTA, and are in the size range for eukaryotic calmodulin, with molecular weights of 14 to 21kDa. Moreover, subsequent work (Laoudj *et al.*, 1994) with these low molecular weight heat-stable proteins has shown that some of these proteins cross react with antibodies raised against a CaM-like protein from the bacterium *Saccharopolyspora erythraea* (Swan *et al.*, 1989), and the mobility of a 17kDa heat-stable protein is retarded when Ca^{2+} is removed. Moreover, in the latter case, the dye, ruthenium red binds to a protein of 17kDa in the absence of Ca^{2+}, but does not bind if Ca^{2+} has been added to the protein before adding the dye (D Laoudj, personal communication). All of these properties are classical features of eukaryotic calmodulin. In N43, a protein of 50kDa is induced following treatment with verapamil, which is not seen in the mutant. This protein could perhaps be concerned with Ca^{2+} buffering or stress-response.

In order to clone and identify the genes whose expression is affected by the treatment of the mutant and parent with EGTA or verapamil, a possible approach would be to construct RNA subtraction libraries. By extracting RNA both from untreated and verapamil/EGTA treated N43 and from N43*verA1*, it is possible to construct a cDNA library based upon those genes which are differentially expressed under the inducing and non-inducing conditions. Alternatively, a promoter library

could be constructed, whereby N43 is transformed with a plasmid encoding a Tn10::*lacZ* promoter probe, allowing random insertion downstream and in frame of a promoter. β-Galactosidase expression can be detected by the formation of blue colonies on X-gal containing media under appropriate conditions. Replica plating could then be used to transfer bacteria onto plates lacking or containing EGTA or verapamil, to identify for subsequent cloning, genes whose expression is affected by EGTA or verapamil.

With all of this albeit indirect evidence to indicate a perturbation in the regulation of the $[Ca^{2+}]_i$ in N43*verA1*, it came as a great surprise to find that plasmid pLG701, required for full complementation of the mutant phenotype, contains three genes, none of which has any known link with calcium metabolism, (and certainly does not contain genes encoding ion channels). The genes contained on pLG701, and required for complementation of the *verA* phenotype were identified as *hns*, *galU* and initially designated by us as *rrx* in view of its sequence indicating a response regulator (the latter is also known as *hnrG* (Conteras, 1992)). The three genes are located at minute 27.25 on the *E. coli* linkage map. The phenotype of N43*verA1* was found to be remarkably complex, since attempts using complementation analysis, to identify which of the three genes encoded by pLG701 that was required to complement the *verA* phenotype, proved unsuccessful. Subcloning of pLG701 to form pLG702, that contains the genes *galU* and *rrx*, restored growth at 42°C, but instead of restoring verapamil sensitivity, greatly increased the levels of verapamil resistance both in the mutant, and also in the parent, N43. No single gene restored growth at 42°C, although weak growth was detected when plasmid pLG708 containing *galU* alone, was transformed into the mutant. However, by subcloning the genes individually, it was possible to establish some of the phenotypic effects associated with each gene. Initially, no obvious phenotype could be ascribed to *rrx*, since its presence on pLG701 did not appear to affect temperature sensitivity or verapamil resistance, although in conjunction with *galU*, it did enhance growth at 42°C. Curiously, whereas the *galU* gene alone was shown to result in hyper-resistance to verapamil, *hns* was found to cause hyper-sensitivity to the drug. Most remarkable was the finding that plasmid pLG709, containing the genes *hns* and *galU* was required to restore growth at 42°C, verapamil sensitivity and rapid growth rate (in fact, pLG709 rendered N43*verA1* slightly more sensitive to verapamil than N43). This then raised the question of the role of *rrx*, since apparently, only *hns* and *galU* together were needed restore growth at the non-permissive temperature, and verapamil sensitivity. The role of *rrx* became more apparent after examining the effects of the different gene combinations on Ca^{2+} sensitivity (see later). All of this complementation data clearly indicates that the three genes are interacting in a complex way.

Further evidence regarding the apparent interaction of *hns*, *galU* and *rrx* emerged from *in-vitro* coupled transcription-translation (Zubay) experiments and "*in-vivo*" experiments with minicells. When plasmid pLG701 was expressed in Zubay reactions, one of the three proteins encoded by this plasmid (excluding the vector-encoded proteins) was found to be either not expressed at all, or at the very least, expressed at very low levels - this was subsequently shown to be the *rrx* gene product. However, when plasmid pLG702, lacking *hns*, was incubated in a Zubay reaction, the *rrx* gene product was clearly visible. Moreover, when the two plasmids pLG701 and pLG702 were incubated together in a Zubay reaction, the *rrx* gene product again failed to be expressed, indicating that the *hns* gene product is capable of repressing *rrx* in *trans*. Essentially similar results were obtained with minicells and these experiments therefore demonstrated that the *hns* gene-product negatively regulates *rrx* expression.

A complex picture also emerged when the effects of the *hns*, *galU* and *rrx* genes were studied in relation to the apparent hypersensitivity of N43*verA1* to Ca^{2+}. As for verapamil, transformation of the mutant with pLG708 encoding *galU* only, resulted in a remarkable degree of Ca^{2+} hyper-resistance. However, unlike the result with verapamil, the additional presence of *hns*, included on the plasmid pLG709, did not reduce the level of resistance to Ca^{2+}. In contrast, the additional presence of *rrx* (pLG702; *galU* and *rrx*) did largely mitigate the enhanced Ca^{2+} resistance conferred by *galU* alone. Transforming N43*verA1* with plasmid pLG707 (*rrx* alone) however, had no effect on the Ca^{2+} MIC. Meanwhile, *hns* alone or in conjunction with *rrx* raised the Ca^{2+} MIC of the mutant slightly but was still more sensitive than the parent strain. The only combination of the three genes which restored the Ca^{2+} MIC of the mutant to exactly the same level as the parent strain was when pLG701, containing all three genes, was transformed into the mutant. This again reinforces the emerging conclusion that the three genes *hns*, *galU* and *rrx* are interacting, and are all required to correctly regulate the sensitivity of *E. coli* to both Ca^{2+} and verapamil.

Further evidence for an interaction between genes / proteins of the *verA* locus emerged from observations regarding the effects of plasmid pLG703 on growth of the mutant and parent strain. As discussed later, it is well recognised that *hns* is difficult to clone due to its toxicity in high copy-number plasmids (Higgins *et al.*, 1990). Plasmid pLG703 which only contains *hns* caused both N43*verA1* and N43 to grow very slowly on solid media. Interestingly however, transforming plasmid pLG709, which contains *hns* and *galU*, enabled them to grow extremely well under all conditions, with no hint of H-NS associated toxicity. This finding raises the interesting speculation that the *galU* gene product is somehow down-regulating *hns* expression or activity.

The next problem that needs to be addressed is what exactly are the roles of the genes constituting the *verA* locus. The *hns* gene encodes the intensively studied

histone-like DNA binding protein, H-NS (for reviews, see Higgins *et al.*, 1988; 1990; Hulton *et al.*, 1990; Owen-Hughes *et al.*, 1992). A reflection of the importance of H-NS in bacterial gene-regulation is illustrated by the number of names that have previously been ascribed, and represent different alleles, for example *osmZ*, *bglY* (May *et al.*, 1990); H1, H1a (Higgins *et al.*, 1990; Hulton *et al.*, 1990); *drdX* (Göransson *et al.*, 1990), *pilG* (Higgins *et al.*, 1988) and VirR in *Shigella flexneri* (Dorman *et al.*, 1990). H-NS appears to be primarily involved with the regulation of genes concerned with responding to environmental stimuli. The mechanism(s) by which this protein controls gene expression are very poorly understood, although two features have been observed: H-NS contributes to the regulation of the level of supercoiling of the DNA (Higgins *et al.*, 1988; 1990; Dorman *et al.*, 1990; Hulton *et al.*, 1990), and secondly, through its apparent affinity for curved DNA sequences, is capable of binding to the promoters of numerous genes containing sequences which form curved motifs (including its own promoter) (Tanaka *et al.*, 1991; Yamada *et al.*, 1991; Owen-Hughes *et al.*, 1992). Upon binding to such promoters, H-NS is thought to block transcription (Göransson *et al.*, 1990). The types of system and stimuli influenced by H-NS include osmoregulation, temperature, pH changes, anaerobicity, fimbrial phase variation, entry into stationary phase, nutrient limitation and virulence. As more genetic systems are identified in which H-NS is found to be involved, it has been becoming clearer that H-NS functions essentially as a repressor of gene expression. How H-NS may be implicated in DNA supercoiling and nucleoid compaction is far less clear, certainly, no evidence has been presented that H-NS is able to compact the nucleoid in the same manner as observed with eukaryotic histones. H-NS does not possess any of the features of a classical topoisomerase (see Luttinger, 1995) but it has been previously speculated that changes in the levels of DNA supercoiling could be mediated through H-NS interactions with the *gyrA*/*gyrB* and/or *topA* promoters of the DNA gyrase and topoisomerase I genes (Higgins *et al.*, 1988; 1990). However, this hypothesis was discounted because of apparent differences in the phenotypes of *hns* and topoisomerase mutants (Higgins *et al.*, 1990). Subsequent to the above reports, information has emerged which, in my view, does support the hypothesis that H-NS regulates the level of supercoiling through interactions with either the gyrase or topoisomerase I promoters, thereby changing the equilibrium of their opposing activities. For example, transferring bacteria from low to high osmolarity (Higgins *et al.*, 1988), or from low to high temperature (Porter and Dorman, 1994; Camacho-Carranza *et al.*, 1995) results in a change in the level of DNA supercoiling, which is controlled by H-NS. However, these changes in supercoiling can be inhibited by treating the bacteria with a gyrase inhibitor (Dorman *et al.*, 1988; Camacho-Carranza *et al.*, 1995).

In addition to its role in adaptations to environmental changes, recent publications have linked H-NS with the control of genes including the stationary-phase-specific sigma factor, σ^S, required for entry into stationary phase, (Barth *et al.*, 1995; Yamashino *et al.*, 1995). This is of particular interest, since bacteria, as they enter stationary phase, become more thermotolerant (Yamashino *et al.*, 1995). Apparently, in *hns* mutants, the levels of σ^S are elevated and this results in enhanced thermotolerance during exponential phase. However, in the case of N43*verA1*, this mutant is actually temperature sensitive, and the reintroduction of *hns* only (pLG703), does not affect the ability to grow at 42°C. Thus, confirming the more complicated basis of this mutant.

As indicated earlier, N43*verA1* is hypersensitive to the antibiotics nalidixic acid and rifampicin. A possible explanation for this is linked to the absence of H-NS. Perhaps because H-NS is not present, gyrase and/or topoisomerase A expression is affected and therefore affects the sensitivity of this strain to nalidixic acid. Rifampicin acts upon RNA polymerase and altered levels of supercoiling in an *hns* mutant will affect the transcription of numerous genes directly and indirectly. Since the binding of RNA polymerase to certain promoters is dependent upon secondary structure, which could be altered in the mutant, initiation of transcription, could be prevented by low levels of rifampicin.

The *galU* gene encodes the enzyme UTP:α-D-glucose-1-phosphate uridylyltransferase (Weissborn *et al.*, 1994), also known as UDP-glucose pyrophosphorylase (UDPGP) (Hossain *et al.*, 1994) and catalyses the following reaction: UTP + glucose--> UDP-glucose. Two of the well established roles of UDPGP are in lipopolysaccharide synthesis (Jiang *et al.*, 1991; Weissborn *et al.*, 1992) and in osmoregulation where UDP-glucose is a precursor in the biosynthesis of the osmoprotectant, trehalose (Giaever *et al.*, 1988; Strom and Kaasen, 1993). Recently however, some evidence indicated that UDPGP may also be involved in regulating entry into stationary phase (Böhringer *et al.*, 1995), through negatively regulating the levels of σ^S. As with H-NS, apparently, inactivation of *galU* also results in elevated levels of σ^S and σ^S-controlled genes, and should therefore result in enhanced thermotolerance. Again, this is in contrast to the *ts* character of *verA1*. Another interesting feature of the *galU* mutant in the article by Böhringer *et al* (1995) is that its growth rate was not slower than the *galU*$^+$ isogenic strain. This is also the case in N43*verA1* when *hns* and *rrx* are restored by the introduction of pLG711. It has been reported that UDPGP requires Mg^{2+} ions for activity (Hossain *et al.*, 1994), but to my knowledge, no work has been undertaken to test whether Ca^{2+} could also activate UDPGP.

The third gene of the *verA* locus, *rrx* was previously sequenced but until this study, was uncharacterised. An examination of its amino acid sequence indicates that it is a member of the two-component response regulator super-family (Stock *et al.*, 1989), showing strong homology in its 13kDa N-terminal portion with the conserved domain found in the response regulator components of this family (see **Appendix 1**). Two-component response regulators consist (usually) of a membrane-bound sensor-kinase and a cytoplasmic DNA-binding reponse regulator protein. Following interaction of the sensor domain with the appropriate stimulatory ligand, the kinase domain autophosphorylates at a histidine residue which then phosphorylates a highly conserved aspartate residue of the appropriate response regulator protein(s). In the great majority of cases so far studied, phosphorylation of the response regulator results in a conformational change which enables it to bind to specific promoter sequences, resulting in gene activation. However, in the case of the chemotaxis response regulator, CheY, the phosphorylated response regulator is thought to interact directly with the flagellar motor (Stock *et al.*, 1989). The sensor-kinase for *rrx* is unknown, and an examination of the DNA sequence upstream of *rrx* failed to identify putative histidine kinases. The nearest histidine kinase gene to *rrx*, located 14kb upstream of *rrx* on the chromosome, is the *narX* gene which, in conjunction with its neighbouring response regulator gene, *narL*, is concerned with the regulation of nitrate metabolism and transport (Bösl, 1993). As indicated in **Chapter 7**, transcriptional response regulators require a metal ion (usually Mg^{2+}) for activity *in vitro*, but Ca^{2+} can also bind and confer activity (J. Stock, Princeton Univ., N.J., personal communication). Indeed this has been clearly demonstrated with the chemotaxis response regulators *in vivo* (Tisa and Adler, 1992; Tisa *et al.*, 1993; Watkins *et al.*, 1995) and *in vitro* with the OmpR-EnvZ two-component system (Rampersaud *et al.*, 1991). In the experiments by Rampersaud *et al*, Ca^{2+} was found both to enhance the phosphorylation of the sensor kinase, and to stimulate the transfer of the phosphate from the kinase to the response regulator.

According to Bösl (1993), there is no transcriptional terminator between *rrx* and *galU* and he postulates that they could form part of a complex transcriptional unit. Indeed, in Northern blot experiments probing for *galU* expression, in addition to the *galU* mRNA transcript which appears to be very abundant, two additional bands of higher molecular weights were often seen (data not shown) and could correspond to transcripts initiating from *rrx* and from the uncharacterised gene, *orf34* upstream of *rrx* (Bösl, 1993). On the basis of the Ca^{2+} MIC measurements, where Rrx appears to be down-regulating the activity and or production of UDPGP resulting in the elimination of the UDPGP-mediated Ca^{2+} hyper-resistance, it seems possible therefore, that the *galU* gene-product either regulates the cells' permeability to Ca^{2+}, or that it somehow

regulates the $[Ca^{2+}]_i$, and that the *rrx*-encoded protein and its presumed membrane sensor is responsive to the $[Ca^{2+}]_i$ and regulates *galU* activity in some way. It is also possible that the UDPGP protein is able to chelate Ca^{2+}, in the manner of the eukaryotic Ca^{2+}-buffering protein parvalbumin (Means and Rasmussen, 1988). Indeed, a possible Ca^{2+}-binding site was identified in UDPGP following an examination of its protein sequence (A. Danchin, personal communication). Overexpressing *galU* results in a remarkable phenotype, whereby the cells form long, fat, filaments of irregular girth, interspersed by chains of minicells, but only as the culture enters stationary phase (see **Appendix 2**). Although much of this effect is probably due to the filamentous protein structures running through the cells, interfering with the septation machinery, the possibility that this excess of *galU* could be chelating the Ca^{2+} ions and interfering with the normal triggering of cell cycle events is nevertheless extremely tantalising.

Whilst this thesis was in prepation, we received new information concerning at least one possible function for *rrx* (R. Hengge-Aronis, personal communication). Thus, null mutations of *rrx* result in complete stabilisation of RpoS (normal $t^1/_2$ is 1.4 min (Lange and Hengge-Aronis, 1994)) and a consequent 10-fold increase in RpoS levels in exponentially growing cells, with only a small (10-20%) increase in generation time. Enhanced RpoS synthesis, apparently resulting from increased translation of *rpoS* mRNA during osmotic shock is unaffected, indicating that *rrx* operates exclusively in the regulation of the stability of RpoS. As expected, the expression of the genes controlled by RpoS, *osmY*, *otsBA* and *dps* is enhanced in the absence of *rrx*, which is now designated *rssB* (regulator of sigma S). A possible candidate for the degradation of RpoS is FtsH (Tomoyasu *et al.*, 1995), with RssB involved in targetting RpoS for proteolysis, but the precise role of RssB in this process remains unclear. The 38kDa protein RpoS is the alternative sigma factor required for the transcription of the stationary phase regulon. This complex regulon is required for adapting bacteria to survival under adverse conditions that are unsuitable for growth. Induction of the stationary phase regulon enables bacteria to survive periods of starvation, osmotic stress, desication, low pH, oxidative stress and high temperatures (Kolter *et al.*, 1993). RpoS regulation is complex, and has been shown to be regulated at the levels of transcription, translation and post-translationally (Lange and Hengge-Aronis, 1994). Recent studies have thus shown all three genes from the *verA* locus are implicated in the regulation of the stationary phase regulon (Barth *et al.*, 1995; Böhringer *et al.*, 1995; Yamashino *et al.*, 1995). H-NS regulates RpoS apparently at the levels of transcription and protein stability, since in *hns* mutants, transcription of *rpoS* is elevated 2-fold during exponential phase and the $t^1/_2$ of RpoS is increased by a factor of ~10. The *galU* gene product, UDPGP apparently negatively regulates the expression of RpoS-regulated genes and also affects the stability of RpoS itself

(Böhringer *et al.*, 1995). Finally, as indicated above, *rrx* (*rssB*) may be more specifically involved in controlling RpoS stability. Because of the role of the stationary phase regulon in the survival of bacteria under adverse conditions, it comes as no surprise that this regulon is also important in the survival of pathogenic bacteria within their hosts (Fang *et al.*, 1992; Heiskanen *et al.*, 1994; Lee *et al.*, 1994; Iriarte *et al.*, 1995; Robbesaule *et al.*, 1995). H-NS is already well known to be involved in the regulation of virulence genes through the mechanisms described earlier (see for example Dorman, 1995). H-NS and the other two genes *galU* and *rrx* (*rssB*) could therefore also play an important role in bacterial virulence.

Whilst trying to determine the location and nature of the *verA1* mutation, since the mutant is *ts*, it was expected that a single point mutation would be the basis behind the phenotype. A sequence of experiments eventually revealed that in fact, a deletion of the three genes constituting the *verA* locus had occurred. Since the diltiazem-resistant mutants were found to have a similar phenotype to N43*verA1*, and were complemented by plasmid pLG701, a Southern blot analysis of these mutants was conducted and demonstrated that these strains also have major genetic lesions at this locus. Indeed two of these strains were also found to contain deletions of the entire *verA* locus, the other two mutants appeared to contain major rearrangements or insertions.

Since it appears to be comparatively easy to obtain *ts* mutants, resistant to eukaryotic VOCC inhibitors, and that all the mutants are affected at a specific genetic locus, resulting either from a deletion or gross rearrangement of the three genes *hns*, *galU* and *rrx* and ultimately leading to their inactivation, it seems that such an event is unlikely to be random or fortuitous. A possible hypothesis that could explain the basis for these observations is that treating cells with VOCC inhibitors triggers a significant and toxic increase in *hns* expression. The balance of factors regulating global gene expression is delicate, such that for a given set of growth conditions, a certain combination of parameters such as levels of supercoiling will be set. If something were to affect the levels of supercoiling without a change in the growth conditions, global gene expression would be affected, possibly resulting in the inappropriate and perhaps deleterious induction or repression of certain genes. On the basis of this hypothesis, experiments were conducted to measure levels of expression from an *hns* promoter-*lacZ* fusion introduced into N43 and N43*verA1*. The β-galactosidase activity in each strain was measured before and after treatment with verapamil and EGTA. The cultures treated with EGTA showed no significant change in p*hns* activity, but low concentrations of verapamil (0.75mM and 0.35mM for N43*verA1* and N43, respectively) resulted in a 2-fold increase in p*hns* activity indicating that indeed, verapamil *does* cause an increase in H-NS levels. In N43, after a rise in p*hns-lacZ*

activity, there was a subsequent decrease, presumably due to autoregulation (Dersch *et al.*, 1993; Falconi *et al.*, 1993). Since N43*verA1* does not contain an *hns* gene, autoregulation cannot occur, and indeed, no decrease was observed. Treating N43*verA1* with higher, lethal concentrations of verapamil resulted in much higher levels of expression of p*hns-lacZ*, but also caused the cells to lyse (data not shown). According to Higgins *et al* (1990), attempts to clone the *hns* gene even into low copy-number vectors is deleterious to cell growth, resulting in an accumulation of mutations at a high frequency. Moreover, overexpression of H-NS results in a lethal hyper-compaction of the nucleoid (Spurio *et al.*, 1992). In this work, it was also found that *hns* could not be cloned in high copy-number vectors, and when the low copy-number plasmid, pLG703 containing *hns* only, was transformed into either N43 or N43*verA1*, the transformants grew very slowly, although when the growth rate of the mutant containing this plasmid was subsequently measured, it was found to grow almost as rapidly as the parent, indicating the possible appearance of a secondary mutation. All these results apparently suggest that overexpression of *hns* is toxic, although whether this toxicity specifically results from changes in DNA supercoiling and whether this is specifically able to explain the killing effects of verapamil, remains to be established.

An interesting angle on H-NS toxicity was taken by McGovern *et al* (1994). McGovern *et al* found as described by Spurio *et al* (1992), that overexpression of H-NS results in hypercompaction of the nucleoid. This was found to be accompanied by a global shut-down of transcription and major changes in the supercoiling of reporter plasmids. However, not all gene expression was found to be shut down. The levels of expression of the *pyrBI* operon, are inversely proportional to the rate of RNA polymerase elongation, such that for example, if pyrimidine becomes limiting, expression of the *pyrBI* operon increases (cited in McGovern *et al.*, 1994). Indeed, expression of the *pyrBI* operon increased following overexpression of *hns*. Most interesting was the observation that following removal of the inducer of *hns* expression, no changes in growth rate, gene expression or DNA supercoiling were observed for 6 hours. However, just prior to recommencement of growth, the DNA supercoiling levels and global gene expression returned to normal. By re-inducing *hns* overexpression, global gene expression was again silenced and nucleoid compaction returned. McGovern *et al* (1994) suggested that these experiments provide a mechanism by which gene expression can be silenced during stationary phase. They also describe possible models for the arrangement of the nucleoid into transcriptionally active and inactive domains, whereby the inactive highly compacted regions in the nucleoid core are where most of the H-NS is found. Unfortunately in this paper, no measurements were made of the expression of stationary phase genes or of the stationary phase regulator, RpoS. McGovern *et al* also provide an explanation for

Spurio *et al* (1992) observations of apparent lethality caused by overexpression of *hns*, as the silencing of the β-lactamase gene on the plasmid encoding the overexpressing *hns* construct, followed by plating the bacteria onto media containing ampicillin.

The gross rearrangements in the *verA* locus observed in the verapamil-resistant mutants might be sufficient to explain the resistance to drugs which induce H-NS expression. However, even with this scenario, it is not clear whether selection for drug resistance immediately identifies mutants with the entire *hns-rrx(rssB)* region deleted or whether an initial deletion of *hns* is followed by a subsequent deletion, since hyper-deletion activity is a characteristic of *hns* mutants (Hulton *et al.*, 1990; Lejeune and Danchin, 1990). An interesting question to be addressed is how *E. coli* can survive, even in the laboratory, having lost a gene such as *hns* which is clearly so important and heavily involved in the general maintenance of normal cell growth and metabolism. One possibility is that *E. coli* carries the car-equivalent of a "spare tyre". Indeed, there is a gene called *stpA* (Zhang and Belfort, 1992), mapping at minute 58 on the *E. coli* linkage map, encoding a peptide of 134 amino acids (H-NS consists of 135-137 amino acids, depending upon the species (Higgins *et al*, 1990)), and shares 58% identity with H-NS. Since mutations in *hns* are so abundant as indicated by the quantity of publications now emerging, it seems highly plausible that a "reserve" gene should exist. In order to test the importance of this, deletions of *stpA* will be needed in N43*verA1*, N43 and N43Δ*hns* strains.

Can any inferences be made regarding the treatment of *E. coli* with VOCC inhibitors and the apparent consequences for H-NS expression? Although this is complete speculation, one possible model to explain the above observations is that verapamil is indeed blocking VOCCs and thereby interfering with the $[Ca^{2+}]_i$. The gene product of *rrx* (*rssB*) which, together with its presumed partner kinase, could be involved in governing the intracellular $[Ca^{2+}]$ and therefore the levels or activity of UDPGP (the product of the *galU* gene), which in turn elevates H-NS expression. Testing this model is technically difficult because of the problems of measuring the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in bacteria. Loading *E. coli* with the fluorescent indicator dyes such as Fura-2 involves a considerable amount of pretreatment (Gangola and Rosen, 1987; Rosen and Gangola, 1987), which could potentially affect the results. Alternatively, *E. coli* can be transformed with expression vectors containing the gene that encodes the chemiluminescence protein, aequorin (Watkins *et al.*, 1995). Aequorin is produced in the jelly fish, *Aequorea victoria*, and produces light of an intensity that is proportional to the $[Ca^{2+}]_i$. Using this system, N43 and N43*verA1* could be challenged to different concentrations of extracellular Ca^{2+}, EGTA or verapamil and the level of light emitted from the aequorin, measured. This would quickly indicate whether N43*verA1* is defective in regulating its $[Ca^{2+}]_i$.

Using the aequorin system, measurements of $[Ca^{2+}]_i$ could also be made following transfer of the mutant from 30 to 42°C. Using the sophisticated microscope and computer technology now available, it should be possible to investigate the Ca^{2+} distribution in the filamentous cells resulting from overexpression of *galU*, to find out if the excess UDPGP is chelating Ca^{2+}, and thereby affecting cell division. To test whether *rrx* (*rssB*), and its postulated sensor-kinase are involved in regulating the levels of $[Ca^{2+}]_i$, experiments would need to be done to test for the ability of these proteins to bind ^{45}Ca, and to measure phosphorylation of RssA and the transfer of the phosphate to RssB in the presence of different metal ions. If RssA and RssB are regulating the $[Ca^{2+}]_i$, one might expect that the levels of phosphorylation increase with increasing $[Ca^{2+}]$ to a certain point, and then diminish. Experiments could also be performed to test whether UDPGP also binds ^{45}Ca. By fusing the promoter of *galU* to the *lacZ* gene, levels of *galU* expression could be measured in an *rrx*$^+$/*rrx*$^-$ background, and to measure levels of *galU* expression following treatment of these strains with verapamil, $Ca^{2+}$and EGTA. More experiments also need to be conducted with N43 and N43*verA1* containing the p*hns-lacZ* constructs to test for regulation of *hns* expression by UDPGP, and Ca^{2+}. Another area which is now developing rapidly, is the regulation of the stationary phase regulon. The stationary phase regulator RpoS is proving to be a remarkably interesting protein, whose regulation is controlled at the levels of transcription, translation and post-translationally, via rapid turn over (Lange and Hengge-Aronis, 1994). It cannot be a coincidence that within the last few months, all three genes constituting the *verA* locus (*hns*, *galU* and *rrx*(*rssB* + *rssA*?)) have been found to regulate RpoS and other stationary phase genes.

In a search for the *E. coli* equivalent of calmodulin (CaM), Chen *et al* (1991) used a similar strategy to the one used in this project, using a CaM inhibitor, compound 48/80. A *ts* mutant, resistant to the compound 48/80, *feeB*, was isolated that was shown to be affected in a rare $tRNA^{leu}_3$, recognising the codon CUA. This particular codon is very rarely used in *E. coli*. At the time of publication, no clear explanation for the basis of this mutant could be established, although it was postulated that perhaps there are cell cycle proteins whose corresponding mRNA might contain a large number of these codons. An examination of the *rrx* gene revealed that there are in fact two CUA codons (there are none in the other two genes). It therefore raises the interesting possibility that a point mutation in the rare $tRNA^{leu}$, recognising the codon CUA could affect the expression of *rrx*, and therefore affect the regulation of the $[Ca^{2+}]_i$. This possibility is further supported by the demonstration that some of the genes that are constitutively derepressed, and further induced by EGTA in N43*verA1*, show striking biochemical resemblances to CaM.

10.2 Conclusions

Although the initial aim of trying to isolate and clone the prokaryotic equivalent of eukaryotic voltage operated Ca^{2+}-channels proved to be unsuccessful, what was in fact obtained proved to be arguably even more exciting. It appears that the genes *hns*, *galU* and *rrx* (*rssB*) encode proteins that may play a major role, directly or indirectly in controlling the $[Ca^{2+}]_i$ in *E. coli* and cell division, thereby providing evidence to support the hypothesis that Ca^{2+} regulates the cell cycle in this organism. Moreover, strong evidence now links all three genes with the regulation and control of stationary phase gene expression. Outside the test-tube, (something that many researchers tend to forget about) bacteria spend a majority of their life in a dormant state, where conditions can be extremely hazardous. It is the primary role of the stationary phase regulon to ensure that the bacterial cell is physiologically prepared for these conditions, which include starvation, desiccation, osmotic shock, low pH, exposure to free-radicals and heat. It is also becoming clearer that this regulon is of the utmost importance in pathogenic bacteria. Pathogens that lack key components of the stationary phase regulon are markedly less virulent, and this is suspected to be due to a reduced ability to resist the host's defences.

All the drugs used in this and other studies in this laboratory against calmodulin or Ca^{2+}-channels affect cell division (and in some cases, nucleoid segregation). All the corresponding mutants display some form of cell division defect, providing evidence to support the hypothesis that Ca^{2+} regulates the cell cycle in *E. coli*. Clearly many of the interpretations of the results are still speculative but with the work that is currently being undertaken, I am confident that much of this speculation will be proved to be correct. Such is the complexity of the *verA* locus, dissecting out the roles of each gene in relation to the control of the cell cycle will provide a formidable challenge, especially as these genes appear to be interacting with each other and involved in many other regulatory circuits. However, with the currently emerging data, this group of genes will probably be shown to play many other important roles, such as the control of virulence determinants in pathogenic bacteria.

10.3 Other considerations regarding the phenotype of the verapamil and diltiazem resistant mutants.

Throughout this work, great emphasis has been placed upon Ca^{2+} and its role in the regulation of the cell cycle. To this end, a detailed examination of the mutant, N43*verA1* has shown that indeed, it appears to be defective in its ability to regulate its $[Ca^{2+}]_i$, and indeed, its cell division is affected at the non-permissive temperature.

However, little work has been undertaken to test whether other ions might also affect the phenotype of the mutant, or whether the mutant is hypersensitive to these ions. Important ions which should be considered include Ba^{2+}, Mg^{2+}, Fe^{3+}, Ni^{3+} and Co^{2+}.

Another aspect which has not been addressed is the fact that since H-NS is a global regulator of gene expression, its absence will inevitably affect many aspects of cellular metabolism and the observed phenotypes could be a compound effect of a number of genes being inappropriately expressed. Moreover, *galU*, which catalyses the synthesis of UDP-glucose from UTP + glucose, will also be fulfilling an important role in the regulation of many cellular functions, since UDP-glucose is a central component of many biochemical reactions, in addition to regulating RpoS (see for example, Böhringer *et al.*, 1995). Therefore the absence of this compound will affect osmoregulation, possibly heat shock protection through loss of trehalose synthesis as observed in *S. cerevisiae* (P. Meacock, personal communication), the interconversion of sugars feeding into different biochemical pathways and expression of the stationary phase regulon. The absence of RssB (Rrx) protein which normally regulates RpoS stability, will also affect the expression of numerous genes. Thus, the deletion of this entire locus will result in a complex phenotype which needs to be examined in a number of different contexts in order to define more critically, the roles of these genes.

The *verA* mutant is only 2-fold more resistant to verapamil and diltiazem than the parent strain, whereas it is at least 4 times more sensitive to rifiampicin, and 2-fold more sensitive to nalidixic acid. However, the mutant has an identical MIC to the parent for the drugs chloramphenicol and trifluoperazine (see **Chapter 6**). This therefore rules out the possibility of a permeability change being the basis for drug resistance. A slight change in resistance to verapamil and diltiazem might be expected from a subtle change in the drugs target. However, the actual target for verapamil is unknown. It is also possible that deletion of H-NS could result in elevated expression of the verapamil binding protein, thereby raising the verapamil MIC. Identification of the drugs target will be necessary to test this possibility.

Appendix 1
Sequence analysis of response regulator protein, Rrx

The amino acid sequences from 18 response regulators have been aligned to the protein sequence of Rrx (see top line of each group), using the Clustal V computer program. The conserved aspartate (D) and lysine (K) residues have been highlighted (see Stock *et al*, 1988), and amino acid residues with 100% conservation throughout the group have been indicated by an asterisk. From this alignment, a dendrogram showing the phylogenetic relationship between the different proteins was compiled. The dendrogram (see **Fig. 50**) permits a visual comparision of amino acid sequence of functionally related proteins (*eg* see the nitrate regulators NtrC from *E. coli* and *Klebsiella pneumoniae*). It therefore becomes apparent that Rrx is functionally unrelated to the other response regulator proteins, at the level of amino acid sequence.

```
ECRRX           MTQPLVGKQI LIVEDEQVFR SLLDSWFS.S LGATTV.LAA DGVDALELLG
BSCHEB          MAH.R....I LIVDDAAFMR MMIKDILV.K NGFEVVAEAE NGAQAVEKYK
BSDEGU          MTK....VNI VIIDDHQLFR EGVKRILDFE PTFEVVAEGD DGDEAARIVE
BSPHOP          MNK....K.I LVVDDEESIV TLLQYNLE.R SGYD.VITAS DGEEALKKAE
BSSP0A          MEK....IKV CVADDNRELV SLLSEYIEGQ EDMEVIGVAY NGQECLSLFK
BSSP0F          MMNEK....I LIVDDQYGIR ILLNEVFN.K EGYQTF.QAA NGLQALDIVT
ECARCA          MQT...PH.I LIVEDELVTR NTLKSIFE.A EGYD.VFEAT DGAEMHQILS
ECEVGA          MNA.......  IIIDDHPLAI AAIRNLL.IK NDIEILAELT EGGSAVQRVE
ECHYDG          MTHDNID..I LVVDDDISHC TILQALLR.G WGYNV.ALAN SGRQALEQVR
ECNARL          MSNQE.PATI LLIDDHPMLR TGVKQLISMA PDITVVGEAS NGEQGIELAE
ECNTRC          MQRGIV....  WVVDDDSSIR WVLERALA.G AGLTC.TTFE NGAEVLEALA
ECRCSC          .........I LVVDDHPINR RLLADQLG.S LGYQ.CKTAN DGVDALNVLS
KPNTRC          MQRGIA....  WIVDDDSSIR WVLERALT.G AGLSC.TTFE SGNEVLDALT
PAPILR          MSRQKA....  LIVDDEPDIR ELLEITLG.R MKLDT.RSAR NVKEARELLA
PSCOPR          M......K.L LVAEDEPKTG IYLQQGLR.E AGFN.VDRVV TGTDAVDQAL
RCPETR          MMSASPPH.L LIVDDDERIR GLLQKFLI.R NGFL.VTAGR DAAHARRLLS
STCHEY          ADKEL...KF LVVDDFSTMR RIVRNLLK.E LGFNNVEEAE DGVDALNKLQ
STOMPR          MQENY..K.I LVVDDDMRLR ALLERYLT.E QGFQ.VRSVA NAEQMDRLLT
                             . .*        .   .

ECRRX           GFTPDLMICD IAMPRMNGLK LLEHIRNRGD QT..PVLVIS ATENMADIAK
BSCHEB          EHSPDLVTMD ITMPEMDGIT ALKEIKQIDA Q..ARIIMCS AMGQQSMVID
BSDEGU          HYHPDVVIMD INMPNVNGVE ATKQLVELYP ESK..VIILS IHDDENYVTH
BSPHOP          TEKPDLIVLD VMLPKLDGIE VCKQLRQ..Q KLMFPILMLT AKDEEFDKVL
BSSP0A          EKDPDVLVLD IIMPHLDGLA VLERLRESDL KKQPNVIMLT AFGQEDVTKK
BSSP0F          KERPDLVLLD MKIPGMDGIE ILKRMKVIDE N..IRVIIMT AYGELDMIQE
ECARCA          EYDINLVIMD INLPGKNGLL LARELRE..Q AN.VALMFLT GRDNEVDKIL
ECEVGA          TLKPDIVIID VDIPGVNGIQ VLETLRKRQY SGI..IIIVS AKNDHFYGKH
ECHYDG          EQVFDLVLCD VRMAEMDGIA TLKEIKALNP A..IPVLIMT AYSSVETAVE
ECNARL          SLDPDLILLD LNMPGMNGLE TLDKLREKSL SGR..IVVFS VSNHEEDVVT
ECNTRC          SKTPDVLLSD IRMPGMDGLA LLKQIKQRHP M..LPVIIMT AHSDLDAAVS
ECRCSC          KNHIDIVLSD VNMPNMDGYR LTQRIRQLGL ..TLPVIGVT ANALAEEKQR
KPNTRC          TKTPDVLLSD IRMPGMDGLA LLKQIKQRHP M..LPVIIMT AHSDLDAAVS
PAPILR          REPFDLCLTD MRLPDGSGLD LVQYIQQRHP Q..TPVAMIT AYGSLDTAIQ
PSCOPR          NEAYDLLILD VMMPGLDGWE VIRRLRT..A GQPVPVLFLT ARDGVDDRVK
RCPETR          GLEFNLIVLD VMMPGEDGLS LTRDLRT..K MAT.PILLLT ARGETRERIE
STCHEY          AGGFGFIISD WNMPNMDGLE LLKTIRADSA MSALPVLMVT AEAKKENIIA
STOMPR          RESFHLMVLD LMLPGEDGLS ICRRLRS..Q SNPMPIIMVT AKGEEVDRIV
                          *   ..   *        .            .   .
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ECRRX           ALRLGVEDVL LKPVKDLNRL REMVFACLYP SMFNSRVEEE ERLFRDWDAM
BSCHEB          AIQAGAKDFI VKPF.QADRV LEAINKTL.. .......... ..........
BSDEGU          ALKTGARGYL LK........ .......... .......... ..........
BSPHOP          GLELGADDYM TKPFSP.... .......... .......... ..........
BSSP0A          AVDLGASYFI LKPFD..... .......... .......... ..........
BSSP0F          SKELGALTHF AKPF.DIDEI RDAVKKYLP. .......... ..........
ECARCA          GLEIGADDYI TKPFNP.... .......... .......... ..........
ECEVGA          CADAGANGFV SK........ .......... .......... ..........
ECHYDG          ALKTGALDYL IKPL.DFDNL QATLEKALAH THSID.AETP AVTASQFGMV
ECNARL          ALKRGADGYL LK........ .......... .......... ..........
ECNTRC          AYQQGAFDYL PKPF.DIDEA VALVERAISH YQEQQQPRNV QLNGPTTDII
ECRCSC          CLESGMDSCL SKPVT.LDVI KQSL..TLYA .......... ERVRKSRDS.
KPNTRC          AYQQGAFDYL PKPF.DIDEA VALVDRAISH YQEQQQPRNA PINSPTADII
PAPILR          ALKAGAFDFL TKPV.DLGRL RELVATALR. .LRNPEAEEA PVDNR...LL
PSCOPR          GLELGADDYL VKPFAL.... .......... .......... ..........
RCPETR          GLEAGADDYL PKPFEP.... .......... .......... ..........
STCHEY          AAQAGASGYV VKPFT.AATL EEKL...... .......... NKIFEKLGM.
STOMPR          GLEIGADDYI PKPFNP.... .......... .......... ..........
                    *       *

ECRRX           VDNPAAAA.. KLLQELQPPV QQVISH.... ......CRVN YRQLVAADKP
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          ..EMDADTLI EAVKVVA... .......... EGGSYLHPKV THNLVNEFRR
BSPHOP          ......REVN ARVKAILRRS EIAAPSSE.. .......... ..........
BSSP0A          .....MENLV GHIRQVSGNA SSVTHRAPSS Q.....SSII RSSQPEPKKK
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          ......RELT IRARNLLSRT MNLGTVSE.. .......... ..........
ECEVGA          ..KEGMNNII AAIEAAK... .......... NGYCYFP... .....FSLNR
ECHYDG          GKSPAMQHLL SEIALVAPSE ATVLIHGDSG TGKELVARAI HASSARSEKP
ECNARL          ..DMEPEDLL KALHQAA... .......... AGEMVLSEAL TPVLAASLR.
ECNTRC          GEAPAMQDVF RIIGRLSRSS ISVLINGESG TGKELVAHAL HRHSPRAKAP
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          GEAPAMQDVF RIIGRLSRSS ISVLINGESG TGKELVAHAL HRHSPRAKAP
PAPILR          GESPPMRALR NQIGKLARSQ APVYISGESG SGKELVARLI HEQGPRIERP
PSCOPR          ......SELL ARVRTLLRRG SSLQVQTS.. .......... ..........
RCPETR          ......KELL LRINAILRRV PEAVTAGP.. .......... ..........
STCHEY          .......... .......... .......... .......... ..........
STOMPR          ......RELL ARIRPVLRRQ ANELPGAP.. .......... ..........

ECRRX           GLVLDIAA.. ..LSENDL.. ....AFYCLD VTRAGH.... .NGVL.....
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          LATSGVSAHP QH....EVYP EIR....... .......... ..........
BSPHOP          ...MKNDEME GQIVIGDL.. .......KIL PDHYEAYFKE SQLELTPKEF
BSSP0A          NLDASITSII HEIGVP...A HIKG.Y.... .......... ....LYLREA
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          ...ERRSVES YKFNGWEL.. .......DIN .SRSLIGPDG EQYKLPRSEF
ECEVGA          FVGSLTSDQQ K......... .......... .......... ..........
ECHYDG          LVTLNCAALN ESLLESELFG HEKGAFTGAD KRREGRFVEA DGGTLFLDEI
ECNARL          ......ANRA TT....E... .......... .......... ..........
ECNTRC          FIALNMAAIP KDLIESELFG HEKGAFTGAN TIRQGRFEQA DGGTLFLDEI
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          FIALNMAAIP KDLIESELFG HEKGAFTGAN TVRQGRFEQA DGGTLFLDEI
PAPILR          FVPVNCGAIP SELMESEFFG HKKGSFTGAI EDKQGLFQAA SGGTLFLDEV
PSCOPR          ...LQIGDLQ VDL....L.. .......KRR ATR.....GG KRIELTAKEF
RCPETR          ...K....Y. ..LSLGPL.. .......RYD LDRGELSQGD QPVRLTATEA
STCHEY          .......... .......... .......... .......... ..........
STOMPR          ...SQEEAV. ..IAFGKF.. .......KLN LGTREMFRED EPMPLTSGEF
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```
ECRRX           .......AAL LLRAL..... .......... .......... ..........
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          .........R PLHILTRREC EVL....... .......... .QMLADGKSN
BSPHOP          E......... LLLYLGRHKG RVLT...... .........R DLLLSAV.WN
BSSP0A          ISM....... .......... .........VY NDIELLGSIT KVLYPDIAKK
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          R......... AMLHFCENPG KIQS...... .........R AELLKKMT.G
ECEVGA          .......... .LDSLSKQEI SVM....... .......... .RYILDGKDN
ECHYDG          GDISPMMQVR LLRAIQEREV QRVGSNQIIS VDVRLIAATH RDLAAEVNAG
ECNARL          .........R DVNQLTPRER DIL....... .......... .KLIAQGLPN
ECNTRC          GDMPLDVQTR LLRVLADGQF YRVGGYAPVK VDVRIIAATH QNLEQRVQEG
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          GDMPLDVQTR LLRVLADGQF YRVGGYAPVK VDVRIIAATH QNLELRVQEG
PAPILR          ADLPMAMQVK LLRAIQEKAV RAVGGQQEVA VDVRILCATH KDLAAEVGAG
PSCOPR          A......... LLELLMRRQG EVLS...... .........K SLIASQV.WD
RCPETR          A......... LMRIFAAHAG EVIG...... .........R TELIEELGRD
STCHEY          .......... .......... .......... .......... ..........
STOMPR          A......... VLKALVSHPR EPLS...... .........R DKLMN.LARG

ECRRX           ..FNGLLQEQ LAHQNQRLPE L......... .......... .......GAL
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          RGIGESLFIS EKTVKNHVSN ILQKMNVNDR TQAVVVAI.. ..........
BSPHOP          YDFAG....D TRIVDVHISH LRDKIENNTK .KPIYIKTI. ..........
BSSP0A          FNTTASRVER AIRHAIEVAW SRGNIDSISS L......... ..........
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          RELKPHD... .RTVDVTIRR IRKHFESTPD .TPEIIATI. ..........
ECEVGA          NDIAEKMFIS NKTVSTYKSR LMEKLECKSL MDLYTFA... ..........
ECHYDG          R.FRQDLYYR LNVVAIEVPS LRQRREDIPL LAGHFLQRFA ERNRKAVKGF
ECNARL          KMIARRLDIT ESTVKVHVKH MLKKMKLKSR VEAAV..... ..........
ECNTRC          K.FREDLFHR LNVIRVHLPP LRERREDIPR LARHFLQVAA RELGVEAKLL
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          K.FREDLFHR LNVIRVHLPP LRERREDIPR LARHFLQIAA RELGVEAKQL
PAPILR          R.FRQDLYYR LNVIELRVPP LRERREDIPL LAERILKRLA GDTGLPAARL
PSCOPR          MNFDS....D TNVIEVAIRR LRAKIDDDFE .VKL.LHTC. ..........
RCPETR          RSASAEEAAG DRAVDVQITR LRRKIEPDPR .EPRYLQTV. ..........
STCHEY          .......... .......... .......... .......... ..........
STOMPR          REYSAME... .RSIDVQISR LRRMVEEDPA .HPRYIQTV. ..........

ECRRX           LKQVNHLLRQ ANLPGQFPLL VGYYHR.... .........E LKNLI.....
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          .......... .......... .......... .......... ..........
BSPHOP          .......... .......... .......... ........RG LGYKLEEPKM
BSSP0A          .......... .......... .......... .......... FGYTVSMTKA
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          .......... .......... .......... ........HG EGYRFCGD.L
ECEVGA          .......... .......... .......... .......... ..........
ECHYDG          TPQAMDLLIH YDWPGNIREL ENAVERAVVL LTGEYISERE LPLAIASTPI
ECNARL          .......... .......... .......... .......... ..........
ECNTRC          HPETEAALTR LAWPGNVRQL ENTCRWLTVM AAGQEVLIQD LPGELFESTV
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          HPETEMALTR LAWPGNVRQL ENTCRWLTVM AAGQEVLTQD LPSELFETAI
PAPILR          TGDAQEKLKN YRFPGNVREL ENMLERAYTL CEDDQIQPHD LRLADAPGAS
PSCOPR          .......... .......... .......... ........RG MGYMLEAQD.
RCPETR          .......... .......... .......... ........RG LGYMLAPD..
STCHEY          .......... .......... .......... .......... ..........
STOMPR          .......... .......... .......... ........WG LGYVFVPDGS
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```
ECRRX           .......... ....LVSAGL NATLNTGEHQ V..QISNGVP LGTLGNAYLN
BSCHEB          .......... .......... .......... .......... ..........
BSDEGU          ........KN GW........ .......... .......... ..........
BSPHOP          NE........ .......... .......... .......... ..........
BSSP0A          KPTNSEFIA. ....MVAD.. ..KLRLEHKA .......... ..........
BSSP0F          .......... .......... .......... .......... ..........
ECARCA          ED........ .......... .......... .......... ..........
ECEVGA          .......... .......... .......... .......... ..........
ECHYDG          P......... .....LGQSQ DI......QP LV.....EVE KEVILAALEK
ECNARL          .......... .W........ .......... .......... ..........
ECNTRC          AESTSQMQPD SWATLLAQWA DRALRSGHQN LLSEAQPELE RTLLTTALRH
ECRCSC          .......... .......... .......... .......... ..........
KPNTRC          PDNPTQMLPD SWATLLGQWA DRALRSGHQN LLSEAQPEME RTLLTTALRH
PAPILR          QEGAASL... .......... ......SEID NLEDYLEDIE RKLIMQALEE
PSCOPR          EG........ .......... .......... .......... ..........
RCPETR          .......... .......... .......... .......... ..........
STCHEY          .......... .......... .......... .......... ..........
STOMPR          KA........ .......... .......... .......... ..........

ECRRX           QLSQRCDAWQ CQIWGTGGRL RLM..LSAE
BSCHEB          .......... .......... ........N
BSDEGU          .......... .......... ..VEMR...
BSPHOP          .......... .......... .........
BSSP0A          .......... .......... ........S
BSSP0F          .......... .......... .....LKSN
ECARCA          .......... .......... .........
ECEVGA          .......... .......... ...QRNKIG
ECHYDG          TGGNKTEAAR QLGITRKTLL AKLSR....
ECNARL          .......... .......... ..VHQERIF
ECNTRC          TQGHKQEAAR LLGWGRNTLT RKLKELGME
ECRCSC          .......... .......... .........
KPNTRC          TQGHKQEAAR LLGWGRNTLT RKLKELGME
PAPILR          TRWNRTAAAQ RLGLTFRSMR YRLKKLGID
PSCOPR          .......... .......... .........
RCPETR          .......... .......... .........
STCHEY          .......... .......... .........
STOMPR          .......... .......... .........
```

Protein	Organism	Function
Rrx	*E. coli*	?$[Ca^{2+}]_i$ / RpoS
CheB	*B. subtilis*	Chemotaxis
DegU	*B. subtilis*	Extracellular protease production
PhoP	*B. subtilis*	Phosphate metabolism
Spo0A	*B. subtilis*	Sporulation
Spo0F	*B. subtilis*	Sporulation
ArcA	*E. coli*	Switching between aerobic/anaerobic metabolism
EvgA	*E. coli*	Homologous to *Bordetella pertussis* virulence regulator
HydG	*E. coli*	Hydrogenase activity.

Protein	Organism	Function
NarL	*E. coli*	Nitrate metabolism
NtrC	*E. coli*	Nitrate metabolism
RcsC	*E. coli*	Capsule synthesis
NtrC	*K. pneumoniae*	Nitrate metabolism
PilR	*P. aeruginosa*	Pilus synthesis
CopR	*P. aeruginosa*	Copper resistance
PetR	*R. capsulatus*	Photosynthetic/ respiratory growth
CheY	*S. typhimurium*	Chemotaxis
OmpR	*S. typhimurium*	Osmoregulation/ virulence

Table 16. Two-component response regulator proteins used in the comparison of amino acid sequence homologies with Rrx, and their respective functions.

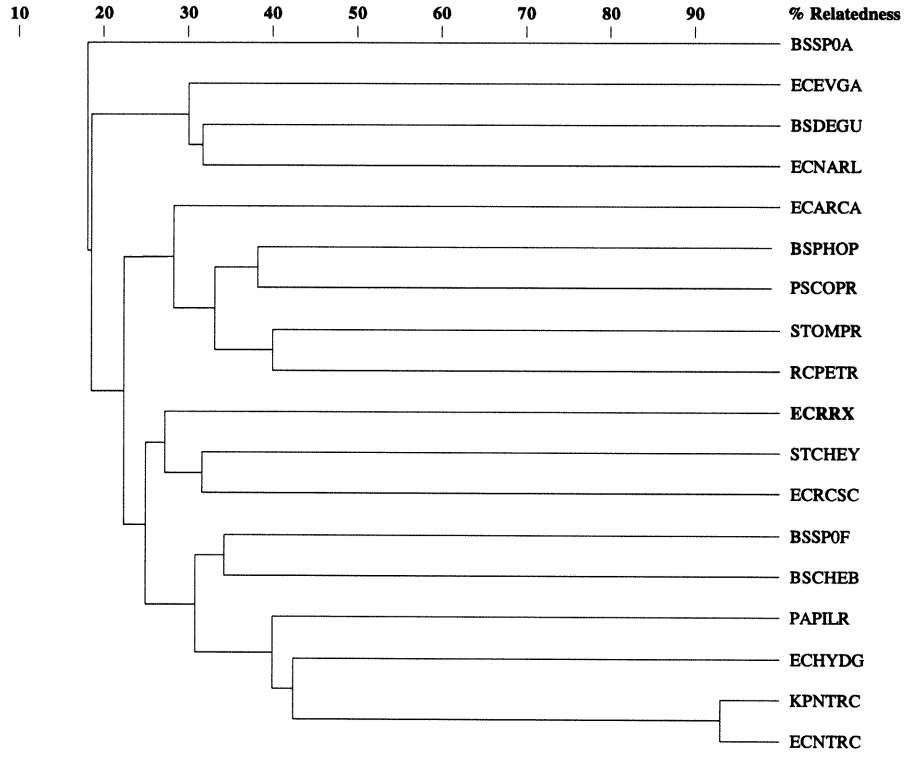

Fig 50. Dendrogram showing relatedness between different two-component response regulator proteins, calculated using the Neighbour Joining algorithm of Saitou and Nei supplied with the Clustal V sequence comparison program.

Appendix 2

Analysis of the cell division defects induced by the overexpression of *galU*

As described in **Chapter 5**, and illustrated in **Fig. 26**, during the preparations to make minicells, and to study the expression of the genes *galU* and *rrx*, these genes were cloned both singly, and together into the high copy-number vector, pUC19. Plasmid pLG704 (see **Fig. 51**), containing *galU* and *rrx* was constructed by digesting the M13-based plasmid, pLG712f containing these genes (see **Chapter 4**), that was originally used to sequence the *verA* locus, with the restriction endonucleases *Xma*I and *Eco*RI (cut in the multiple cloning site of pUC19), and the 3.17kb fragment purified and cloned into the *Eco*RI-*Xma*I sites in pUC19. The *galU* gene was cloned into pUC19 by digesting pLG704 with *Sal*I, and cloning the 1.7kb fragment into the *Sal*I site of pUC19, thus generating plasmid pLG705. The *rrx* gene was cloned into pUC19 by digesting pLG704 with *Xmn*I and *Sph*I, and the 1.3kb fragment ligated into the *Sph*I-*Sma*I sites in pUC19, to obtain plasmid pLG706. During the construction of these plasmids, the ligations were transformed into DH5α. It was noted that DH5α containing either plasmids pLG704 or pLG705 grew very poorly after overnight incubation at 37°C in nutrient broth containing ampicillin. In contrast, DH5α containing pLG706 grew normally. A microscopic examination of the strains carrying pLG704 or pLG705 indicated that their ability to divide normally had been severely impared, resulting in the formation minicells, long fat filaments and "chains of sausages" (see **Fig. 26, Chapter 6**). It was noted that the bacteria containing either pLG704 or pLG705 appeared virtually normal (only slightly filamentatous) during exponential growth, and only began to develop the aberrant division phenotype as the cultures entered stationary phase.

An article was subsequently published (Okada *et al.*, 1994), in which a gene, *cafA*, which forms part of the *mre* cell shape determining operon, was found to cause precisely the same cell division defects following overexpression, as described above. In the article by Okada *et al.*, the most extraordinary discovery was the observation of axial cytoplasmic filaments running through the cells, when examined by electron microscopy. These filaments appeared to have a regular hexagonal cross-section. Because of the remarkable similarity in appearance of the cells when examined by light microscopy with DH5α (and subsequently, N43 and N43*verA1*) containing pLG704 and pLG705, it was decided to investigate whether overexpression of *galU* also causes the formation of axial cytoplasmic filaments.

5ml overnight cultures of N43, N43*verA1* and N43*verA1* containing pLG705 (*galU* only) were incubated in NB at 30°C. 4.0 A_{600} units of each culture were taken, and kindly prepared for transmission electron microscopy by Mr S. Hyman, Electron

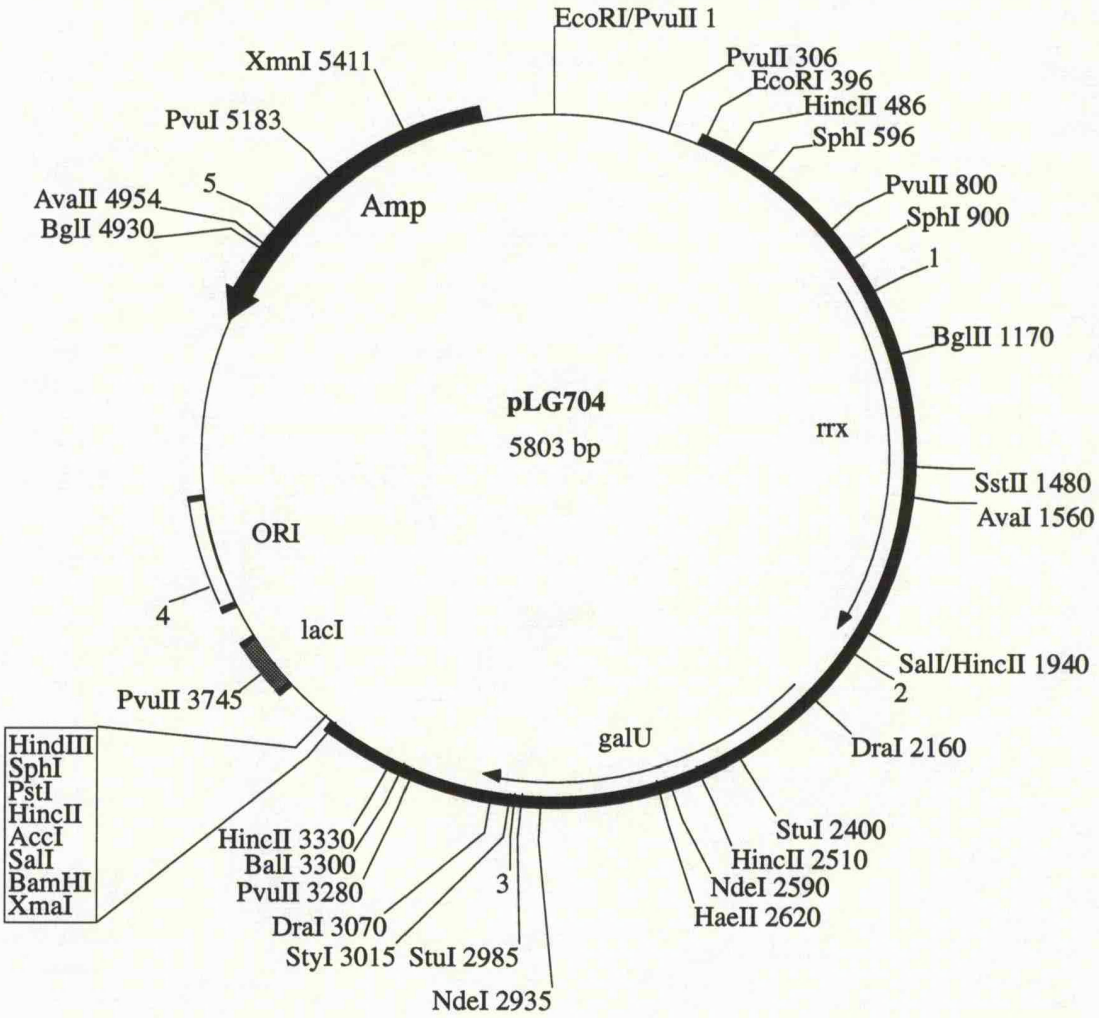

Fig. 51. Restriction map of pLG704.

Microscopy Laboratory, Leicester University, as described in Materials and Methods. The following electron micrographs are examples of the N43 (**Fig. 52**), N43*verA1* (**Fig. 53**) and N43*verA1* + pLG705 (**Figs. 54a-h**). It is clear that like CafA, overexpression of the *galU* gene product, α-D-glucose-1-phosphate uridylyl transferase (UDPGP) also results in the formation of cytoplasmic axial filaments with a hexagonal cross-section. A number of interesting features can be seen in the photographs. Firstly, the filaments appear to have a fibrous structure, that sometimes spread-out slightly at the ends, the ends are often associated with lighter staining material which might be the chromosome, but further work needs to be carried out to test this. Also visible, at the points where the cell is trying to divide, a 'collar-like' structure forms.

In order to verify that the protein filaments visible by electron microscopy were in fact UDPGP, samples of the bacteria sent for electron microscopy, were also boiled in SDS-PAGE sample buffer, loaded onto an 11%$^{v}/_{v}$ polyacrylamide gel and stained with coomassie blue (see **Fig. 53**). In the lane loaded with protein from N43*verA1* + pLG705, a very strongly staining band corresponding to a protein with an apparent molecular weight of 38kDa can be seen. As described in **Chapter 5.3**, UDPGP, which has a predicted molecular weight of 33kDa migrates aberrantly, with an apparent molecular weight of 38kDa. As a final confirmation that the strongly staining protein was UDPGP, some of the protein extract from N43*verA1* + pLG705 was kindly analysed by Dr E. Cavanagh, Nucleic Acids and Protein Chemistry Laboratory, Leicester University, and N-terminal amino acid sequence of the strongly staining protein obtained as follows: **Ala Ala Ile Asn Thr**. This amino acid sequence is 100% identical with the N-terminal sequence of UDPGP (see **Chapter 4**).

Due to time constraints, a more detailed investigation of this phenomenon was not undertaken here. The most obvious possibility is that the UDPGP filaments are in fact inclusion bodies, resulting from the overexpression of *galU*. However, this is unlikely, since inclusion bodies normally form amorphous globular masses within the cells, and the filaments described here have a very definite structure. UDPGP does not possess an α-helical coiled-coil motif (D. Clark, personal communication), characteristic of eukaryotic filamentous proteins such as myosin heavy chain, paramyosin and kinesin heavy chain (cited in Niki *et al.*, 1991), or the *E. coli* MukB protein involved in nucleoid segregation (see **Chapter 1**) (Niki *et al.*, 1991). One possibility is that UDPGP is associating with another protein that does form a filamentous structure. This could be tested by purifying the filaments on sucrose density gradients, followed by loading onto an SDS-PAGE gel to find out whether the filamentous protein is pure UDPGP. Also, it might be possible to examine the purified filaments by electron microscopy.

Whatever the basis of the UDPGP filaments, an important question is raised: Why do the filaments cause the division mechanism to fail *ie.* even though the filaments prevent completion of the septum, the incomplete septa are not regularly spaced. Long filaments are sometimes interspersed by a chain of minicells, followed by another filament. If for example, calcium ions are important for correct septation, the filaments might disrupt the correct localisation of these ions or some other factor within the cell. Alternatively, UDPGP might bind Ca^{2+} and thus, the filaments coud be acting as a reservoir of Ca^{2+} ions. Apparently, UDPGP possesses a possible Ca^{2+}-binding motif (A. Danchin, Institut Pasteur, Paris, personal communication), although if such a motif exists, it is not a classical EF-hand as seen for example in calmodulin, (see **Chapter 1.4.2.2**), and no experimental evidence exists to corroborate this.

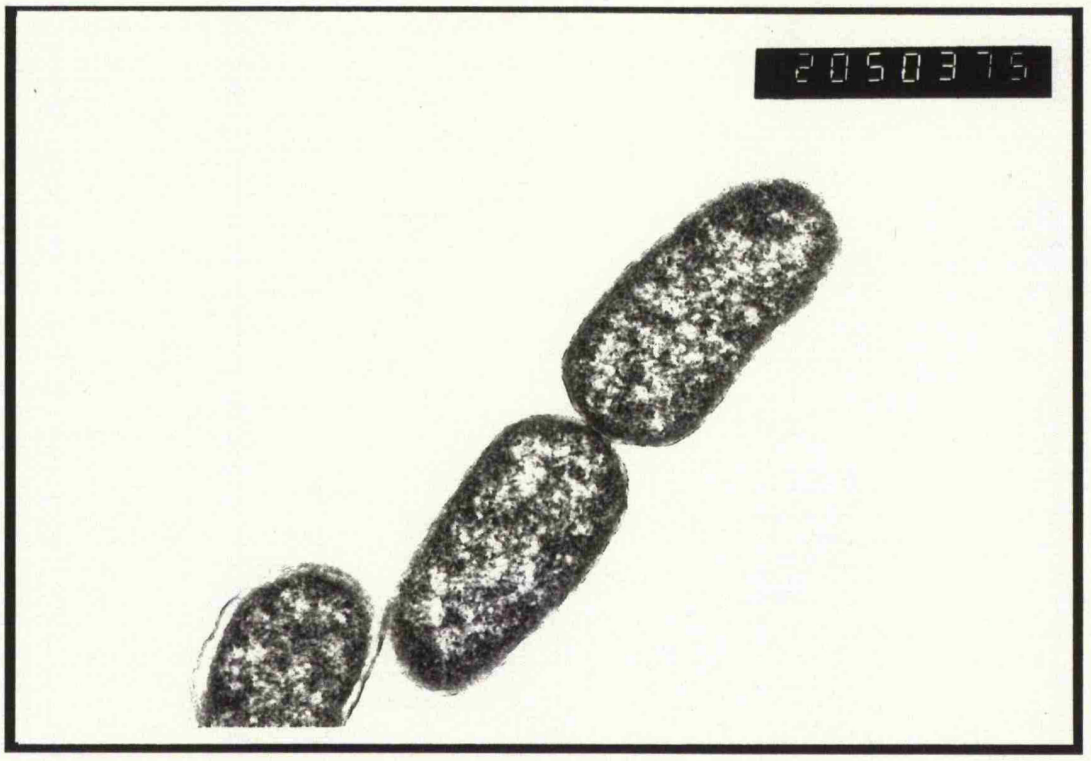

Fig. 52. N43 (Magification x20,000)

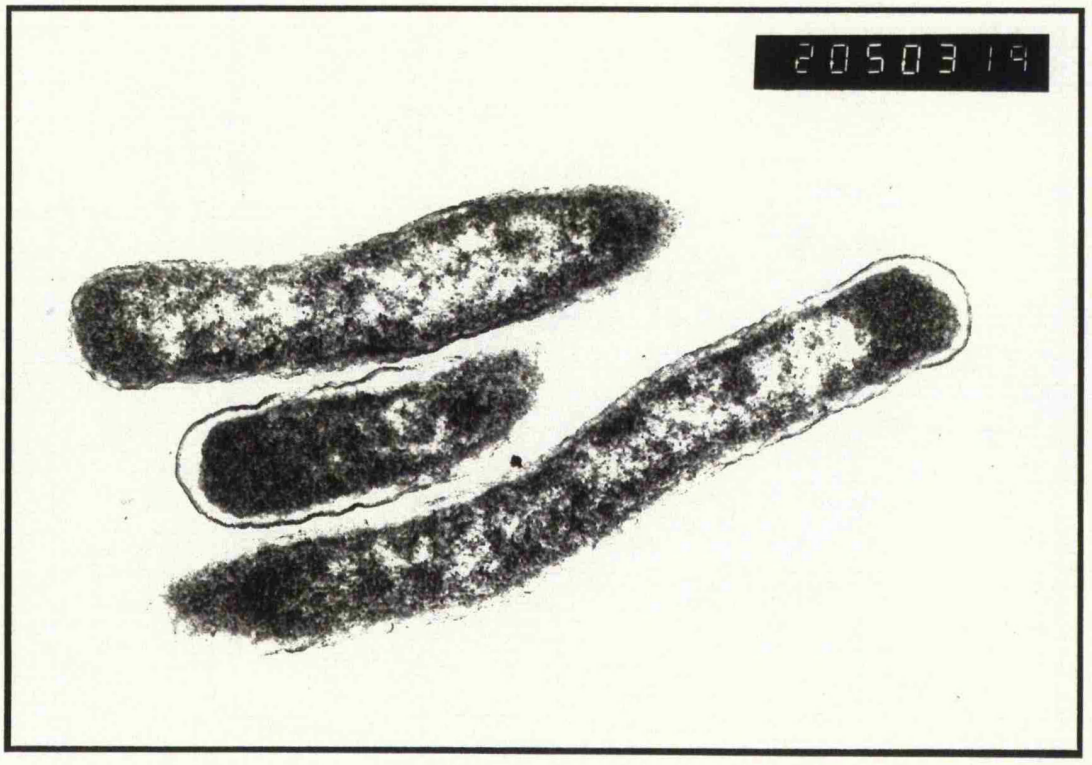

Fig. 53. N43*verA1* (Magnification x20,000)

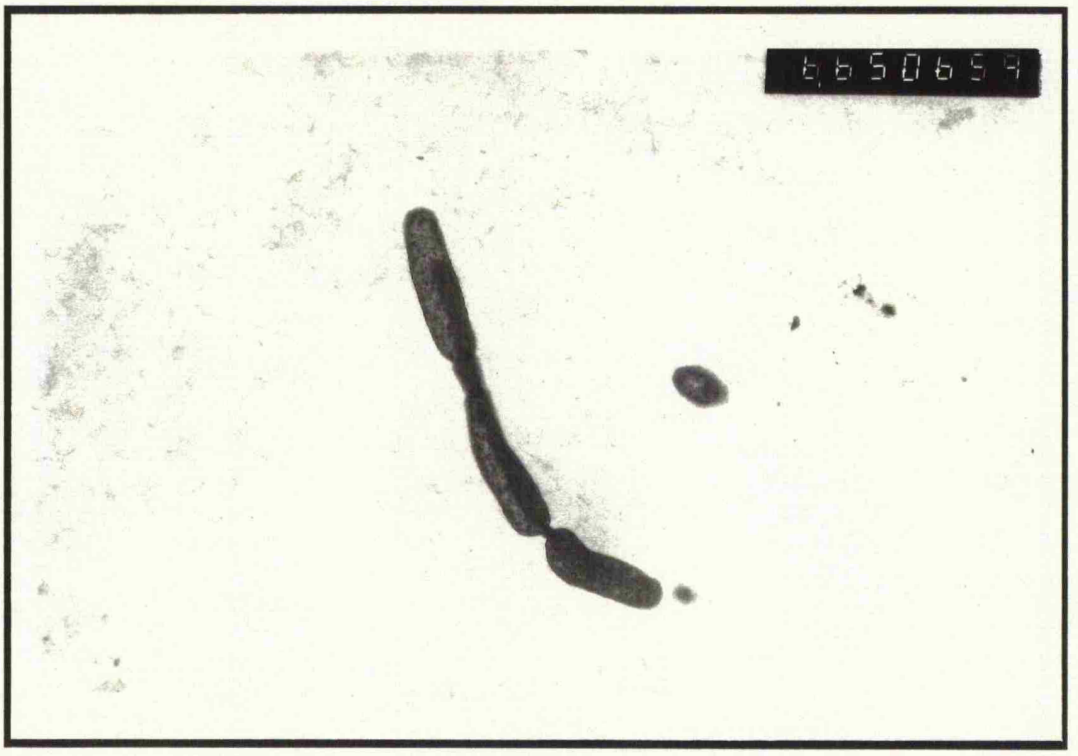

Fig. 54a. N43*verA1* + pLG705. (Magnification x6000)

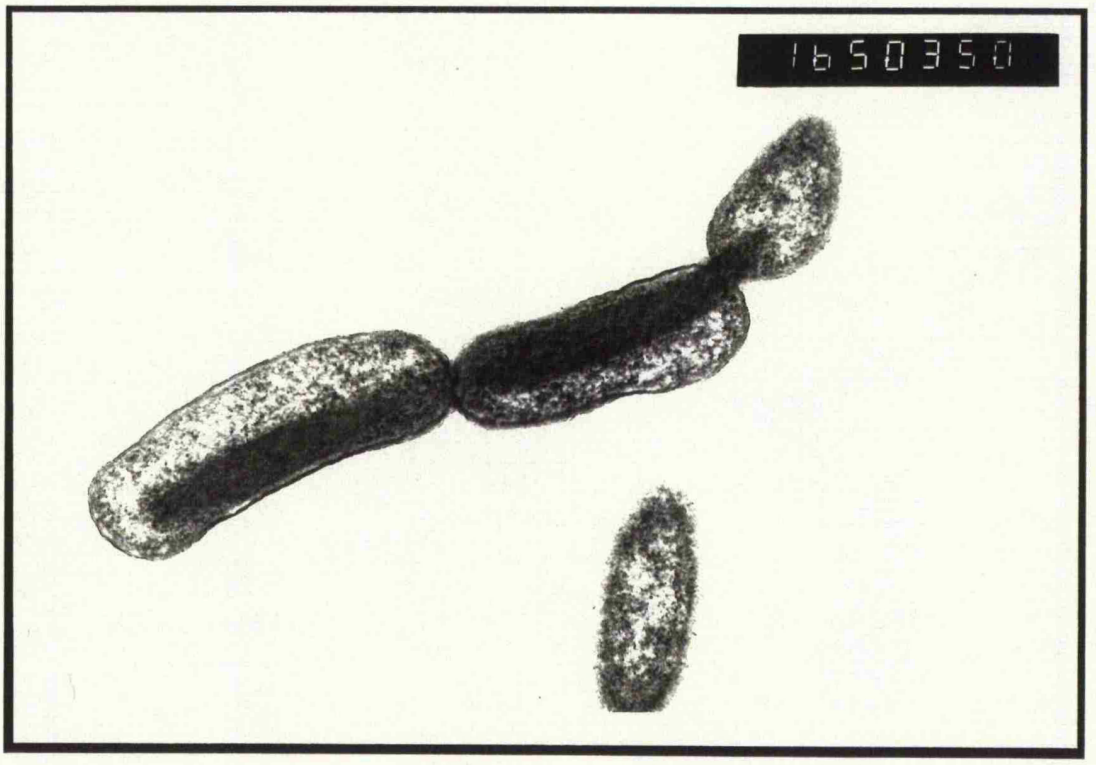

Fig. 54b. N43*verA1* + pLG705. (Magnification x16000)

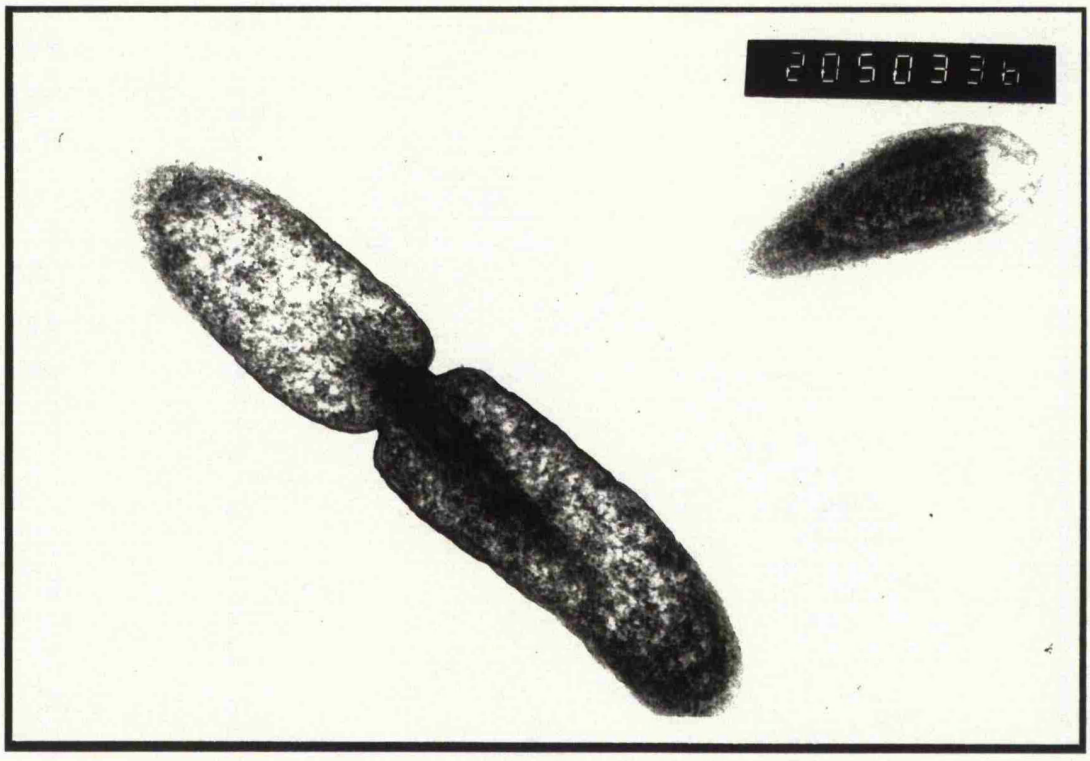

Fig. 54c. N43*verA1* + pLG705 (Magnification x16,000)
Note the way the filaments appear to grow from the septum into the cells.

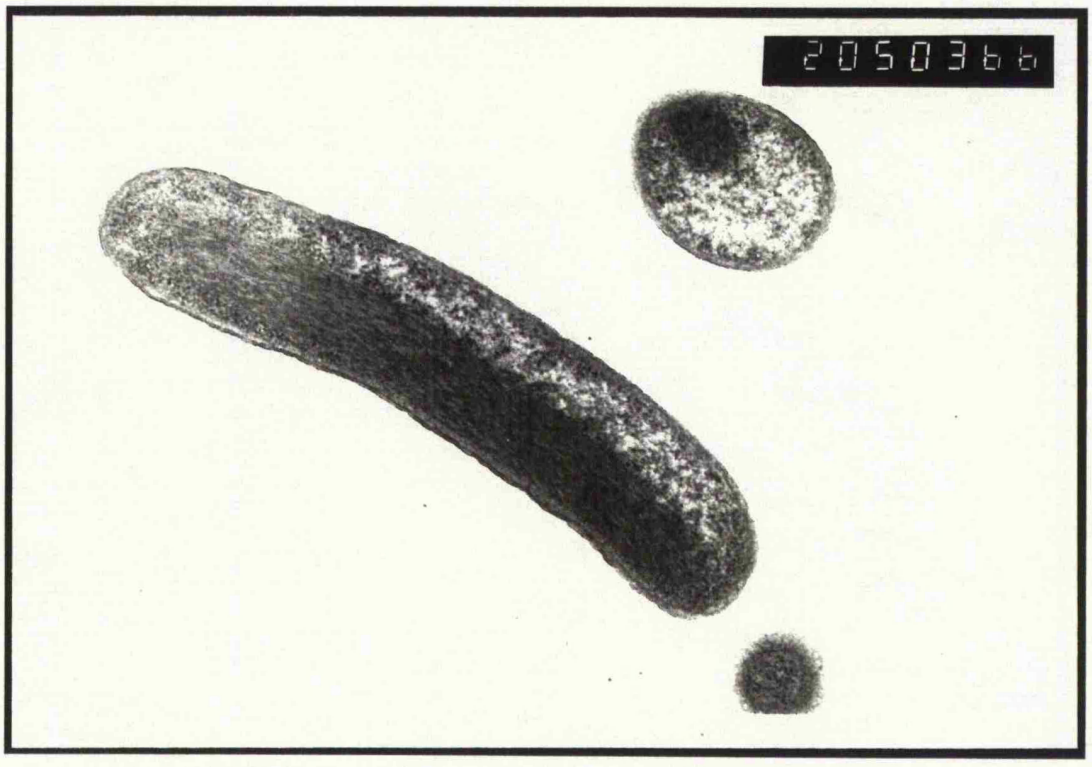

Fig. 54d. N43*verA1* + pLG705 (Magnification x20,000)
Note the off-centre location of the filaments and fibrous appearance.

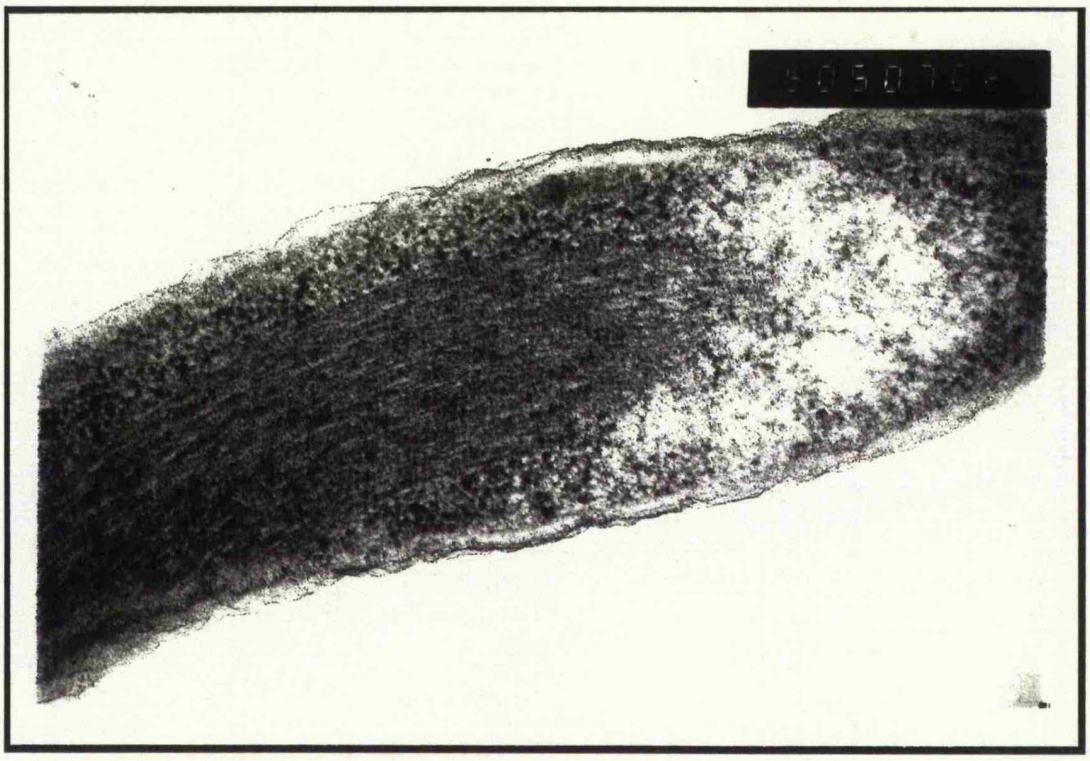

Fig. 54e. N43*verA1* + pLG705 (Magnification x50,000)

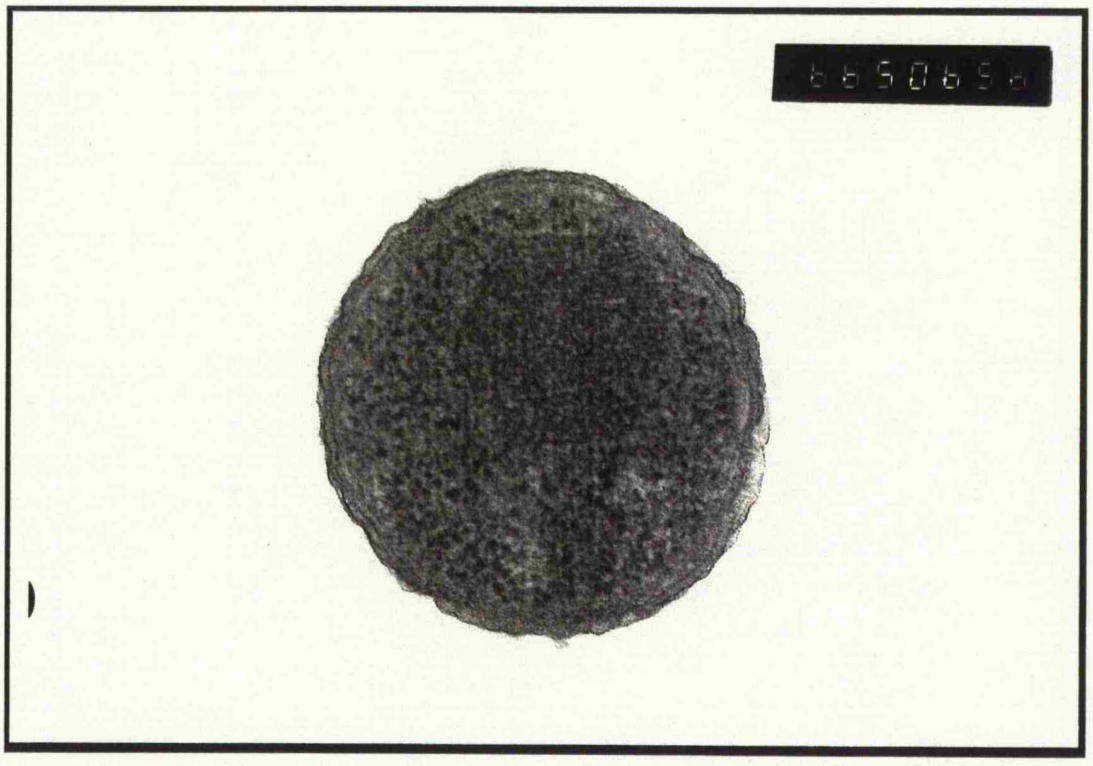

Fig. 54f. Cross section of N43*verA1* + pLG705 (Magnification x66,000)
Note the hexagonal, structured appearance of the filament.

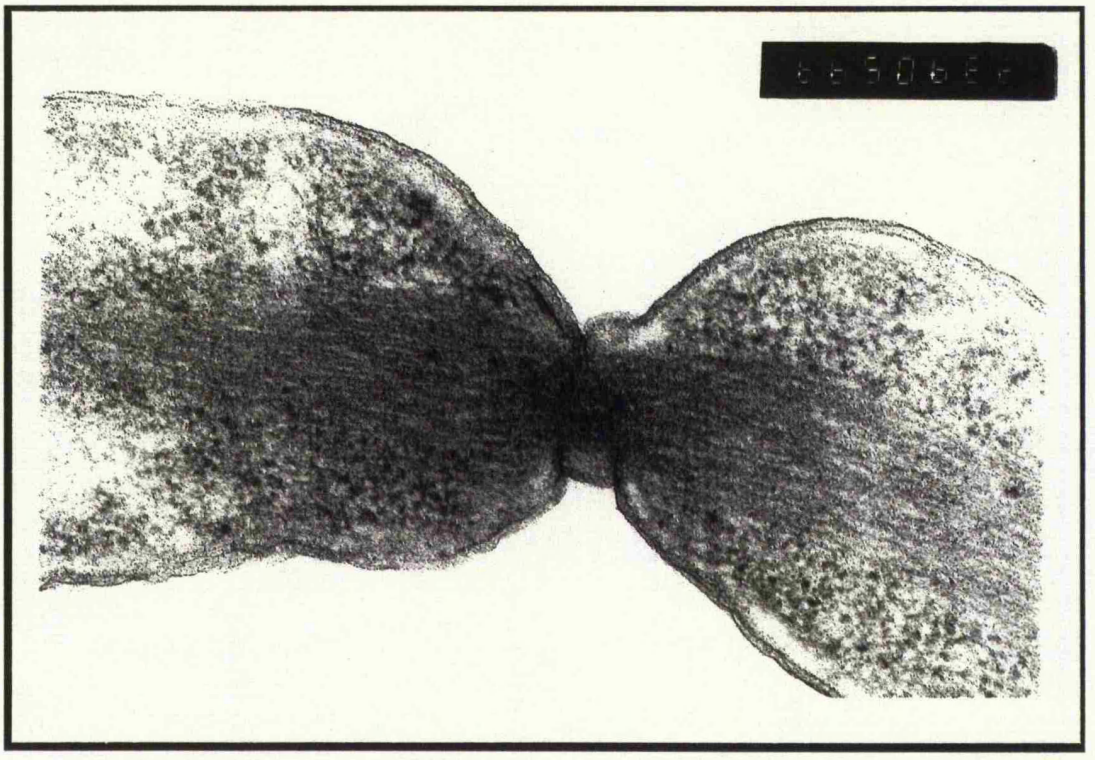

Fig. 54g. Division site of N43*verA1* + pLG705 (Magnification x66,000)
Note the formation of a "collar" and the fibrous nature of the filament.

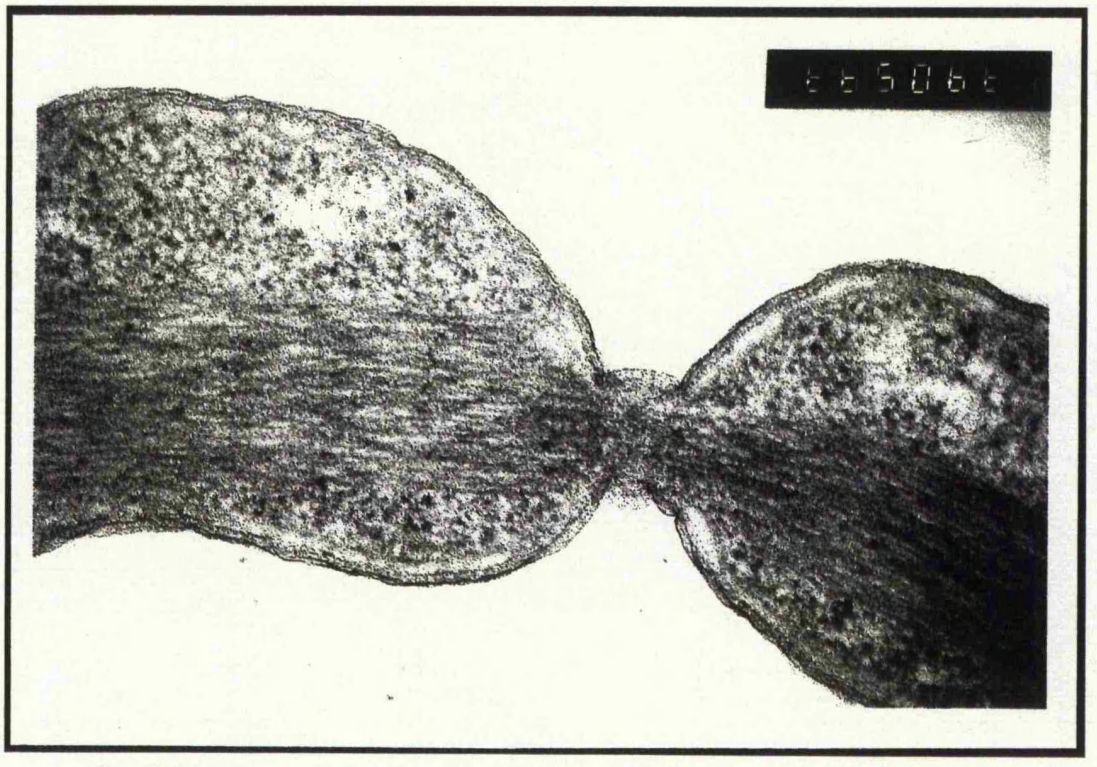

Fig. 54h. Division site of N43*verA1* + pLG705 (Magnification x66,000)
Again, note the formation of a "collar" and the fibrous nature of the filament.

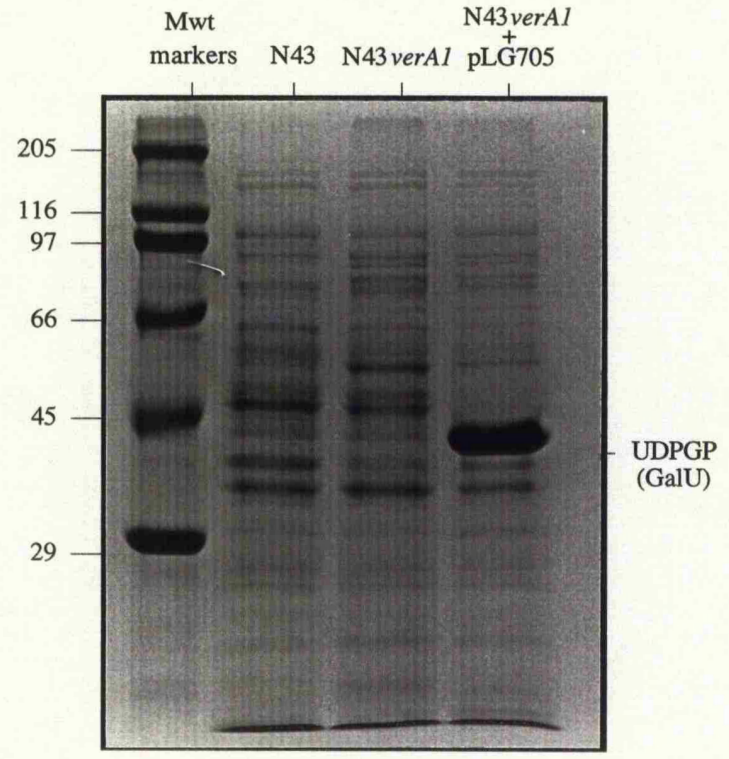

Fig. 55. Coomassie blue-stained SDS-polyacrylamide gel showing the overexpression of the *galU* gene product, UDPGP.

Protein extracts from overnight cultures of N43, N43*verA1* and N43*verA1* + pLG705 (pUC19 containing *galU*) were prepared and loaded onto an 11%$^{v}/_{v}$ SDS-polyacrylamide gel. Following electrophoresis, the gel was stained with coomassie blue (see Materials and Methods) and photographed.

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