TRANS-KINGDOM PLASMID TRANSFER FROM BACTERIA TO YEAST

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by

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Abstract

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Bacterial conjugation is a plasmid-encoded process resulting in the horizontal transfer of DNA from a donor to a recipient cell. However, the process of conjugation is not limited to transferring DNA between related prokaryotes. Indeed conjugation can result in the transfer of DNA to a wide range of organisms, even eukaryotes. Two such trans-kingdom transfer events have been reported; the transfer of the Ti plasmid of *Agrobacterium tumefaciens* to plant cells, and transfer from *Escherichia coli* to the budding yeast *Saccharomyces cerevisiae* the subject of this thesis. The aim of this study was to identify the genetic factors, both in the donor bacterium and the recipient yeast cell, required for trans-kingdom transfer to occur. The evolutionary implications of trans-kingdom transfer will also be discussed.

This work describes the successful development and optimisation of an efficient protocol for *E. coli*-yeast plasmid transfer. Using this optimised protocol the ability of a range of conjugative plasmids to mediate transfer to yeast was compared. Only the promiscuous plasmid RP4 appeared capable of mediating transfer to yeast using this protocol. A genetic analysis of RP4 was carried out to determine the factors required for transfer to yeast and to identify the basis of RP4 promiscuity. This analysis revealed that the same set of genes essential for transfer between *E. coli* cells are also required for transfer to yeast. Therefore, no additional factors are required for the promiscuous transfer of RP4 to yeast. Hence, the promiscuous nature of RP4 is inherent in its transfer machinery.

To determine the yeast factors required for successful transfer, mutants were isolated that displayed an altered ability to receive and establish a plasmid by trans-kingdom conjugation. Five UV induced yeast mutants (*con1-5*) were identified that displayed a decrease in transfer. Genetic complementation of *con1* with a genomic library was successfully carried out. *CON1* is allelic to the previously uncharacterised ORF YHR185c, located on the right arm of chromosome 8. *CON1* encodes a predicated protein of 237 amino acids, and is unique in the yeast genome. The function of CON1p is unclear, it may be required for the cellular interactions between cells or for the establishment of the transferred DNA strand. Analysis of these mutants will provide a tool to determine the molecular basis of trans-kingdom DNA transfer.

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Finally, I would like to dedicate this work to my parents and to Kathryn for their love and support.

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Abbreviations

A	Adenine
ATP	Adenosine 5'-triphosphate
bp, kb	Base pair, Kilo-
BSA	Bovine Serum Albumin
C	Cytosine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetra-acetic acid
G	Guanine
g, mg, µg, ng	Grams, milli-, micro-, nano-
IMS	Industrial methylated spirits
l, ml, µl	Litres, milli-, micro-
M, mM, µM, nM	Molar, milli-, micro-, nano-
MAT	Mating Type Locus
min	Minute
NLS	Nuclear Localisation Signal
nt	Nucleotide
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEG	Polyethylene Gylcol
RNA	Ribonucleic acid
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second
SSC	Standard saline citrate
T	Thymine
Tris	Tris-(hydroymethyl)-methylamine[2-amino-(2-hydroxymethyl)-propan-1.3-diol]
UV	Ultra Violet

CONTENTS

Chapter One

Introduction

Historically, changes in the traits of organisms were thought to occur only between parent and offspring, through so called vertical transmission. However, we now know that mechanisms do exist for the exchange of DNA between unrelated organisms in a population by horizontal transfer. Indeed the initial evidence that the genetic material is DNA, Avery's "Transforming principle", was wholly reliant on horizontal transfer. Three general mechanisms of horizontal transfer have been characterised in prokaryotes, those of transformation, transduction, and conjugation. Of these processes transformation and transduction are chance happenings resulting in the spread of non specific chromosomal sequences. Conjugation is more purposeful in that it transfers specific plasmid DNA between two cells, the donor and recipient. The first conjugative plasmid identified, the fertility factor F, was discovered by Lederberg and Tatum (1946). Serendipitously the F plasmid had integrated into the chromosome of one of their laboratory strains, resulting in a higher level of recombination. Accordingly the majority of research has been aimed at determining the molecular basis of F plasmid transfer. However, the F plasmid is by no means unique as exemplified by the finding of at least 25 plasmid incompatibility groups in *E. coli* (Couturier *et al.*, 1988). The current paradigms for conjugative transfer are the sex factor F and the promiscuous plasmid RP4, of the IncFI and Pα incompatibility groups respectively. Although these plasmids encode discrete transfer systems the mechanistic nature of their transfer is comparable. However, these plasmids do display differences in their ability to transfer to a wide range of organisms (Guiney, 1982)

One of the main driving forces behind research into mechanisms of conjugation is its evolutionary implications and medical importance. This is exemplified by the widespread dissemination of the same antibiotic resistance determinants among diverse bacteria in the last 50 years since the introduction of antibiotics into medicine and animal husbandry. The majority of these resistance determinants are carried on conjugative plasmids thus providing compelling evidence for their spread by conjugation (Amabile-Cuevas & Chicurel, 1992). Such a spread between diverse organisms would require the promiscuous transfer of DNA. However, although the transfer range of conjugative plasmids

are often found to be wider than their associated replication functions differences are apparent in their ability to transfer to diverse organisms (Guiney, 1982).

The wide transfer range of conjugation was confirmed in 1989 with the demonstration that conjugative plasmids could mediate DNA transfer from *E. coli* to the eukaryote *Saccharomyces cerevisiae* (Heinemann & Sprague, 1989). There is no apparent common niche for these two organisms. Hence, it seems unlikely that transfer is the result of a specialised system, unlike T-DNA transfer by the Ti plasmid of *Agrobacterium* to plant cells (reviewed in Kado, 1993). Hence conjugation is capable of mobilising DNA between different kingdoms. Trans-kingdom transfer then could provide a convenient system for studying promiscuous transfer at its most extreme. Transfer between such diverse organisms would be typically unproductive due to the lack of sequences for replication and expression. Hence, productive transfer would rely on the integration of the immigrant DNA into the host genome, although such events are rare in the laboratory over an evolutionary time scale they may become important. In fact there are published examples using horizontal transfer to explain discrepancies in phylogenetic trees (reviewed in Syvamen, 1994; Smith *et al.*, 1992).

The central theme of the work described in this thesis is the elucidation of factors both in donor and recipient cells required for promiscuous transfer between bacteria and yeast. Many questions are raised by this phenomena concerning both yeast biology, the interaction with bacterial cells and uptake of DNA; and on the conjugative process, both the specificity of contacts between donor and recipient cells and the nature of promiscuity. To this purpose this introduction will review our current understanding of conjugation using F and RP4 as the main examples. Examples of promiscuous transfer will also be discussed. It will also cover the evidence that horizontal transfer occurs in nature and has affected the evolution of many species including both prokaryotes and eukaryotes. Finally it will address the aspects of yeast biology pertinent to this study.

1.1 Bacterial Conjugation

Conjugation can be defined as the polar transfer of DNA from a donor to a recipient cell by a mechanism requiring specific cellular contact. The majority

of conjugative systems are plasmid encoded. The exception being conjugative transposons which will not be discussed in this work. The process of conjugation can be considered as two main events. Initially a DNA transport bridge is formed between donor and recipient, through the action of the conjugative pilus. This process is termed mating pair formation. The formation of mating pairs is believed to trigger the processing of plasmid DNA for transfer. A single stranded nick is introduced into the plasmid DNA at a specific region termed the origin of transfer (*oriT*). Subsequent unwinding of the DNA occurs coupled to the transfer of the nicked strand into the recipient cell in a 5' to 3' direction. Hence, the *oriT* region enters the recipient first. Transfer is terminated by the circularisation of the transferred strand in the recipient cell, in a process which requires interactions at the *oriT*. This transfer is usually linked to replacement strand synthesis in both donor and recipient.

1.1.1 Organisation of F and RP4 Transfer Genes

The transfer genes of F are organised into a continuous 33.3kb region of the 100kb plasmid (Figure 1.1). The entire sequence of this region is known (Frost *et al.* 1994). There are ~40 transfer genes (termed *tra* and *trb* genes), only 4 of which are required for the processing of plasmid DNA for transfer. The remainder of the transfer genes encode the mating apparatus and proteins to stabilise mating pairs. The F-*oriT* is situated at the end of this region and is orientated such that the transfer genes enter the recipient cell last, a common feature of many conjugative plasmids. The majority of the genes are expressed from one main promotor, P_Y, which is situated between *traJ* and *traY*. Transcription from this promotor is TraJ dependent (Willetts, 1977). The *traJ* gene has its own promotor which is negatively regulated by an antisence RNA encoded by *finP*. However, regulation by *finP* also requires the *finO* gene product which is believed to stabilise the *finP* RNA (Frost *et al.*, 1989). In F *finO* is disrupted by the insertion of an IS3 element. Hence, *traJ* and therefore the other Tra genes are consitutively expressed, resulting in F being derepressed for transfer (Cheah & Skurray, 1986). There is also evidence for distal promotors in the Tra region, particularly for *traS* and *traT* which appear to be constitutively expressed. The *traS* and *traT* genes are involved in surface exclusion, the process that prevents mating between cells harbouring related plasmids. This function may be important ecologically to prevent successive rounds of mating between cells which could produce membrane damage. There is also evidence that *traI* and *traD* have their own promotors, however, full

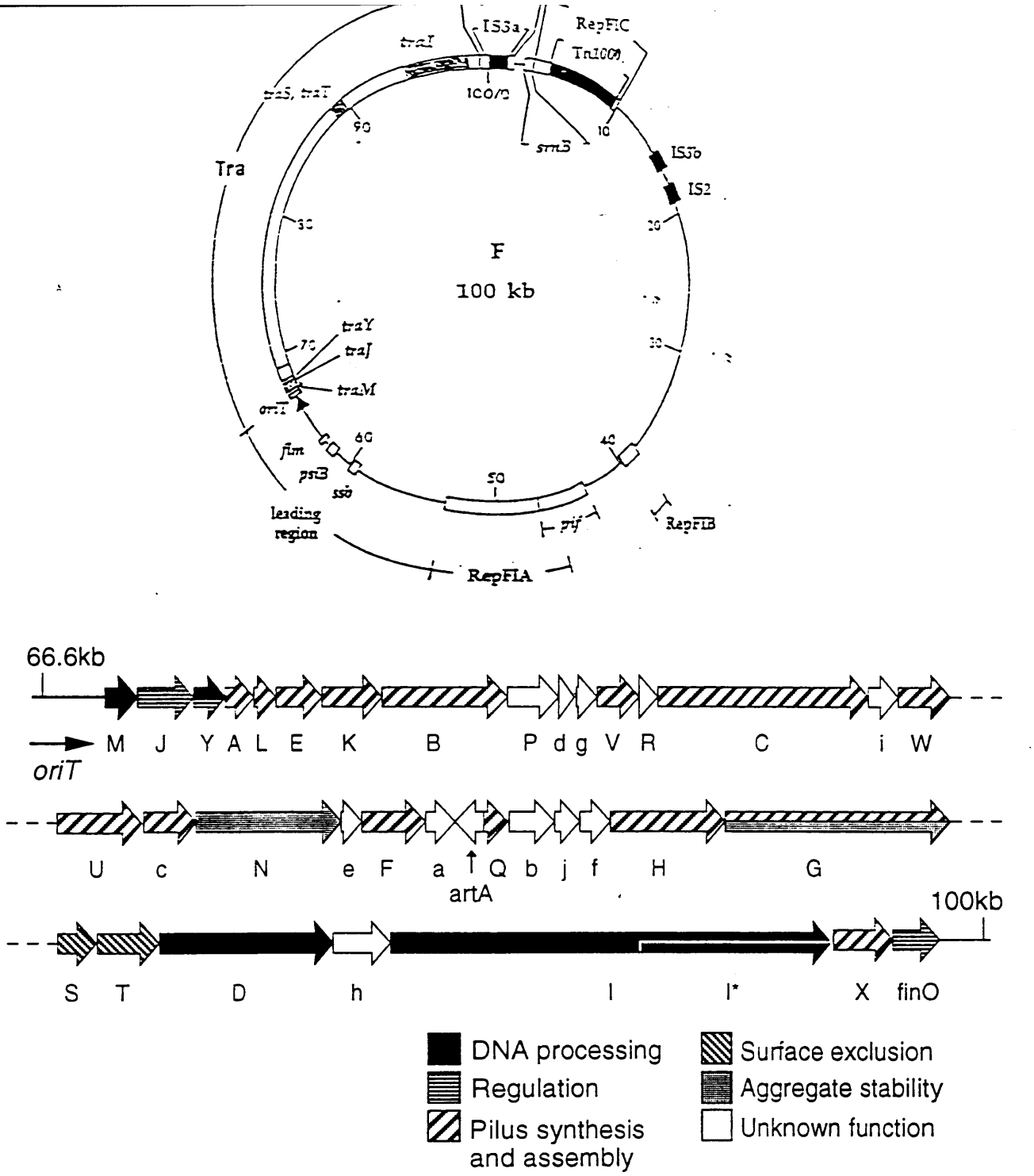


Figure 1.1. Genetic and Physical Map of the F Plasmid.

The circular map details the position of insertion elements and plasmid replicons in the 100kb F plasmid backbone. Leading region genes and the Tra regions are also detailed. The extended section shows the position and orientation of the transfer genes; *tra* genes are denoted in uppercase, *trb* genes in lower case. The proposed functions of the different Tra genes are indicated. and will be discussed in more detail in the text. The position of the *oriT* region is indicated; transfer is orientated such that the Tra genes enter the recipient cell last. The circular map is derived from Willetts & Skurray, 1987; details on the position, orientation, and function of the Tra genes was obtained from Frost *et al.*, 1994.

expression of these genes requires a functional P_Y promotor (Mullineaux & Willetts, 1985).

The transfer genes of RP4 are organised into two regions, termed Tra1 and Tra2. These regions are separated by a multimer resolution system, partitioning site, *aphA* (for kanamycin resistance) gene, and an IS21 element (Figure 1.2; Pansegrau *et al.*, 1994a). The separation of transfer genes in conjugative plasmids is unusual and it has been suggested that separation occurred due to insertions into the RP4 backbone during P plasmid evolution. Analysis of the base composition of RP4 supports this theory (Wilkins *et al.*, 1996). The transfer regions cover almost half of the 60kb genome of RP4 and encode for 27 proteins. The Tra2 region contains a core of 11 genes (*trbB-L*) required for plasmid transfer. The products of these genes are involved in mating pair formation and may also constitute the DNA transport pore (Lessl *et al.*, 1993; Haase *et al.*, 1995). The *trbA* gene is involved in the regulation of expression of various RP4 loci including the Tra2 genes which are expressed as one long operon. The Tra1 region contains 13 genes, however, only 5 Tra1 genes are required for transfer between *E. coli* cells (*traF,G,I,J, & K*). Four of these genes are required for DNA processing, the fifth *traF* is involved in mating pair formation. The *traM* gene is not absolutely required for transfer between *E. coli* cells, however its absence results in a 300 fold reduction in transfer efficiency (Lessl *et al.*, 1993). It has been speculated that the other Tra1 loci may encode ancillary factors which may be important for interspecific matings (Lessl *et al.*, 1992). The origin of transfer is also situated in the Tra1 region between *traJ* and *traK*. Transfer is orientated such that *traK,L, & M* enter the recipient cell first, with the majority of the Tra genes being transferred late. Transcription in the Tra1 region is initiated in the *oriT* region from 2 divergent operons; the leader operon consisting of *traK,L & M*, and the relaxase operon, *traJ* through to *traA* (Greener *et al.*, 1992).

1.1.2 Mating Pair Formation

The conjugative pilus of Gram negative bacteria is a key feature in producing stable mating pairs. These extracellular structures are envisaged to interact, via the tip of the pilus, with a receptor on the surface of the recipient cell. The pilus then retracts by depolymerisation of its subunits into the membrane fraction of both donor and recipient. This results in the cells being drawn into close proximity to form mating pairs. The interactions between mating pairs

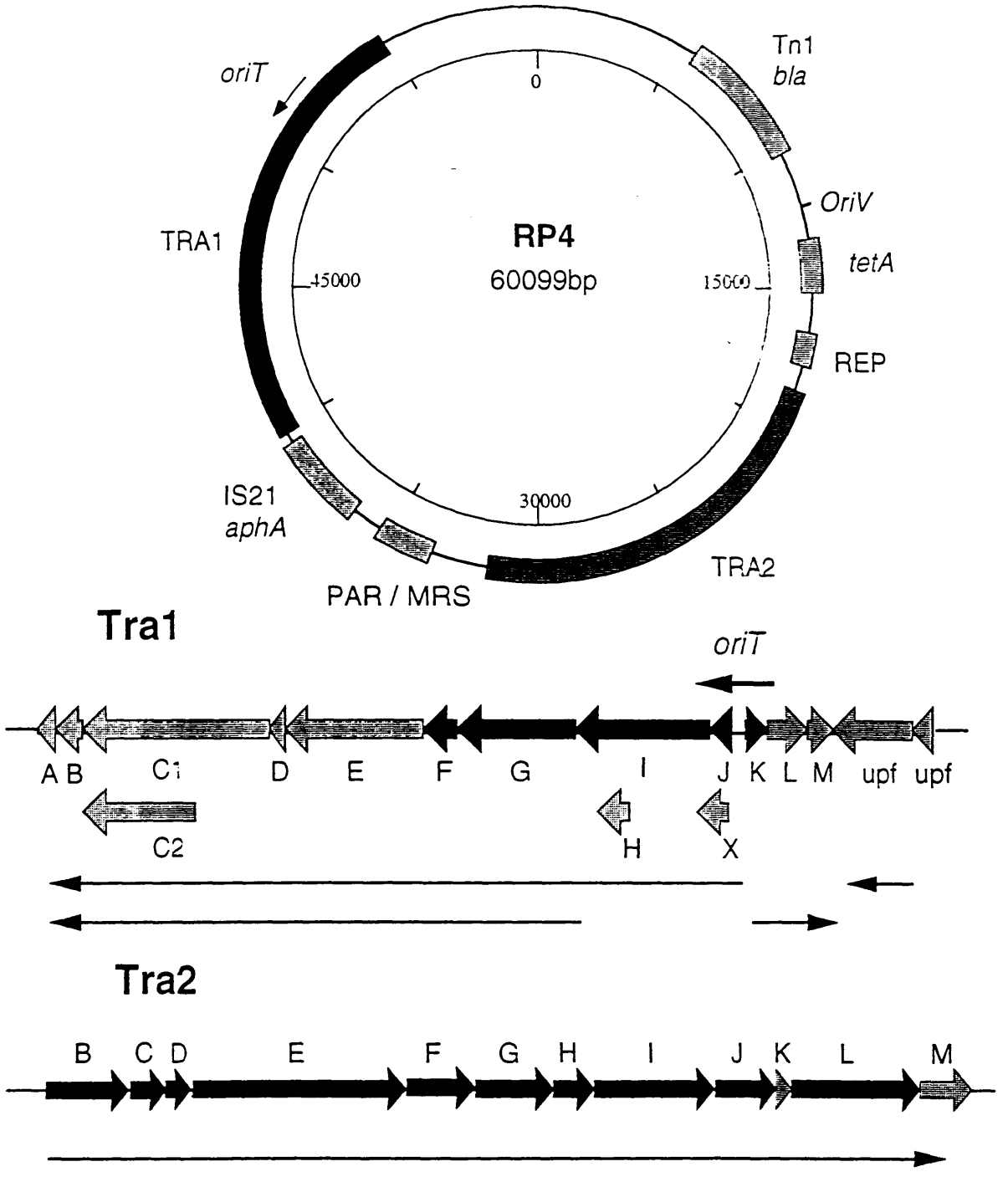


Figure 1.2. Organisation of the RP4 Plasmid.

The circular map indicates the relative position of the major determinants on RP4. Loci indicted include Tn1(*bla*)-providing ampicillin resistance; *tetA*-for tetracycline resistance; IS21(*aphA*)-kanamycin resistance; PAR/MRS-for multimer resolution and partitioning; REP-replication functions; *oriV* -origin of replication. The position of the Tra regions (Tra1 and Tra2) is also indicated. The extended sections indicate the position and orientation of the Tra genes, arrows indicate the transcriptional units. The position of the *oriT* region is shown, transfer is orientated such that the transfer genes enter the recipient cell last. Genes essential for transfer are shaded black, non essential transfer genes are shaded grey.

are then stabilised to form cellular contact. It has been suggested that the pilus, or at least its base, may constitute the actual DNA transport pore. This is supported by the observation that DNA can be transferred through an extended F pilus (Harrington & Rogerson, 1990). Additionally the gene products required for pilus assembly are found in the inner and outer membranes, and the pilus emerges from a zone of adhesion between these membranes. Hence, it could be envisaged that these proteins form a pore for the transfer of DNA through the inner and outer membrane of the donor cell.

There are three morphological forms of pili found in Gram negative bacteria; the thin flexible, thick flexible, and rigid filaments (Bradley, 1980; Frost, 1993). The rigid pili are produced by plasmids of the IncP, N, and W groups. These transfer systems are limited to mating on solid surfaces. The thick flexible pilus is exemplified by the IncF plasmids, it allows efficient mating in liquid as well as on surfaces. The IncI complex of plasmids (incorporating IncB,I1,I2,I5,K, & Z) specifies both a rigid pilus and a thin flexible pilus, accordingly these plasmids are able to mate on surfaces and in liquid. It has been suggested that the thin flexible pilus may act to stabilise mating pairs in liquid matings (Rees *et al.*, 1987).

The production of the thick flexible pilus of the IncF plasmids is best understood. The F pilus is a hollow cylinder typically 20μm in length with a diameter of 8nm and an internal core of 2nm - large enough for the passage of single stranded DNA (Folkhard *at al.*, 1979). The pilus is formed from a single subunit, pilin, the product of the *traA* gene (Minkley *et al.*, 1976). Pilin is arranged in a helical array to produce the pilus with 5 subunits contributing to one turn of the helix. The pilin subunit is initially expressed as a precursor from propilin. The 12kDa propilin polypeptide is processed to form the mature 7kDa subunit. The cleavage site is characteristic of a signal peptidase target site, however, the signal sequence is unusually long and it has been shown that processing of propilin requires the *traQ* gene product (Frost *et al.*, 1984). Mature pilin subunits are also N terminal acetylated, a reaction which requires the *traX* gene product (Moore *et al.*, 1993). As well as *traQ* and *traX* for maturation of pilin at least 12 other Tra genes (*traL,E,K,B,V,C,W,U,F,H, TrbC,* and the N terminal half of *traG*) are required for pilus assembly (Moore *et al.*, 1991). Mutations in these genes result in no pili being formed and therefore abolish plasmid transfer. Of particular interest is a missence mutation in *traC* (*traC1044*, arginine to cysteine) which resulted in no discernible pili being

formed. However, plasmid transfer was not affected in this mutant. It has been suggested that this mutant is defective in pilus elongation, but allows the expression of the pilus tip at the cell surface (Schandel *et al.*, 1987). The *traN* and *traG* products are also essential for DNA transfer and may act to stabilise mating pairs. This has been proposed as mutants produce F pili but do not form stable, SDS resistant, mating pairs (Manning *et al.*, 1981).

The mating pair formation system of RP4 requires the products of 10 genes in the Tra2 region (*trbB,C,D,E,F,G,H,I,J,* & *L*) and *traF* of the Tra1. Defined mutations in these genes have shown them to be essential transfer components. Their involvement in mating pair formation is inferred as they are required for the propagation of donor specific phages, and transfer of the naturally mobilisable IncQ plasmid RSF1010 (Lessl *et al.*, 1993; Haase *et al.*, 1995). The TrbB protein is hydrophilic and contains a weak DNA independent ATPase activity, possibly due to a type A nucleotide binding site in its primary structure. The other genes required all encode hydrophobic proteins several of which contain potential transmembrane domains, or signal sequences for export. Hence, these proteins are liable to be associated with the cell envelope (Pansegrau *et al.*, 1994a). TrbE also contains a type A nucleotide binding site (Lessl *et al.*, 1992b), leading to the hypothesis that TrbB and TrbE may be involved in energising pilus assembly. Interestingly no pili are detected in a *trbK* mutant, however, conjugative transfer is not affected in this mutant. This observation coupled with the facts that pili are rarely seen associated with cells, and that donor specific phages are seen to directly associate with the cell surface, brings the role of the pilus in RP4 transfer into question. Potentially the extended structure may not be required, and can be replaced by a short pilus 'stub' - not discernible by electron microscopy.

In contrast to Gram negative bacteria the conjugative systems of Gram positive bacteria do not use a conjugative pilus. In Gram positive bacteria other mechanisms exist for mating pair formation, one example being the pheromone induced transfer of *Enterococcus facaelis* plasmids such as pAD1 (Clewell, 1993). Recipient strains of *E. facaelis* secrete a hydrophobic polypeptide of 7-8 amino acids which acts as a pheromone for donor cells. In response to the pheromone donor cells produce a proteinaceous adhesin, termed aggregation substance, which facilitates mating aggregate formation. Aggregate formation occurs by binding of aggregation substance to a 'binding substance', possibly lipoteichoic acid, found on the surface of both recipient and donor cells. The

process of aggregation however occurs by chance contact between cells, and not by a chemotaxic response. DNA transfer is believed to occur between aggregates at 'fusion points' of close contact between the cells. Once transfer has occurred the newly formed transconjugant cell shuts down production of the pheromone. However, the transconjugant continues to produce pheromones specific for other plasmids. The production of pheromones by recipient cells is intriguing, presumably these peptides have had, or continue to have, a function and plasmids have evolved to take advantage of their presence.

One of the most mysterious aspects of conjugation is the nature of the cellular contacts formed between cells to potentiate transfer. The main approach to analyse these interactions between cells has been to isolate mutants defective as recipients in conjugation. These studies have identified ConF$^-$ mutants defective in receiving the F plasmid. The mutants were found either to lack the outer membrane protein OmpA, or had altered lipopolysaccharide (LPS) in the cell envelope. However, these mutants were all isolated in liquid matings, when mating was carried out on surfaces a wild type level of transfer was restored. This has led to the hypothesis that the initial interactions in liquid matings are different to mating on surfaces, alternatively mating on surfaces may bypass some of the criteria required for stabilising mating pairs (Achtman *et al.*, 1978). It is now generally accepted that OmpA acts to stabilise mating pairs in contact. However, OmpA's role is confused further by the finding that its requirement is plasmid specific, even among F-like plasmids (Havekes *et al.*, 1977). It has been suggested that LPS mutants can be explained by the inappropriate insertion of OmpA in the membrane. However, the pyrophosphorylethanolamine residue on the first heptose in the inner core of LPS has recently been implicated in F plasmid transfer in liquid (Anthony *et al.*, 1994). This observation together with the fact that ConI$^-$ mutants are also found in LPS (Havekes *et al.*, 1977a), and that normal LPS can inhibit plasmid transfer (Anthony *et al.*, 1994) points towards LPS having a more central role in conjugation in liquid at least. In conclusion our knowledge of the cellular interactions required to form mating pairs is still limited, as is our knowledge of the actual DNA transport pore. The observation that conjugation is possible between divergent organisms, such as Gram negative and Gram positive bacteria, and even to eukaryotes raises the question of the specificity of contacts between donor and recipient cells. Further studies are required to address these issues, of particular interest would be to isolate mutants defective in mating on surfaces.

1.1.3 DNA Processing Reactions During Bacterial Conjugation

The key event in conjugal DNA transfer is the introduction of a site and strand specific nick at the origin of transfer region. This nick is produced by a specific enzyme complex which forms a DNA-protein complex termed the relaxosome. Nicking of the DNA takes place at a specific point, *nic*, and occurs independently of mating pair formation. The nicked DNA is then unwound and passed into the recipient cell in a 5` to 3` direction (Figure 1.3). It is a common assumption that single stranded DNA is transferred, however, this has only been demonstrated for F, R538-1 (IncFII), and R64 (IncI1) plasmid transfer (Ohki & Tomizawa, 1968; Rupp & Ihler, 1968; Vapnek *et al.*, 1971). Additionally, it has only been demonstrated for the F plasmid that transfer occurs in a 5` to 3` direction (Rupp & Ihler, 1968). However, the similarities between different transfer systems in the functional processes of conjugation provides convincing evidence that these observations are common to all conjugative plasmids. Transfer is usually linked to replacement strand synthesis in both donor and recipient. In the donor cell DNA synthesis is believed to occur by a rolling circle process, using the free 3` hydroxyl of the transferred strand. Replacement strand synthesis in the recipient would be characteristic of lagging strand synthesis and would require RNA primers. Finally the ends of the transferred strand are joined, by a process requiring Tra gene products, to form a circular DNA molecule.

Origin of Transfer (*oriT*) sites

The *oriT* region is the only region required *in* cis on a plasmid for transfer to occur. Hence, *oriT* regions can be easily defined by their ability to convert a non transmissible plasmid to a mobilisable plasmid. The sequence of several *oriT* regions is available and has identified several characteristics common to all *oriT's* (reviewed in Lanka & Wilkins, 1995). Extensive homologies between *oriT's* are limited except between closely related plasmids. However, sequence homology can be seen to exist in the *nic* region of *oriT's* (Pansegrau & Lanka, 1991; Waters *et al.*, 1991). This has identified three groups of *nic* regions, related to the IncP, F, and Q plasmids (Figure 1.4). The *oriT* regions themselves are typically short, up to about 500bp, and map such that the majority of the Tra genes are transferred to the recipient cell last. Common features are a high A/T content and a complex secondary structure resulting from direct and inverted repeats. A high A/T content is also characteristic of

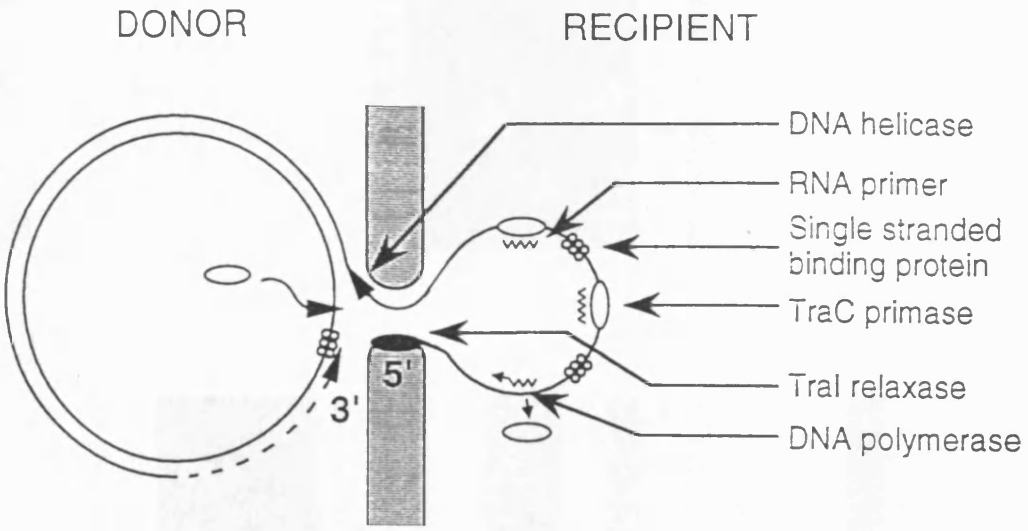


Figure 1.3. General Model for Conjugative Transfer of IncPα Plasmids.

Conjugative transfer is initiated by cleavage at the *oriT* region by the relaxase (TraI) which remains covalently bound to the 5' end of the nicked DNA. DNA transfer then occurs from donor to recipient cell in a 5' to 3' direction. Single stranded DNA is transferred hence a helicase is required to unwind the plasmid DNA, however, the nature of the helicase involved in IncP transfer is unknown. The TraC primase is also transported to the recipient cell where it directs DNA synthesis on the transferred strand. DNA replication in the donor is believed to occur via a rolling circle mechanism using the free 3' hydroxyl on the transferred strand. Transfer is terminated by a reversal of the nicking reaction to produce a covalently closed circular DNA molecule.

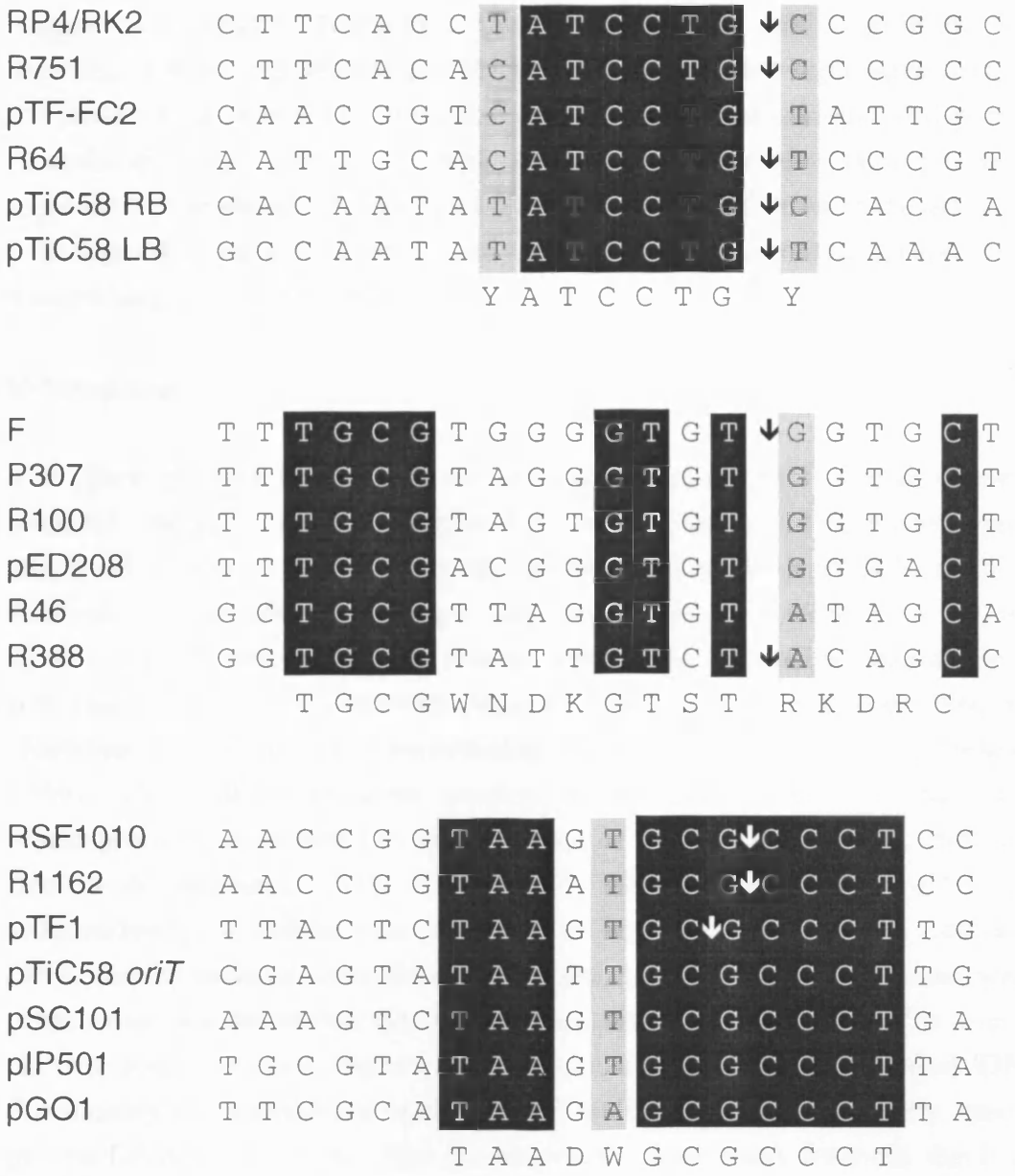


Figure 1.4. Homologies Between Plasmid *nic* Regions.

The alignment of several *oriT* regions are shown, these fall into three groups. The position of the *nic* site, if known, is indicated (↓). Identical nucleotides are blocked in black with conserved nucleotides in grey a consensus is also given. Figure reproduced from Lanka & Wilkins, 1995.

origins of vegetative replication (*oriV*); in fact the *oriT* region of RK2 (essentially RP4) can replace the M13(+) strand *oriV* as a replicative origin in the presence of certain Tra functions, such as *traC* a plasmid-encoded primase (Yakobson *et al.*, 1990). A high A/T content may also facilitate strand separation of negatively supercoiled plasmid DNA In addition to these features *oriT* regions usually have intrinsically bent regions, and contain promotors for transcribing the Tra gene products.

Relaxosomes

The cleavage reaction at *nic* in the *oriT* is carried out by a multicompoment complex, the relaxosome. Specifically, cleavage occurs by the neutrophillic attack of a specific phosphodiester bond (usually between R/Y or Y/R). Analysis of the cleaved product has shown the 3` hydroxyl to be free. However, the 5` end becomes covalently attached to the nicking enzyme, on the side chain hydroxyl of a tyrosine residue. The relaxosome complex was first identified for the naturally mobilisable plasmid ColEI (Clewell & Helinski, 1969). The ColEI relaxosome consists of three proteins 60, 16, and 11kDa, which probably correspond to three ColEI *mbe* genes - *mbeA*, *mbeB*, and *mbeC* (Lovett & Heliniski, 1975; Boyd *et al.*, 1989). Cleavage at *nic* occurs independently of mating pair formation as both nicked DNA and uncleaved DNA can be isolated from donors. If DNA is prepared in conditions where proteins are not denatured, the relaxase complex can be purified. This consists of two forms, nicked supercoiled DNA and uncleaved supercoiled DNA. Presumably the supercoiled nature of the nicked DNA is retained by specific protein/DNA interactions. The formation of relaxosomes for both the F and RP4 plasmids is well understood and will be reviewed here.

Genetic analysis of the F plasmid Tra genes have shown that nicking requires the *traY* gene and the *traI* region. These regions are at separate ends of the F plasmid Tra region, demonstrating that genes for nicking need not be linked (Everett & Willetts, 1980). The *traY* gene product is a plasmid specific *oriT* binding protein. It binds to two regions of DNA between 40-100bp upstream of *nic* in the trailing region of transfer (Frost *et al.*, 1994). There are also sites for integration host factor (IHF) binding in this area (Tsai *et al.*, 1990). *In vitro* studies have shown that TraI alone is required for cleavage in the presence of oriT-DNA and Mg^{2+} ions. However, this cleavage requires a high concentration of TraI, and only results in 50-70% of input DNA being cleaved

(Matson & Morton, 1991, Reygers *et al.*, 1991). An alternative *in vitro* system is more efficient and requires less TraI, this model requires negatively supercoiled *oriT* DNA, Mg^{2+} ions, and TraI, however, TraY and IHF must be added before TraI (Inamoto *at al.*, 1994). Prior to its discovery as relaxase TraI had been designated *E. coli* DNA Helicase I due to its ssDNA dependent ATPase and helicase activity. DNA helicase I acts in a 5` to 3` direction and catalyses the unwinding of 1000bp per second (Geider & Hoffmann-Berling, 1981). This is in excess of the speed of conjugal transfer estimated at 750bp per second for F. This has lead to the hypothesis that TraI may drive DNA transfer, perhaps by being fixed at the cell membrane and unwinding the DNA driving the bound strand into the recipient cell. However, there is as yet no proof that the helicase activity of TraI provides the motive force for conjugation. Other conjugative plasmids do not encode a helicase, presumably in these systems host proteins are involved in the unwinding of plasmid DNA.

The genes required for *oriT* specificity and relaxosome formation for RP4 are contained in a ~5.8kb core region of Tral (Lessl *et al.*, 1993) . A 2.2kb region of Tral surrounding the ~300bp *oriT*, was identified as the region required for heterologous mobilisation by R751 (IncPβ) and relaxosome formation. Isolation of plasmid DNA specifically cleaved at *oriT* requires the region containing *traJ* and the 5` three fifths of *traI*. The actual proteins of the relaxosome have been identified by its *in vitro* reconstruction using purified components (Pansegrau *et al.*, 1990a). Cleavage was found to require *traJ* and *traI* gene products in the presence of Mg^{2+} ions. The TraH protein stabilised the relaxosome complex such that it was discernible after agarose gel electrophoresis, and under the electron microscope. The TraK protein was also found to increase the yield of nicked DNA *in vitro*, as it does *in vivo* (Furste *et al.*, 1989; Waters *et al.*, 1991). The analysis of the RP4 relaxosome has lead to a model for its assembly in a cascade like mechanism. The TraJ protein binds to negatively supercoiled DNA via a specific interaction with a 10bp imperfect palindrome in the right arm of a inverted repeat upstream of the *nic* site (Zeigelin *et al.*, 1989). TraI, which is unable to bind DNA directly, recognises this TraJ-*oriT* complex and binds to it, interacting with 6bp between *nic* and the TraJ binding site. Interestingly the TraJ binding site and *nic* are on the same side of the DNA helix leading to the possibility of close interactions between TraI and TraJ. The TraH protein is believed to stabilise this complex through interactions with TraJ/TraI, as TraH cannot bind DNA directly. However, it should be noted that *traH*, which is internal to *traI* but occupies a different

reading frame, is not an essential Tra gene. The *traK* gene is essential, however, its role in transfer is not clear. The TraK protein has been shown to bind to the *oriT* region adjacent to *nic* in the leading region of transfer. Footprinting studies have shown that TraK protects a large region, up to 200bp, of DNA. It has been suggested that TraK-*oriT* complexes consist of a nucleosome structure with DNA wrapped around a core of TraK molecules (Zeigelin *et al.*, 1992). This complex may assist in the unwinding of DNA, or in producing higher order protein-DNA complexes for transport. The leading region of transfer in RP4 contains the *traK, traL,* and *traM* genes. The *traK* gene as discussed is essential for transfer, where as the *traL* and *traM* gene products are not required for transfer. However, although not essential for transfer, mutations in the *traM* gene result in a 300 fold decrease in transfer efficiency, leading to the hypothesis that this is an accessory protein (Lessl *et al.*, 1993).

Coupling of DNA Processing to Mating Pair Formation and DNA Transport

The fact that DNA processing occurs independently of mating pair formation leads to the question of how these processes are coupled for transfer to occur. Presumably there must be a signal that identifies when a stable mating pair is formed and links the relaxosome to the DNA transport pore. Candidates for proteins that link the unwound DNA to the transport pore include a family of Tra proteins exemplified by F-TraD and RP4-TraG (others include Ti-VirD4, R388-TrwB, & pGO1-TrsK). All these proteins contain two nucleotide binding sites of typeA and typeB, as determined by sequence analysis. Although the typeA site is not as well conserved in some proteins (for example RP4-TraG & Ti-VirD4), the typeB site is well conserved in each protein (Balzer *et al.*, 1994).

The F-TraD protein is found in the inner membrane, although the protein itself is largely hydrophilic, three hydrophobic domains are believed to be responsible for its subcellular location. TraD displays non specific DNA binding as determined by its binding to DNA-cellulose columns in a pH sensitive manner (Panicker & Minkley, 1992). The TraD protein was first implicated in coupling to the transport pore as it was found to be required after aggregation, nicking and initiation of unwinding of the plasmid DNA. It is also required for F specific phage entry, and it is the only protein, other than those

for mating pair formation, that is required for mobilisation of ColEI (Everett & Willetts, 1980; Kingsman & Willetts, 1978; Panicker & Minkley, 1985). Due to the DNA binding ability of TraD it has been suggested that it may mediate DNA transfer directly. Mutants in TraD are also multipiliated which suggests that TraD may have an additional role in either pilus assembly or retraction (Armstrong *et al.*, 1980).

The RP4 TraG protein belongs to the same family as F-TraD. It is the only other Tra product other than the mating pair formation system that is required for mobilisation of RSF1010. However, TraG is not required for donor specific phage propagation (Haase *et al.*, 1995). Mutations in *traG* have shown it to be essential. However, it is not involved in pilus formation or *oriT* cleavage. Recent studies have shown that introducing mutations into the typeA or typeB nucleotide binding sites of TraG affects transfer efficiency, demonstrating the importance of these domains for TraG function (Balzer *et al.*, 1994).

The F-TraM protein also has interesting properties, it is not required for nicking, but it is required for initiation of replacement strand synthesis. For this reason it has been proposed that TraM may act as the signal that a mating pair has been formed and that transfer should begin (Willetts & Wilkins, 1984). The majority of TraM is found in the cytoplasm, however, detectable amounts are seen in the inner membrane, consistent with the idea that TraM forms a signal. TraM has also been observed to bind to its cognate *oriT*. Binding of F-TraM occurs at three large motifs (inverted repeat, and two direct repeats), over 110bp indicating that more than one TraM molecule must be involved (Abo *et al.*, 1991). This phenomenum is reminiscent of RP4-TraK binding to its cognate *oriT*.

Termination of Transfer

In the general model of conjugation, transfer is terminated with the ligation of the ends of the transferred strand to produce a covalently closed circular molecule. This process is mediated by the relaxase molecule in what is believed to be a reversal of the nicking reaction. At present there are two models for the termination of transfer. In the first model transfer is terminated by a reversal of the nicking reaction with the free 3` hydroxyl residue at the trailing end of the transferred strand. However, it is widely thought that replacement strand synthesis in the donor cell occurs by extension of the 3`

hydroxyl residue of the transferred strand; if this is correct then there would not be a free 3` hydroxyl to terminate transfer. For this reason the currently favoured model for termination of transfer is the second cleavage model. This assumes that DNA of greater than unit length is transferred to the recipient and that the relaxase recognises the newly replicated *oriT*; a second cleavage reaction could then occur followed by the subsequent ligation of the DNA termini to produce the unit length plasmid. Support from this model comes from studies using recombinant plasmids carrying more than one *oriT* region separated by a spacer region. After transfer these plasmids only contain one *oriT* and lack the spacer indicating that termination occurred at the second *oriT* region. These studies have also identified the requirement of inverted repeats in the trailing region which are required for termination. Presumably these repeats form a secondary structure which interacts with relaxase (Erickson & Meyer, 1993). This also leads to the question of where these events, and therefore the relaxase are situated. Most models place relaxase at the bridge between donor and recipient, however, there is no proof for this and it is not inconceivable that the relaxase enters the recipient cell.

1.1.4 Conjugative DNA synthesis

The process of replacement strand synthesis in the donor cell is termed donor conjugative DNA synthesis (DCDS). Our understanding of this process has come from *in vivo* studies with temperature sensitive *dna* mutants. A *dnaE* (DNA polymerase III) temperature sensitive mutant donor cell is capable of acting as a donor but is deficient in DCDS. This has two main implications, firstly it demonstrates that DNA polymerase III is the main polymerase for DCDS, additionally it demonstrates that replacement strand synthesis and the transfer process are not coupled (Kingsman & Willetts, 1978).

The nature of the primer for replacement strand synthesis is an intriguing question. It has been speculated that the 3` hydroxyl of the transferred strand could act as a primer for the continuous extension of the plasmid DNA by a rolling circle mechanism. However, although the 3` terminus is free in *in vitro* studies it is not known whether it is accessible *in vivo*. Evidence exists that suggests that replacement strand synthesis actually requires RNA synthesis. This evidence came from studies with rifamipicin treated *dnaB* (DNA Helicase) mutants which suggested that untranslated RNA synthesis was required for DCDS (Willetts & Wilkins, 1984). Additional to these mechanisms some

conjugative plasmids encode their own primases. For example the RP4 primase (TraC) has sites in the *oriT* region for RP4 primase directed initiation of DNA synthesis.

DNA synthesis on the trailing strand in the recipient is termed RCDS (recipient conjugative DNA synthesis) and is independent of Tra gene expression in the recipient cell. If transfer occurs in a 5` to 3` direction DNA synthesis on the transferred strand would be akin to synthesis on the lagging strand in DNA replication and would require multiple primers. Studies with *dnaE* mutants have shown that DNA polymerase III is the main replicative enzyme in RCDS. Rifamipicin treated *dnaB* mutants have also demonstrated that primers are generated by RNA synthesis in a *dnaB* dependent manner (Willetts & Wilkins, 1984). The transferred strand of F contains a single stranded initiation sequence (*ssiE*) which comprises ~200bp in the leading region of *oriT*. The *ssiE* signal allows DnaG (primase) to produce a primer towards the *oriT* site in a primosome independent manner (Nomura *et al.*, 1991).

Some conjugative systems encode their own primases, that are transferred into the recipient cell to act in RCDS. There is no correlation between encoding a primase and any other plasmid phenotype such as promiscuity. However, the majority of primase encoding plasmids express a rigid pilus. Examples of these primases are the *traC* and *sog* gene products of RP4 and ColIB respectively. Both of the primase genes express two polypeptides, specified from separate in frame translation products. The larger *traC* product and both *sog* gene products have been found in the cytoplasmic fraction of the recipient after mating, demonstrating their transfer. However, the amino acid sequences of these proteins lack signals for export, indicating that their transfer occurs by a process distinct from the classic protein export pathway. Primase transfer requires an active DNA transfer system and occurs in the same direction as transfer. As these proteins bind to ssDNA it has been suggested that they may be transmitted bound to the transferred strand (Merryweather *et al.*, 1986; Rees & Wilkins, 1989). Such a priming mechanism may act to render RCDS independent of host enzymes and as such may contribute to the promiscuity of transfer in some systems.

1.1.5 Establishment in the Recipient Cell

The leading region of transfer is the segment of plasmid DNA that enters the recipient cell first. It has been hypothesised that this region may contain 'installation' genes that act to establish the transferred plasmid in the recipient. The leading region of F and the IncI1 plasmid ColIb contain two such genes, *psiB* - which inhibits the SOS response, and *ssb* - encoding a single stranded DNA binding protein. These genes are found in the same orientation in the leading region of nine Inc groups in the F complex, and I1-B-K of the I complex. Interestingly, these genes undergo a transient burst of expression, termed zygotic induction, when they are transferred to the recipient cell.

The plasmid encoded *ssb* gene is homologous to and can substitute for the *E. coli ssb* gene. Single stranded binding (SSB) proteins are important in DNA replication, protecting DNA from nucleolytic cleavage, and determining the specificity of priming (Kornberg & Baker, 1992). The significance of a plasmid-encoded *ssb* gene is hard to assess; especially as it is not required for transfer, and is not transferred to the recipient unlike plasmid encoded primases (Rees & Wilkins, 1990). The *E. coli* SSB protein is not abundant; hence the presence of a plasmid encoded SSB, transiently expressed, may help prevent the harmful effects of SSB starvation.

The *psiB* gene product inhibits the bacterial SOS response (Bagdasarian *et al.*, 1986). The SOS response is signalled by the presence of ssDNA which activates the coprotease function of RecA. Hence conjugative transfer would promote the SOS response, however, this is avoided by the *psiB* product which interacts directly with RecA (Bailone *et al.*, 1988). For inhibition of SOS the *psiB* gene needs to be overexpressed, this would occur during zygotic induction when the gene is first transferred to the recipient cell.

The leading region of ColIb also contains the *ardA* gene, this acts as an antirestriction gene to alleviate restriction of the immigrant DNA by host encoded type I restriction enzymes (Delver *et al.*, 1991; Read *et al.*, 1992). Recent work has demonstrated that *ardA* is also under the control of zygotic induction (B.M. Wilkins, personal communication). It would be interesting to examine the process of zygotic induction further, as the standard expression of genes occurs from dsDNA. However, if expression of *ssb, psiB*, and *ardA* occurred after DNA synthesis then the time when it would presumably be

important for them to be present, before replication, would have past. Therefore it has been suggested that these genes may be expressed from ssDNA.

1.1.6 Surface Exclusion

Whilst the majority of plasmid genes act to promote conjugation there are genes which act to limit conjugation. These genes operate to reduce transfer rates between cells carrying identical or related plasmids, this process is called surface exclusion. The F plasmid carries two such genes, *traT* and *traS*, that act additively in surface exclusion. TraT is an outer membrane lipoprotein which is believed to inhibit mating aggregate formation, potentially through interactions with the pilus tip (Anthony *et al.*, 1994). The TraS protein is located in the inner membrane and acts after mating aggregate formation by inhibiting DNA transfer. It has been suggested that TraS blocks a putative signal from recipient to donor cell indicating that a mating pair has been formed (Frost *et al.*, 1994). The RP4 plasmid contains just one gene for surface exclusion, *trbK*. TrbK is a small hydrophilic lipoprotein located in the inner membrane. It has been shown to act through the mating pair formation system of RP4, and potentially blocks transfer after cell contact (Haase *et al.*, 1995).

1.1.7 Homology between Conjugative Systems

Sequence analysis of the Tra genes of different conjugative systems has uncovered interesting homologies, and has indicated that transfer systems are not as diverse as originally thought. One interesting homology is that between RP4 and the Vir system of the Ti plasmid of *Agrobacterium tumefaciens*. The later is responsible for the transfer of a linear 25kb DNA fragment from *Agrobacterium* to plant cells by a process analogous to conjugation. Six genes in the Tra2 region of RP4 show striking homology to genes in the VirB operon of the Ti plasmid (Lessl *et al.*, 1992b). This homology is manifest in gross operon organisation, amino acid similarity, and the physical properties of the proteins encoded (Figure 1.5). Interestingly five of these genes also share homology with the Ptl operon of *Bordetella pertussis* (Lessl *et al.*, 1993) . The Ptl operon is involved in the export of pertussis toxin. This data would suggest that these proteins belong to a evolutionary conserved superfamily involved in macromolecule export.

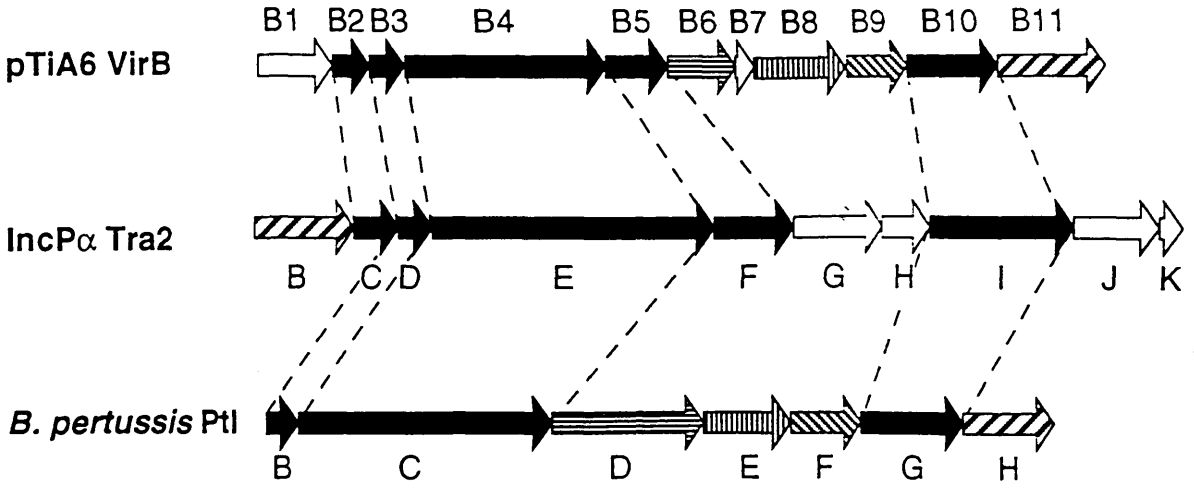


Figure 1.5. Alignment of RP4 Tra2 with Ti VirB and Ptl operons.

Genes encoding proteins that share homology are joined by dashed lines or are shown with identical hatching. Unfilled boxes denote genes with no homology to the other genes indicated. Figure derived from the data of Lessl *et al.*, 1992,1993; Weiss *et al.*, 1993; and Pansegrau *et al.*, 1994. Sequence data from the EMBL database -accession numbers J03216 (VirB), L10720 (Ptl) and M93696 (Tra2).

Homologies also exist between the DNA processing functions of conjugative plasmids. The loci for relaxase and associated accessory proteins are similarly organised between some plasmids, for example the organisation of RP4; *traI, J,K, & L,* is similar to the Ti plasmid; *VirD2,D1,C1, & C2,* and pTF-FC2 (a mobilisable plasmid from *thiobacillus ferrooxidans*); *mobA,B,C, & D* (Pansegrau *et al.*, 1994a) This similarity is illustrated further with the finding of three conserved domains in many relaxases [TraI≡VirD2≡MobA (pTF-FC2) ≡NikA (R64) ≡Rlx (pS194)] (Pansegrau *et al.*, 1993b; 1994a). The homology between these relaxases is also paralleled by the finding that their target *nic* sites also share homology (Figure 1.4). In fact TraI and VirD2 show functional homology in that they can cleave each others *nic* site in *in vitro* studies (Pansegrau *et al*, 1993a).

In many systems recognition of *oriT* by relaxase requires accessory proteins, for example TraJ (RP4), VirD1 (Ti), MobB (pTF-FC2), NikA (R64), and TrwA (R388). However, although these proteins share similar functional roles, very little similarity is seen between them. This lack of similarity may reflect a mechanism for specificity for their cognate *oriT* regions.

Other Tra genes that share homology include the RP4 TraG family which were discussed in an earlier section. This family includes: TraG (RP4), TraD (F), VirD4 (Ti), TrsK (pGO1), TrwB (R388), and MobB (ColDF13) (Pansegrau *et al.*, 1994a); the role of these proteins is believed to be in linking the DNA transport machinery with the mating pair formation system. This family of proteins is one of the few that contain proteins from the F plasmid. The F plasmid Tra proteins share very little homology with RP4 Tra proteins, this is exemplified with the finding that their *nic* regions differ. Exceptions to the lack of homology between F and RP4 include the TraD and TraG proteins discussed above, additionally homology exists between F TraA (propilin) and RP4 TrbC and Ti VirB2 (Shirasu & Kado, 1993).

The IncI1 conjugative plasmids are unusual in that they encode two forms of conjugative pilus, they also provide extra evidence for the interchange of Tra regions between plasmids. This evidence stems from the observation that a sub-region of IncI1 Tra shares homology and organisational similarity to the RP4 TraI region, including the relaxase (Furuya *et al.*, 1991). However, the leading region of transfer and replication functions of IncI1 plasmids are related to their counterparts on the F plasmid (Jones *et al.*, 1992).

1.2 Plasmid Transfer between Bacteria

1.2.1 Transfer between Bacterial Species

One of the major limitations to the promiscuous spread of bacterial plasmids would be their ability to replicate and to be expressed in the new host. Many of the conjugative plasmids that have been isolated from the *Enterobacteriaceae*, such as F and the IncI1 plasmid ColIb-P9, are only maintained in this group of organisms. Such plasmids are termed to have a 'narrow' host range. However, some of the more recently isolated antibiotic resistant plasmids, such as the IncP plasmids, are capable of autonomous existence in a wide range of organisms. For example natural isolates of IncP plasmids have been found in the enterobacteria and many other Gram-negative genera including *Pseudomonas aeruginosa, Alcaligenes eutrophus,* and *Bordetella bronchiseptica.* Furthermore, IncP plasmids are stable in all Gram-negative bacteria tested in the laboratory (Smith & Thomas, 1989). The small mobilisable IncQ plasmids, such as RSF1010, have an extremely broad host range and are capable of autonomous maintenance in both Gram-negative and Gram-positive bacteria . This broad host range is a result of IncQ plasmids encoding functions for their own replication (Scherzinger *et al.*, 1984). Gram-positive replicons also display a wide host range, in fact the rolling circle plasmids of *Staphylococcal aureus* can be maintained in the yeast *Saccharomyces cerevisiae* (Goursot *et al.*, 1982).

The widespread transfer of certain conjugative plasmids is exemplified by the classic example of the spread of the same antibiotic resistance determinants between bacteria, since the general introduction of antibiotics into medicine. This observation is supported by an analysis of the Murray collection of strains, collected in the pre-antibiotic years (Datta & Hughes, 1983). Of the 84 conjugative plasmids identified from these strains none carried antibiotic resistance. The plasmids were mostly from the I1 and F complexes and IncX, interestingly current isolates from these groups are associated with antibiotic resistance. This would suggest that within the last 50 years these plasmids have received antibiotic resistance markers, possibly from transposons, and that these new resistance plasmids have become disseminated among the bacterial population. Interestingly, the Murray collection did not contain plasmids from the IncC, H, M, P, or W groups which are now widely distributed and often associated with antibiotic resistance. The majority of the strains collected were

Salmonella, Shigella, Escherichia, and *Klebsiella.* It is perhaps likely then that the underrepresented plasmids were not associated with these species and their distribution has increased only recently due to the selectional pressure applied by the use of antibiotics. In fact recent sequence evidence would suggest that the IncP plasmids originated in a host such as *Pseudomonas* (Wilkins *et al.*, 1996).

It has frequently been found that conjugative plasmids have a transfer range wider than their associated replication functions. This has typically been demonstrated using hybrid 'shuttle' vectors, these vectors contain two replicons for maintenance in different species and a selectable marker known to be expressed in both. Using this approach it was possible to demonstrate that RP4 could mediate transfer from Gram-negative to a variety of Gram-positive bacteria, including *E. faecalis, S. lactis, S. agalactiae, B. thuringiensis kurstaki, L. monocytogenes, S. aureus,* and *B. sphaericus.* The transfer efficiencies observed were typically low, between $2x10^{-8}$ to $5x10^{-7}$ (transconjugants per donor) and after long periods of co-incubation. However, no attempt to optimise transfer was made therefore these rates cannot be viewed as maximal (Trieu-Cuot *et al.*, 1987).

It has also been shown that RP4 can mobilise the naturally occurring IncQ plasmid RSF1010 to the Gram-positive actinomycetes *Streptomyces lividans,* and *Mycobacterium smegmatis.* This is the first example a naturally occurring plasmid being transferred from Gram-negative to Gram-positive bacteria (Gormley & Davies, 1991). RSF1010 can also be mobilised by RP4 to cyanobacterium (Kreps *et al.*, 1990). RSF1010 was stable in these organisms over several generations. The transfer efficiency was also relatively efficient at approximately 10^{-2} for *M. smegmatis* and the cyanobacterium, however, transfer to *S. lividans* was relatively low at 10^{-6}; these differences may represent differences in the recipient cell, perhaps the presence of a restriction system. The broad mobilisation range of RSF1010 was confirmed in 1987 with the observation that its *mob* and *oriT* functions could, in the presence of the Ti plasmid of *Agrobacterium,* mediate its transfer to plant cells (Buchanan-Wollaston *et al.*, 1987).

The F plasmid also has a transfer range wider than its associated replication functions. Guiney (1982) constructed a hybrid plasmid containing the F origin of transfer and a mini RK2 (essentially RP4) replicon capable of maintenance

in both *E. coli* and *P. aeruginosa*. This plasmid could be effectively mobilised by both F and RK2 between *E. coli* cells. The plasmid could also be mobilised to *P. aeruginosa* by F and RK2. Therefore, the F plasmid, although unable to replicate in *Pseudomonas*, is capable of mediating plasmid transfer to this organism. Interestingly, the level of transfer by the F plasmid was some 10^4 fold lower than that by RK2, demonstrating that there are inherent differences in the ability of conjugative plasmids to transfer promiscuously.

Promiscuous transfer can also be seen from a study of the transfer efficiency of plasmids from *E. coli* to *P. aeruginosa* (Tardif & Grant, 1983). This work tested the transfer of a wide range of enterobacterial plasmids from different Inc groups to *P. aeruginosa*. Of these only the IncP group was able to transfer efficiently, confirming its title as a promiscuous plasmid. Low transfer rates were detected with members of the Inc groups A-C, C,FI,M,N,T,W (10^{-7} to 10^{-8}); whilst the IncIα group demonstrated intermediate transfer levels (~10^{-4}). No transfer was detectable from members of the FII,H2,J, and X groups. However, a mutant of *P. aeruginosa* was isolated (Era) that displayed an enhanced ability as a recipient. This mutant was specific for members of the C,FI,FII,J,N,W, and X incompatibility groups, this demonstrated that all the plasmids tested, with the exception of the IncH2 plasmid, were capable of mediating transfer to *P. aeruginosa*. The nature of the DNA in the transconjugants was also analysed and revealed interesting patterns. Some plasmids from the IncN and IncW groups appear to have undergone some form of rearrangement of their DNA, although the precise nature is unknown as some reacquire their 'normal' size when re-established in *E. coli*. The majority of the transconjugants, however, have no discernible plasmid DNA, these appear to have the plasmid DNA inserted into the chromosome (from Inc groups A-C,J,FI,FII,Iα,M,N,T, & X). The transconjugants from mating with IncP and C groups have plasmid DNA identical to that present in *E. coli* transconjugants characteristic of the ability of these plasmid to replicate in *P. aeruginosa*.

Transfer of plasmid DNA is also possible from Gram-positive to Gram-negative organisms, in fact as genes in Gram-positive bacteria are readily expressed in Gram-negative bacteria, but not vice-versa, transfer in this direction may be more productive in nature. Indeed resistance genes such as *aphA-3*, *ermAM*, and *TetM*, once thought to be specific for Gram-positive organisms have been found in Gram-negative human pathogens like *Campylobacter coli*, and

members of the *Enterobacteriaceae* (reviewed in Courvalin, 1994). The classic laboratory example of transfer between Gram-positive and Gram-negative bacteria is between *E. faecalis* and *E. coli* (Trieu-Cuot *et al.*, 1988). The efficiency of this transfer was low ($5x10^{-9}$ transconjugants per donor). However, similar transfer efficiencies have also been obtained under physiological conditions in the intestinal tracts of mice - even in the absence of selection (Doucet-Populaire *et al.*, 1992). The natural transfer of Tn916, a conjugative transposon, also occurs from *E. faecalis* to a variety of Gram-negative organisms, where it can subsequently be transferred back to Gram-positive bacteria (Bertram *et al.*, 1991).

1.2.2 'Promiscuity' Determinants

The concept of plasmids being promiscuous in their ability to transfer to a diverse range of organisms leads to the question of specialisation. Do promiscuous plasmids carry specific genes required for their promiscuity, so called 'promiscuity determinants'. Only five of the genes in the Tra1 of IncPα plasmids are absolutely required for transfer between *E. coli*, this has lead to the suggestion that the remaining Tra1 genes may be required for transfer between different organisms (Lessl *et al.*, 1993). Indeed, two RP4 genes have been implicated in promiscuity through the phenotype of mutants created by transposon mutagenesis.

The *traC* gene encodes the RP4 primase (see section 1.1.4), it was initially shown by Tn7 insertion mutagenesis to be required for RP4 transfer and the efficient mobilisation of R300B (an IncQ plasmid similar to RSF1010) from *E. coli* to *S. typhimurium* and *P. mirabilis* (Lanka & Barth, 1981). Subsequently it has been shown to be required for transfer from *P. aeruginosa* to *P. stutzeri*, however, it was not required for transfer within *P. aeruginosa* and *P. stutzeri*, or even for transfer from *P. stutzeri* to *P. aeruginosa* (Krishnapillai, 1988). The primase has been seen to transfer to the recipient (Merryweather *et al.*, 1986), where it directs host independent priming. Presumably, when the primase is absent proteins in the recipient cell can substitute to allow productive transfer. However, although RP4 TraC can be classed as a promiscuity determinant, there is no correlation with plasmids that encode their own primase and plasmids which demonstrate a broad transfer range. For example the narrow host range IncI1 plasmid ColIb-P9 encodes its

own primase, however, members of the broad host range IncN and W groups do not (Wilkins & Lanka, 1993).

The RP4 gene *upf54.4* has also been implicated as a promiscuity determinant by analysis of Tn7 insertion mutants. Two mutants of *upf54.4* have been identified that exhibit different phenotypes (Krishnapillai, 1988). The first mutant was defective for plasmid transfer from *P. aeruginosa* to *E. coli* C, *S. typhimurium*, and *P. maltophilia*. This mutant was rescued by supplying wild type *upf54.4* in the recipient cell, indicating the action of Upf54.4p in the recipient. The second mutant was specifically affected in transfer from *P. aeruginosa* to *P. stutzeri*. However, in contrast to the first mutant this mutant was rescued by supplying *upf54.4* in the donor cell. The finding that these mutants can be rescued by *upf54.4* either in the recipient or donor cell suggests that Upf54.4 may have more than one function. The biochemical function(s) of Upf54.4, however, remain to be elucidated.

The TraH protein of RP4 also has a minimal effect on RP4 transfer between diverse organisms. Site directed mutagenesis was carried out to convert the ATG start codon to ACG to knockout *traH*, this change however did not affect the amino acid composition of TraI whose coding region overlaps that of *traH*. The TraH knockout exhibited a two to three fold decrease in transfer from *E. coli* to *P. stutzeri, P. putida,* and *P. aeruginosa*. Transfer from *K. pneumoniae* or *P. stutzeri* to an *E. coli* recipient was reduced seven and three fold respectively (Cole *et al.*, 1993). These effects are only minor and may be attributed to the action of TraH in stabilising the relaxosome. However, it is possible that under physiological conditions such small enhancements in transfer efficiency could have a more pronounced effect.

Recently the RP4 factors required for transfer between *E. coli* and *S. lividans* have been investigated (Geibelhaus *et al.*, 1996). These workers used a bipartite system for analysing RP4 where the Tra1 and Tra2 functions are carried on different compatible plasmids (Lessl *et al.*, 1992a); this system was used successfully to determine the minimal components required for transfer between *E. coli*. It was demonstrated that transfer to *S. lividans* requires the same set of genes required for transfer between *E. coli* cells. With the exception of *trbF* which is absolutely required for transfer of RP4 in *E. coli* but only reduced transfer to *S. lividans*. Thus for transfer between *E. coli* and *S. lividans* there are no specific promiscuity determinants required.

Interestingly *E. coli* and *S. lividans* are seen to form tight junctions between cells in the absence of any known conjugation system. However, these junctions were not able to substitute for the mating pair formation system. Hence, as the genes for pilus expression were still required for transfer to *S. lividans* this would suggests a direct role for the pilus in DNA export.

1.3 Ti Plasmid Mediated T-DNA Transfer to Plant Cells.

One of the most striking examples of horizontal transfer is that from *Agrobacterium tumefaciens* to plant cells. *Agrobacterium* is a Gram negative bacterium that causes tumours at plant wound sites. Tumourogenesis is caused by the transfer of a specific segment of DNA, the T-DNA (~25kb), from the large (~200kb) Ti plasmid of *Agrobacterium* (reviewed in Kado, 1993). The T-DNA carries *onc* genes which encode plant growth hormones (Auxin and Cytokinin) that promote plant cell division leading to tumour formation. The region also encodes proteins required for opine synthesis. Opines are acid-basic amino acid conjugates that *Agrobacterium* can utilise as a novel carbon and nitrogen source. The T-DNA region can largely be replaced by other DNA, hence this system has been employed in plant genetic engineering to deliver foreign DNA into plant cells.

The process of transfer, which has a striking resemblance to bacterial conjugation, is reliant on the plasmid encoded *vir* gene products contained on a 35kb segment of the plasmid. Activation of the transfer system is mediated by VirA which senses compounds released at plant wound sites (such as acetosyringone). VirA activates the rest of the *vir* genes by a classic two component system via the VirG transcriptional activator (see Kado, 1991).

The T-DNA is prepared for transfer by nicking at sites flanking the T-DNA termed the left and right border (Albright *et al.*, 1987; Wnag *et al.*, 1987). These 25bp direct repeats show homology to the *nic* site in the IncP plasmids *oriT*. Nicking of the border sequences requires two proteins, VirD1 and VirD2. VirD2 is the actual relaxase and has homology to the RP4 TraI relaxase, VirD2 nicking requires VirD1 which fulfils a role similar to RP4 TraJ (Lessl & Lanka, 1984). The nicking reaction occurs predominantly at the right border, probably due to the enhancory effect of VirC binding to a sequence adjacent to RB (Toro *et al.*, 1988). As in bacterial conjugation the VirD2 protein remains covalently attached to the 5' end of the nicked strand. The T-DNA is subsequently

transferred to the plant cell in a linear single stranded form, with VirD2 covelently attached (Tinland *et al.*, 1994). The VirE2 protein is also transferred to the plant cell. VirE2 is a non specific single stranded DNA binding protein, and is thought to coat the T-strand (Citovsky *et al.*, 1989) . However, VirE2 transfer occurs independently of T-strand transfer in a VirE1 dependent manner (Sundberg *et al.*, 1996). Both VirD2 and VirE2 contain active nuclear localisation signals which act to pilot the bound T-strand to the nucleus (Tinland *et al.*, 1992; Citovsky *et al.*, 1992).

The T-DNA integrates into the genome of the plant by illegitimate recombination The mechanism of integration is unknown, but VirD2 may be involved (Gheyson *et al.*, 1991). Integration usually results in either small deletions or truncation of the border sequences, however the right border is not effected, presumably due to VirD2 being bound.

The T-DNA is transferred to plant cells through an undefined DNA transport pore, consisting of the 11 products of the VirB operon. The VirB genes show homology to the Tra2 genes of RP4 as well as the Ptl of *B. pertussis* (see section 1.1.7; reviewed in Lessl & Lanka, 1994). Most of the VirB proteins are located in the membrane, and VirB mutants are avirulent but competent for T-DNA processing and are required for mobilisation of RSF1010; supporting their presumed role as the DNA transport pore.

1.4 Plasmid Transfer to Yeast.

Heineman and Sprague in 1989 demonstrated that conjugation could traverse the kingdom barrier with the observation that DNA could be mobilised from bacteria to the lower eukaryote *S. cerevisiae*. This was accomplished by mobilising an *E. coli* / Yeast shuttle vector (YEp13) capable of maintenance and selection in both organisms. This vector was mobilised in *trans* by a derivative of R751, an IncPβ plasmid. This derivative as well as carrying the cognate *oriT* and *tra* functions of R751 also carried the *mob* region of the naturally mobilisable plasmid ColEI. Mobilisation of YEp13 was possible as this shuttle vector is based on a ColEI replicon and carries the ColEI *bom* site (basis of mobility), equivalent to an *oriT*. Transfer to yeast was possible after co-cultivation of donor bacteria and recipient yeast on yeast minimal media selective for transconjugants. Transfer efficiencies were low and varied considerably. However, as mating conditions were not standardised and no

attempt was made to optimise the protocol, a range of efficiencies would be expected. Transfer to yeast could also be mediated by the F plasmid. The actual transfer process shared the same physical properties as bacterial conjugation. Transfer was not due to transformation as demonstrated by treating cells both by adding exogenous DNA and adding DNaseI to mating cells. The requirement for viable donor cells and cellular contact was also demonstrated. Conjugative functions such as the *mob* genes were also required for transfer. Curiously though no strict requirement for the *bom* site could be demonstrated for mobilisation. These observations suggest that transfer to yeast occurs via a process analogous to conjugation, however, further work is required to confirm this.

Since the initial observation that transfer was possible to *S. cerevisiae* other workers have shown that transfer is also possible to *S. pombe* (Sikorski *et al.*, 1990), *K. lactis, P. angusta, P. tannophilus* (Hayman & Bolen, 1993), and *S. kluyveri* (Inomata *et al.*, 1994). Some of these workers reported structural rearrangements in ~30% of the transferred plasmids. However, the donor strains in all these cases were recombination competent, therefore, rearrangements could have occurred in the donor cell between homologous regions on the conjugative plasmid and shuttle vector. This argument is supported by Nishikawa *et al* (1990) who reported no structural rearrangements of the transferred plasmid, these workers used a recombination defective (*recA*) bacterial donor strain. These workers also demonstrated that nalidixic acid could inhibit *E. coli*-yeast transfer and that maximal transfer efficiencies were achieved after twelve hour co-incubation of donor and recipient, with a minimum of eight hours required for transfer to reach detectable levels.

The fact that transfer can occur between *E. coli* and yeast is intriguing but the implications of transfer are difficult to assess. Any such transfer occurring in nature would be typically unproductive due to the lack of sequences for replication. Hence productive transfer would only occur if the transferred DNA is integrated into the host chromosome. Integration of transferred DNA has been demonstrated in the laboratory (Nishikawa *et al.*, 1992). Integration occurred after the transfer of a plasmid lacking a ARS (autonomous replication sequence) element. Integration occurred at an approximately 1000 fold reduced rate than transfer by an *ARS* containing plasmid. The majority of integration occurred by homologous recombination, resulting in a gene replacement or double cross over event of the selectable marker. It would be interesting to

determine what would happen if the immigrant DNA lacked homology, whether it could integrate by illegitimate recombination or not.

Recently it has been shown that *Agrobacterium* can mobilise its T-DNA to yeast cells (Bundock *et al.*, 1995; Peirs *et al.*, 1996). These workers used a binary system where the *vir* gene functions were contained on one plasmid, and another plasmid carried the left and right border DNA. Between the two border sequences a yeast replication origin and selectable marker (*URA3*) were cloned. Transfer to form URA^+ yeast transconjugants required activation of the *vir* genes by the presence of acetosyringone, present naturally at plant wound sites. Of the transconjugants formed a minority resulted from the precise joining of the left and right border sequences, possibly carried out by the VirD2 protein. A third of the transconjugants were slightly smaller than expected, these were most likely the result of intra T-DNA homologous recombination and hence resulted in the border sequences being lost. Interestingly the remainder of the transconjugants all contained the entire plasmid carrying the left and right border, these probably resulted by the failure to nick at left border and transfer therefore occurring only from RB alone.

The ability of the T-DNA to integrate was also analysed, interestingly this integration occurred at the same efficiency as transfer of a replicating vector. Of the integrants tested the majority recombined via homologous recombination in contrast to transfer to plants where integration occurs by illegitimate recombination. For a small number of integrants there was no proof that they had recombined with the yeast genome leaving the possibility that integration may have occurred with mitochondrial DNA. It has recently been shown that T-DNA transfer to yeast cells can result in illegitimate recombination (Bundock *et al.*, 1996). This was demonstrated using a URA3 containing T-DNA transferred to a URA3Δ recipient. URA^+ transconjugants isolated resulted from the illegitimate recombination of the transferred DNA with different chromosomes. Analysis of the integration point revealed microhomology in some cases but not all. Integration also resulted in deletions ranging from 3-65bp of yeast genomic DNA, similar deletions have been seen at plant integration points. Interestingly one of the integrants analysed appeared to have been integrated into the mitochondrial genome.

Requirement of specific *vir* genes for transfer to yeast has also been demonstrated (Bundock *et al.*, 1995). The VirD4 and VirB proteins were

required for transfer to yeast, as was the relaxase and accessory proteins, VirD2 and VirD1. Intriguingly a mutant in the nuclear localisation sequence of VirD2, still capable of nicking, was found to be incapable of transferring to yeast, implicating this signal as being important for transfer to yeast. Mutants in the single stranded binding protein VirE2 demonstrated a reduced transfer efficiency. This could be due to several reasons, the nucleolytic breakdown of the T-strand in the yeast cell, or the lack of the VirE2 nuclear localisation signal.

1.5 Yeast Cell Surface Interactions

DNA transfer from *E. coli* to yeast by conjugation would require cellular interactions to form between the bacterial cell and the yeast cell wall. The cell wall of yeast constitutes 30% of the dry weight of the cell and is responsible for cellular integrity. Its roles include osmotic protection, maintenance of morphology, permeability, and enzyme support. The cell wall also acts in specialised functions such as sexual aggregation, budding, and flocculation. The wall is composed of an outer layer of manoproteins, covering a inner glucan layer responsible for the mechanical strength of the cell. However, the distinction between inner and outer layers is not discreet and mannoproteins are interwoven into the glucan layer. The inner layer itself can be separated into two layers. The inner glucan layer consists of fibres of β(1,3)-glucan and is responsible for the mechanical strength of the cell. The outer layer is more amorphous and is enriched for β(1,6)-glucan. Chitin is also found in the cell wall and is found linked to β(1,3)-glucan and β(1,6)-glucan (reviewed in Klis, 1994; Stratford, 1994).

Discussion of the composition of the yeast cell wall may lead to the suggestion that it is a static structure, however this is not the case. The cell wall is a dynamic structure constantly changing in response to the extracellular environment. Moreover, the wall changes in response to the cell cycle and cell wall synthesis is tightly regulated. Glucan synthesis is regulated during the cell cycle and is found to drop during G_1. Of the glucan produced during the first half of the cell cycle most is alkali soluble switching to alkali insoluble glucan in the later half of the cell cycle; this switch is probably caused by cross linking to chitin (Biely, 1978; Hayashibe *et al.*, 1977). Chitin synthesis is also highly regulated, three chitin synthases have been identified and these act at different points in the cell cycle, for instance the chitin ring at the base of the bud is laid

down in late G_1, the primary septum is formed in late M phase, and chitin synthesis in the lateral walls is maximum after primary septum formation (reviewed in Cid *et al.*, 1995).

Yeast mating is a specialised case of cell wall remodelling. Haploid yeast cells secrete pheromones (a and α), and express a receptor for these on their cell surface. Binding of the relevant pheromone to its receptor results in cell cycle arrest in G1 via a mechanism involving G protein activation of a MAP kinase pathway. At higher pheromone levels polarised growth of the yeast cell occurs towards the partner cell by sensing of a pheromone gradient (Madden & Snyder, 1992); the elongated cell produced is termed a schmoo. Many proteins are found to accumulate at the tip of the schmoo, including the pheromone receptor (Jackson *et al.*, 1991), sexual agglutinins (Watzele *et al.*, 1988), and *FUS* gene products required for fusion of mating cells (Brizzio *et al.*, 1996). Chitin is also deposited at the tip of the schmoo (Lipke *et al.*, 1976). Mating contacts are initiated by the sexual agglutinins. These highly glycosolated cell wall proteins produced by haploid cells interact to allow cells of the opposite mating type to adhere, forming the first cellular contact between mating cells (Terrance *et al.*, 1987). However, these contacts are not essential for mating but they may act in liquid to prolong cellular contact to enable mating to occur (Lipke *et al.*, 1989). As mentioned earlier, in response to pheromone, the agglutinins and pheromone receptor accumulate in the tip of the schmoo. Hence interaction between agglutinins localised at the shmoo tip may result in a localised high concentration of pheromone which is required for cellular fusion. During cellular fusion the cell wall remodels to produce a seal between cells that is resistant to osmotic shock, the intervening wall is then broken down to allow the fusion of plasma membranes (Brizzio *et al.*, 1996). Finally the two haploid nuclei fuse and immediately go through a division cycle to form two diploid nuclei, one of which enters a newly formed bud.

Yeast flocculation is promoted by cell wall interactions. Flocculation is defined as the process by which yeast cell form clumps (flocs) which then sediment from the media. Flocculation is induced by several different mechanisms including nutrient/oxygen depletion and growth state (Straver *et al.*, 1993). It occurs through a lectin interaction, by binding of carbohydrate on neighbouring cell walls by specific lectins (Miki *et al.*, 1982). In *S. cerevisiae* the lectin involved is on the outer chain branches of the N-glycosylated mannans. The major genes involved in flocculation are *FLO1* (Teunissen *et al.*,

1993) encoding a cell wall protein, and homologous genes *FLO5, FLO9,* and *FLO10.* The expression of these genes in regulated by *FLO8, TUP1,* and *SSN6* (Kobayashi *et al.*, 1996; Teunissen *et al.*, 1995). However, the actions of Flo1p and Flo5p to promote flocculation are unclear, they are unlikely to be the lectins themselves and may act in a later step of flocculation possibly stabilising flocs (Stratford & Carter, 1993).

The surface interactions that occur between bacteria and yeast during trans-kingdom conjugation are unknown. Potentially cell wall proteins, such as those involved in mating, flocculation, or cell wall synthesis, may be involved. Alternatively, the study of *E. coli*- yeast transfer may identify novel cell wall proteins.

1.4 Evidence for Horizontal Transfer During Evolution.

The role of horizontal transfer in molecular evolution is a hotly debated subject. Few doubt that it has been instrumental in the evolution of prokaryotes, however, suggestions that transfer can occur between prokaryotes and eukaryotes are controversial. The field of bioinformatics is quickly growing due to the large amounts of sequence data currently being produced. This data can be used to construct phylogenetic trees that map the history of life and largely confirm our classical views on the relationship between different organisms. However, a growing number of unusual trees are being produced where sequences appear in radically unexpected positions. Horizontal transfer events have been proposed by several workers to explain some of these odd placements (reviewed in Smith *et al.*, 1992; Syvanen, 1994). Instances of horizontal transfer in evolution are difficult to prove. Ideally, the unusual tree produced should be 'normal', i.e. comparable to the species tree, except for the one unusual placement. Several tree building methods should be used and all produce a similar aberrant tree. Other sequences from the same species should also be analysed and produce a standard tree. Finally, it is encouraging when the organisms between which transfer is proposed to have occurred would have had the opportunity to come into contact.

One of the major pitfalls in analysing potential horizontal transfer is the misclassification of paralogous genes as orthologous. Paralogous genes diverged after a gene duplication event in a common ancestor, whereas genes are termed orthologous if they diverged after a speciation event. The problem

arises when the common ancestor branched to produce the two or more extant species. If both gene pairs are still found in the extant species their nature can be easily deduced. However, if one of the gene pair has been lost during evolution, and different paralogous genes exist in the two species then these may be misclassified as orthologous pairs. When a gene tree is constructed these two genes would then cluster together in a pattern that does not conform to the species tree.

Convergent evolution must also be considered when analysing the possibility of horizontal transfer. However, there is a distinct lack of evidence at the sequence level for convergent evolution. Even in the most widely cited example, langur lysozyme (Stewart *et al.*, 1987), the convergent changes in sequence where not sufficient to produce an aberrant tree and hide the true affinity of the langur to other primates. The next sections will consider the cases of a few of the best documented examples of horizontal transfer. It remains to be determined if these cases are exceptional or the first indications of a wider web of gene transfer.

Glyceraldehyde-3-Phosphate Dehydrogenase. *E. coli* contains two genes for glyceraldehyde-3-phosphate dehydrogenase (Gapdh). One of these genes is more similar to eukaryotic Gapdhs than any other prokaryotic Gapdhs. The second represents an ordinary phylogeny, and is prokaryotic in nature. Two methods of tree building clustered the former Gapdh with the eukaryotic sequences. Additionally, meeting the criteria set out above, other sequences from the same organisms produce a normal phylogeny (Doolittle *et al.*, 1990). With the finding of eukaryotic like Gapdhs in other eubacteria the possibility of transfer from a eukaryotic donor to an ancestor of the eubacteria is raised (Nelson *et al.*, 1991; Lawrence *et al.*, 1991). However, the finding of a Gapdh in *Anabaena*, a cyanobacterium, orthologous to a plant cystolic Gapdh brings doubt on the direction of transfer (Martin *et al.*, 1993).

Glutamine Synthetase. Two forms of glutamine synthetase (GSI & GSII) are found in nature. GSI is a dodecamer of identical subunits, found only in eubacteria and archaebacteria. The second form, GSII, consists of an octomer of subunits and was believed only to occur in eukaryotes. However, the GSII form has been found in *Agrobacterium* and *Rhizobium*. The amino acid sequence of the GSII of *Bradyrhizobium japonicum* has a high similarity to plant GSII sequences, and exists as a octomer. This has lead to the hypothesis

that the GSII gene was transferred from plants to *B. japonicium*. The symbiotic nature of these organisms made the possibility of transfer even more attractive (Carlson & Chelm, 1986). A study of the rate of change of GSI and GSII sequences upholds the theory of horizontal transfer. However, the proposed date of transfer was before the divergence of plants, animals and yeast thus abolishing the idea of transfer during the symbiotic relationship between *B. japonicium* and plants (Pesole *et al.*, 1991). Given this confused state, Smith *et al.* (1992) re-examined the proposed transfer using a larger data set. No transfer events were required to explain the phylogeny produced. However, as the sequences are very similar the authors still favour horizontal transfer.

Glucose Phosphate Isomerase. The case for horizontal transfer of the glucose phosphate isomerase (*gpi*) gene stems from the finding that the *E. coli gpi* gene is 88% similar to the same gene in *Clarkia ungulata*. GPI acts in glycogenisis in animals and carbohydrate synthesis in plants. The similarity between *E. coli* GPI and *C. ungulata* was considered too high for normal descent, particularly as *gpi* is a dispensable gene. In fact some *E. coli* strains do not posses a *gpi* gene. Glucose is metabolised in these strains by the pentose phosphate shunt. It has therefore been proposed that *E. coli* lost its own copy of *gpi*, but acquired a new copy from plants (Smith & Doolittle, 1992). Despite the large sequence similarity some authors have argued that the results can be explained by the existence of multiple isoforms of GPI (Hattori *et al.*, 1995). However, a recent study on GPI, using all the GPI sequences available and several tree building methods, was entirely consistent with the horizontal transfer model (Katz, 1996).

Fe-Superoxide Dismutase. The sequences for Fe-Superoxide dismuatse (Fe-SOD) are available for a wide range of organisms, and typically produce standard phylogenetic trees. The exception being the Fe-SOD of *Entamoeba histolytica*, a eukaryotic protist, which shows a 60% similarity to prokaryotic Fe-SOD's (e.g. *E. coli* Fe-SOD). Other sequences from *E. hitolytica* produce standard phylogenies demonstrating that the similarity of the Fe-SOD is an exception (Tannich *et al.*, 1991). In fact besides the chloroplast Fe-SOD's *E. histolytica* is only the second eukaryote known to have a Fe-SOD. The second example being *Tetrahymena* which can fashion a Fe-SOD from a Mn-SOD. However, in the case of *E. histolytica* it would appear that the Fe-SOD was derived from bacteria. As *E. histolytica* engulf bacteria an obvious chance for transfer to occur is apparent.

Aldolase. Two distinct forms of fructose-bisphosphate aldolase are found in nature, type I and II, both are discreet enzymes with a unique mechanism. The type I aldolases generate a standard phylogenetic tree, and to date no examples have been found in fungi, eubacteria, or archaebacteria. At the time of analysis only four examples of type II enzymes were known, three from eubacteria and one from yeast. Interestingly the *E. coli* and yeast sequences group together away from the other prokaryotes. More sequences are required for a thorough analysis, but from this initial data it would appear likely that transfer of the type II aldolase occurred from *E. coli* to yeast (Schelberger *et al.*, 1989).

Isopenicillin-N-Synthetase. Most prokaryotic species can produce β-lactam antibiotics. However, this ability is only held by a few fillamentous fungi in eukaryotes. The pathway for their production in prokaryotes and eukaryotes is identical. Additionally, the genes involved in their synthesis are found clustered together, and show high seqeunce homology. It has therefore been proposed that the ability of filamentous fungi to produce β-lactams stems from the ancient horizontal transfer of the genes involved. An alternative hypothesis is that these IPNS (isopenicillin-N- synthetase) genes are subject to slow evolution. The horizontal transfer model is the most supported as it has been shown that evolution rates of IPNS genes in fungi is not appreciably slower than other genes. Additionally there is no evidence for IPNS genes having a selective advantage in fungi. Therefore constraints on sequence divergence would not be apparent (reviewed in Aharonowitz *et al.*, 1992). The original analysis was carried out on a small unrooted data set. However, analysis of a larger rooted data set produced a conventional tree which argued for a gene duplication event before the prokaryotic-eukaryotic divergence (Smith *et al.*, 1992). However, Buades and Moya (1996) inspected this tree and found that the branches were considerably shortened. From the tree the estimated branching date for the prokaryotic and eukaryotic IPNS genes is 850 million years (MY) ago, much earlier than the proposed 2400MY branching date for prokaryotes and eukaryotes. Hence the current model for the evolution of IPNS genes involved both a gene duplication event before the prokaryote eukaryote split, with subsequent horizontal transfer to eukaryotes.

The examples described above are the most documented cases of potential horizontal transfer during evolution. In some of the cases discussed the direction of transfer can be deduced, however, the actual mechanism of transfer

remains unknown. One example where the mechanism of transfer appears apparent is the finding of a tobacco homologue of the Ri plasmid *rolc* gene. The tobacco homologue (*trolc*) is between 69-87% similar to various Ri plasmid *rolc* genes. It constitutes a small gene family in tobacco species derived from the *tomentosiformis* ancestor of modern tobacco. Hence it would appear that the *trolc* gene resulted from the *A. rhizogenes* Ri plasmids ancient transfer to the tobacco progenitor. Interestingly *trolc* is expressed in plants and is regulated by the growth hormones auxin and cytokinin, hence, it would appear that a gene of bacterial origin can be expressed and have a function in a modern plant (Meyer *et al.*, 1995).

1.7 Aims

Conjugative transfer between *E. coli* and yeast raises the possibility that horizontal transfer of DNA may be possible between many diverse organisms. *E. coli* and yeast are specifically useful for examining promiscuous conjugative transfer (in this case trans-kingdom) as transfer is unlikely to be the result of a specialisied system. Analysis of promiscuous transfer may provide insights into the mechanism of conjugation particularly one of the most mysterious aspects of conjugation, that of mating pair formation. It would also be interesting to determine what plasmid-encoded factors are required for transfer, especially if additional factors are required for promiscuity. An appealing theory is that specific Tra proteins may be transferred to the recipient cell with the DNA where they aid its establishment in the new host. Trans-kingdom transfer to yeast also raises many questions concerning yeast biology, including the nature of cellular interactions with bacterial cells, and the delivery of the transferred strand to the nucleus and its replication.

The objective of the work described in this thesis is to analyse the process of conjugative transfer from *E. coli* to the bakers yeast *S. cerevisiae*. More specifically to determine the factors required for transfer in both donor and recipient cells. Specific aims are summarised below.

1. Development and optimisation of a protocol for *E. coli* - yeast conjugation. Of paramount importance to further studies is determining the efficiency of transfer to yeast, and deducing over what time period transfer can occur. To prove that any transfer detected is due to conjugative transfer the physical and the general genetic requirements of transfer will be determined.

2. The bacterial factors required for transfer will be investigated. Initially the ability of different conjugative plasmids to transfer to yeast will be determined. The requirement of individual Tra genes will also be addressed using defined mutants. It will be especially interesting to assess if any additional factors are required for transfer to diverse organisms, thereby providing an insight into the molecular basis of plasmid promiscuity.

3. The aspects of yeast biology important for transfer to occur will also be investigated. Initially physiological studies will be undertaken to assess the conditions important for productive transfer. These studies will include monitoring transfer during yeast growth, the cell cycle, and to different yeast strains including both haploids and diploids. Further analysis of the yeast factors required for transfer will involve the isolation and characterisation of yeast mutants defective in their ability to receive a plasmid by conjugation. Such mutants are liable to possess altered cell surfaces or be defective in the uptake of DNA to the nucleus.

Chapter Two

Materials and Methods

2.1 Standard Buffers, Solutions and Materials

0.5M EDTA

0.5M Diaminoethanetetra-acetic acid disodium salt. The pH was adjusted as required with sodium hydroxide.

1M Tris-HCl

1M Tris base. The pH was adjusted as required with concentrated hydrochloric acid.

TE Buffer

10mM Tris-HCl pH8.0

1mM EDTA pH8.0

50x Denhardt's Solution

1% Ficoll

1% Polyvinylpyrrolidone

1% Bovine Serum Albumin (BSA) (Pentax Fraction V)

Made up in distilled water and stored in aliquots at -20°C.

20x Standard Saline Citrate (20xSSC)

3M NaCl

0.3M Sodium Citrate.

Phenol Chloroform

Tris equilibrated phenol containing 0.4% hydroxyquinoline was combined with chloroform and iso-amyl alcohol at a ratio of 25:24:1 respectively, and stored in the dark at 4°C

Salmon Sperm DNA

10mg/ml salmon sperm DNA (type III sodium salt) was made up in distilled water and sheared. The solution was boiled for 10 minutes and stored at -20°C in aliquots. For subsequent use the solution was boiled for 5 minutes and then allowed to cool on ice.

RNase A

10mg/ml pancreatic RNase was made up in 10mM Tris-HCl pH7.5; 15mM NaCl. The solution was boiled for 15 minutes to remove any contaminating DNase activity, aliquots were stored at -20°C.

Phosphate Buffer (PB)

KH_2PO_4	$3gl^{-1}$	$NaHPO_4$	$7gl^{-1}$
NaCl	$4gl^{-1}$	$MgSO_4.7H_20$	$0.1gl^{-1}$

Oligo Labelling Buffer (OLB)

0.25M Tris-HCl pH8 — 100µM dATP, dTTP, dGTP
25mM MgCl2 — 1M Hepes pH6.6
50mM mercaptoethanol
30 OD units Hexadeoxynucleotides

TAB (Universal Restriction Buffer)

0.33M Tris-Acetate pH7.9 — 0.66M Potassium Acetate
0.1M Magnesium Acetate — 1mg/mL BSA
70mM β-mercatoethanol

2.2 Growth Conditions and Maintenance of *E. coli*

The strains of *E. coli* used in this study are shown in Table 2.1.

2.2.1 Media

Luria-Bertani Medium (LB) : 1%Bacto-tryptone
0.5% Bacto-yeast extract
0.5% NaCl

The pH was adjusted to 7.2 with sodium hydroxide.
For solid media bacto agar was added to a concentration of 2%.
When required antibiotics were added to the media at the concentrations shown below.

Antibiotic	Stock	Final Concentration
Ampicillin †	10mg/ml in 50%Ethanol	100µg/ml
Chloramphenicol	2.5mg/ml in 50%Ethanol	25µg/ml
Kanamycin	40mg/ml in distilled water	50µg/ml
Naladixic acid	10mg/ml in 50%Ethanol	25µg/ml
Tetracycline	7.5mg/ml in 50%Ethanol	7.5µg/ml
Streptomycin	200mg/ml in distilled water	200µg/ml

† Methicillin was also added to the media with ampicillin to avoid satellite growth. 10mg/ml methicillin was made up in 50% Ethanol, and used at a final concentration of 100µg/ml.

Strain	Genotype	Reference
J08	*recA1, leu, deoB, rpsL, dna*$^{+}$, *colI*^{r}	Chatfield *et al.*, 1982.
DH5α	*endA1, hsdR17* (r_k^-, m_k^+), *SupE44, thi-1, recA1, gyrA96, relA1, deoR, Δ(lacZYA-argF)-U169, Ø80dlacZΔM15*	Hanahan, 1983.
HB101	*thi-1, hsdS20* (r_B^-, m_B^-), *SupE44, recA13, ara-14, leuB6, proA2, lacY1, rpsL20, xyl-5, int1-1*	Boyer & Roulland-Doussiox 1969.
BW97N	*leu, thyA, deoB, rpsL, civ, Δchl-uvrB, nal*^{R}	Boulnois & Wilkins, 1979.
BW96	*leu, deoA, deoC, tdk, rpsL, civ, Col*^{R}, *rpoB*	Boulnois & Wilkins, 1979.
SCS1	*recA1, endA1, gyrA96, thr-1, SupE44, relA1, hsdR17* (r_k^-, m_k^+)	Hanahan, 1983.
DH1	*recA1, endA1, gyrA96, thr-1, SupE44, relA1, hsdR17* (r_k^-, m_k^+)	Hanahan, 1983.
MH1598	As DH1 + IncW plasmid R388::Tn(HIS3)	Sedgwick & Morgan 1994
MH1578	*recA1, endA1, gyrA96, thr-1, SupE44, relA1, hsdR17* (r_k^- ,m_k^+), *rpsL*	Sedgwick & Morgan 1994

Table 2.1 *E. coli* strains used in this study.

2.2.2 Growth Conditions

All *E. coli* strains were grown at 37°C. Liquid cultures were grown with continuous shaking.

2.2.3 Determination of Cell Numbers

The growth of a liquid cultures was monitored by measuring its optical density (OD) at 600nm. An OD_{600} of 0.5 is approximately equal to a cell density of $2x10^8$ cells/ml.

2.2.4 Preservation of *E. coli* stocks

For long term storage of strains 1ml of fresh culture (OD_{600}=1) was mixed with glycerol at a final concentration of 17.5% and frozen rapidly in a dry ice ethanol bath before storage at -70°C.

2.3 Growth Conditions and Maintenance of *S. cerevisiae*

The *S. cerevisiae* strains used in this study are shown in Table 2.2.

2.3.1 Media

YEPD : 1% yeast extract
2% Bactopeptone
2% glucose.

Synthetic medium (SD) : 0.67% Difco yeast nitrogen base
2% glucose
Amino acid supplements were added as appropriate.

Amino Acid/Base	Stock Concentration	Final Concentration
Adenine Sulphate	2mg/ml	20µg/ml
L-Histidine	8mg/ml	20µg/ml
L-Leucine	12mg/ml	30µg/ml
L-Tryptophan	8mg/ml	20µg/ml
Uracil	2mg/ml	20µg/ml

Presporulation medium : 0.8% Bacto-yeast extract
0.3% Bactopeptone
10% glucose

Strain	Genotype	Reference
S150-2B	Mat a, [cir$^+$], *leu2-3*, *leu2-112*, *his3*-Δ, *trp1-289*, *ura3-52*	Mcleod *et al.*, 1984.
8HB	Mat α, [cir$^+$], *leu2-3*, *leu2-112*, *ade2-1*, *ura3-52*, *trp1-289*	M. Pockliton.
MC16	Mat α, [cir$^+$], *leu2-3*, *ade2-1*, *his4-712*^{FS}, *SUF2*	Beggs, 1978.
C1	Mat a, [cir$^+$], *lys2*, *trp1*	CSH Laboratories
C2	Mat α, [cir$^+$], *lys2*, *leu2-3*	CSH Laboratories
RTY235	Mat α, [cir$^+$], *his4-519*, *leu2-3*, *leu2-112*, *trp1-289*, *uar3-52*	Williams & Trumbly, 1990.
842	Mat a/α, [cir$^+$], *leu2/leu2*, *ade1/ade1*, *his3/his3*, *ura3/ura3*, *trp1-1/trp1-1*	K. Nasmyth.
DS8H	Diploid of S150-2B and 8HB Mat a/α, [cir$^+$], *leu2-3/leu2-3*, *leu2-112/LEU2-112*, *his3*-Δ/HIS3, *trp1-289/trp1-289*, *ura3-52/ura3-52*, *ADE2-1*, *ade2-1*	This Work
DSMC	Diploid of S150-2B and MC16 Mat a/α, [cir$^+$], *leu2-3/leu2-3*, *leu2-112/LEU2-112*, *his3*-Δ/HIS3 HIS4/ his4-*712*^{FS}, *ADE2-1/ade2-1*, *trp1-289/TRP1*, *ura3-52/URA3*, *SUF2/SUF2*	This Work

Table 2.2 *S. cerevisiae* strains used in this study.

Sporulation medium : 1% Potassium acetate
0.1% Bacto-yeast extract
0.05% glucose

Glucose : A stock solution of 40% glucose was prepared for use as a carbon source at a final concentration of 2%.

For solid media, Bacto agar was added at a final concentration of 2% prior to autoclaving.

2.3.2 Sterilisation

All media without a carbon source was autoclaved at 15 p.s.i. for 15 minutes. Media containing a carbon source and supplements for SD media was autoclaved at 10 p.s.i. for 15 minutes.

2.3.3 Determination of Cell Numbers

Cell density of liquid cultures was measured by counting the number of cells, in a known volume, using a counting chamber marked with a thoma ruling. A 10µl sample of culture, diluted if necessary, was placed over the grid and covered with a cover glass. The density of the culture was determined from an average of 12 readings.

2.3.4 Preservation of *S. cerevisiae* Strains

All strains were viable for 6-8 weeks on YEPD or SD plates at 4°C. For long term preservation of strains a fresh colony was picked into 20% glycerol, suspended then frozen in a dry ice-ethanol bath prior to storage at -70°C.

2.3.5 Growth Conditions

All *S. cerevisiae* strains were grown at 30°C. Liquid cultures were grown with continuous shaking.

Matings were carried out between haploid strains of the opposite mating-type on YEPD plates, cells were then replica-plated onto SD medium selective for vegetatively growing diploid cells.

Presporulation was carried out by patching diploid cells onto presporulation media and incubating at 30°C for 2 days.

Sporulation was carried out by patching cells from presporulation plates onto solid sporulation medium and incubating at 24°C. After incubation for 5-7 days, spore development was checked microscopically.

Synchronous Cultures of *MAT a* strains were produced by the addition of 750µg/ml of α factor to exponentially growing cultures at a density of $1x10^7$ cells/ml. After incubation at 30°C with continuous shaking for 1½ cell doubling's the α factor was removed by filtering the cells onto a cellular acetate disc (25mm, 45µm pore size), and washing with prewarmed YEPD media. Synchronous cells were then suspended in prewarmed YEPD and incubated with shaking at 30°C to allow growth to continue. The degree of synchrony of the culture can be followed by determining the proportion of budded cells in the culture. Using this method cultures showing at least two rounds of synchronous growth could be produced.

2.3.6 Genetic analysis

Tetrad dissections

Tetrad dissections were carried out using the Singer MSM system. A loop full of sporulated cells were resuspended in 150µl of sterile distilled water. 5µl of β-glucuronidase (Sigma) was added and the cell suspension incubated at room temperature for 5 minutes. A loop full of the suspension was then streaked across one side of a YEPD plate, as outlined in the instructions for the Singer MSM system. Up to twenty dissections can be carried out on one plate. Plates were incubated at 30°C until growth of the haploid spores was observed. To ensure even growth of the spores excess adenine was added to the plates before pouring. The plates were poured on an adjustable stand, made flat by the use of a spirit level.

Random spore analysis

A loop full of sporulated cells was resuspended in 300µl of sterile distilled water. Glass beads (425-600µm) were added to the level of the meniscus and the suspension was vortexed for two minutes to physically separate the spores. The spores were then analysed microscopically to check for separation and for viability, diluted and plated onto YEPD plates to a concentration of approximately 200 spores per plate. Plates were incubated at 30°C for 3-4 days until spores had grown up. In all cases, one of the parental strains carries the *ade2* mutation. Pink coloured colonies are therefore haploid and so picked from the plates and screened.

2.3.7 Assay for Zymolyase Sensitivity

Exponentially growing cells from an overnight culture were diluted to $5x10^6$ cells/ml in YEPD and allowed to continue growing to $2x10^7$ cells/ml. Cells were then collected by centrifugation (3200 r.p.m. for 4 minutes) and resuspended in 10mM Tris pH7.5 at an A_{600} of 0.4. 2.4ml of this cell suspension was transferred to a 3ml cuvette and 20µl 5mg/ml zymolyase-100T added (≡5units). The decrease in A_{600} as cells lyse was followed at 5 minute intervals.

2.4 Extraction of DNA

2.4.1 Plasmid DNA Extraction

Small-scale plasmid DNA preparations

The alkali lysis protocol used is essentially that of Birnboim and Doly (1979).

1ml of an overnight culture of *E. coli*, grown under conditions selective for plasmid containing cells, was transferred to an microcentrifuge tube. The cells were then harvested by centrifugation in a microfuge at 13000 r.p.m and resuspended in ice cold lysis buffer (50mM Glucose, 25mM Tris pH8, 10mM EDTA pH8). 200µl of freshly prepared 0.2M NaOH/ 1% SDS solution was then added to the cells and the tube mixed carefully by inversion. After a 5 minute incubation on ice 150µl of ice cold 3M potassium acetate pH4.8 was added and the solution held on ice for a further 10 minutes. The tube was centrifuged and the clear supernatant transferred to a new tube containing 400µl of phenol/chloroform. After mixing the tube was centrifuged for 5 minutes, and the upper phase transferred to a new tube containing 400µl of isopropanol. The tube was then incubated for 10 minutes at room temperature prior to centrifugation for 15 minutes. The supernatant was then removed and the resulting pellet washed in 200µl of 70% ethanol, air dried and resuspended in 30µl of Q water.

Large-scale plasmid DNA preparations

To obtain large amounts of pure plasmid DNA, Qiagen midi columns were used (© Diagen GmbH; Qiagen Inc.). This system uses an alkali lysis method to produce a particle free lysate, and an affinity column to bind plasmid DNA. After washing the DNA can be eluted from the column and precipitated by the addition of 0.7 volumes of isopropanol. Using this method 70-100µg of plasmid DNA can be obtained from either a 100ml

culture for low copy number plasmids, or a 25ml culture for high copy number plasmids.

2.4.2 Preparation of Total Yeast DNA

10^8 yeast cells from exponentially growing cultures were harvested, washed in 1.2M sorbitol, 10mM sodium phosphate buffer pH6.5, and 50mM DTT, resuspended in the same buffer containing 50µg/mL zymolyase-100T, and incubated at 37°C until the formation of spheroplasts was complete. Spheroplasts were then lysed in 10mM sodium chloride, 50mM TRIS pH7.8, 100mM EDTA, 1% SDS, and 10µg/mL proteinase K, and incubated at 65°C for 20 minutes. The lysates were then extracted with an equal volume of phenol/chloroform three times, and precipitated with 2 volumes of ethanol. The precipitate was resuspended in TE and treated with 1mg/mL RNase A at 37°C for 30 minutes. Genomic DNA was recovered by precipitation with 2M ammonium acetate and 2 volumes of ethanol, pelleting and resuspending in 40µL TE.

2.4.3 Small-scale yeast DNA preparations

The following method, essentially that of Hoffman and Winston (1987), was used for the rescue of plasmid DNA from yeast for its subsequent transformation into *E.coli.*

1ml of an overnight culture grown in YEPD was spun in a microfuge and resuspended in 200µl of lysis solution (100mM NaCl; 10mM Tris-HCl pH8.0; 1mM EDTA; 0.1% SDS). Sterile glass beads (Sigma, acid washed 425-600µm) were then added to just below the level of the liquid. Samples were then vortexed for 2 mins. 200µl of phenol/chloroform was added followed by a further 2 min vortex. Samples were then microfuged for 2 mins and the aqueous layer transferred to a clean eppendorf tube. A further 200µl of phenol/chloroform was added, the sample briefly vortexed and microfuged for a further 2 mins. The aqueous layer was again transferred to a clean eppendorf and 1/10th volume 3M sodium acetate, 2x volumes ethanol added. The DNA was precipitated for 10 mins on ice. The samples were microfuged for 10 mins, the pellet washed with 70% ethanol and resuspended in 50µl TE. 2.5µl of the DNA solution was then used in each subsequent *E.coli* transformation.

2.5 Analysis of DNA

2.5.1 Restriction Analysis

Restriction endonucleases purchased from Gibco BRL Ltd were used to analyse DNA. The conditions and buffers used were as recommended by the suppliers. For digests using two or more enzymes that would require different buffers TAB universal buffer was used (O'Farrel *et al.*, 1980).

Plasmid DNA was digested in a 20µl reaction volume for 1 hour with 2 units of enzyme per reaction. Yeast genomic DNA digestions were carried out in 20µl for 3 hours with 15 units of enzyme. This was followed by further digestion with an additional 10 units for 2 hours. The reaction volume was increased to 30µl with restriction buffer and distilled water.

2.5.2 Agarose Gel Electrophoresis

DNA was identified and fragments were purified from gels made in 1x Tris-acetate electrophoresis buffer (TAE) containing 0.5µg/ml ethidium bromide.

50 x TAE : 2M Tris-base
1M Sodium acetate (trihydrate)
0.5M EDTA
pH was adjusted to 8.2 with glacial acetic acid.

For most purposes, such as Southern analysis and restriction mapping, a 0.7% (w/v) Seakem HGT agarose gel was used. For probe preparation and purification a 1% (w/v) Seakem LMT agarose gel was used.

DNA sample sizes of between 10 and 30µl were subjected to electrophoresis after adding 0.1 volumes of 10 x loading buffer. Gels were ran overnight at 20 Volts or for 2 to 3 hours at 70-90 Volts.

10 x Loading Buffer : 0.4% Bromophenol Blue
0.4% Xylene Cyanol
50% Glycerol

2.5.3 Recovery of DNA Fractionated on Agarose Gels

The method used for recovering DNA from agarose gels depended on both the subsequent use of the DNA and the size of the DNA fragment.

Probe preparation

The DNA, after digestion with appropriate restriction enzymes, was subjected to electrophoresis in a low melting temperature agarose gel. The DNA fragment of interest was recovered after visualisation using UV light. The band was cut out with a scalpel and placed into an Eppendorf tube together with distilled water to produce a final DNA concentration of approximately 1.5µg/ml. The tube was placed at 65°C for 5 minutes to melt the agarose. The DNA solution was aliquoted and stored at -20°C.

Centrifugation through polymer wool

To recover DNA fragments under 6kb for ligation restricted DNA was initially run on a 0.6% LGT agarose gel. After visualisation the band of interest was removed from the gel with a clean razor blade, and centrifuged through polymer wool at 13000rpm in a microfuge for 10 minutes. The resulting crude DNA solution was collected in a fresh microcentrifuge tube and it's volume increased to 200µl. The DNA was extracted using a equal volume of phenol/chloroform and then precipitated with 1/10 volume 3M potassium acetate pH4.8 and 2 volumes of 100% ethanol. The resulting pellet was resuspended in 15µl of Q water, and the DNA concentration established by visualisation of an aliquot on an agarose gel.

Electroelution of DNA

To recover DNA fragments over 6kb in length for subsequent use in ligation reactions electroelution was used. Restricted DNA was fractionated on a 0.7% HGT agarose gel. After visualisation the band of interest was electroeluted at onto dialysis membrane. The DNA was recovered from the membrane by washing with 2x 40µl Q water, and subsequently ethanol precipitated. The resulting DNA pellet was resuspended in 15µl Q water, and 3µl ran on an agarose gel to check the concentration.

2.5.4 Recovery of DNA from enzymatic reactions

. DNA can be quickly recovered from enzymatic reactions by adding sufficient EDTA to collate divalent cations. The DNA can then be

precipitated with 2 volumes of isopropanol in the presence of 2M ammonium acetate; under these conditions proteins do not co-precipitate with the DNA.

2.5.5 Phosphatase treatment

If the vector in ligation reactions was only to be cut with a single enzyme phosphatase treatment was used to prevent religation of the restricted vector. To restriction endonuclease digests 0.1units of Shrimp alkaline phoshatase was added for the final 30 minutes of the reaction. Shrimp alkaline phosphatase was used as it has the benefit of being heat sensitive.

2.5.6 Ligations

Ligation reactions were carried out in 20µl using 1 unit of T4 DNA ligase and 2µl 10x ligase buffer [0.5M Tris-HCl pH7.5, 0.1M $MgCl_2$, 0.1M DTT, 10mM spermidine, 10mM ATP, 1mg/ml BSA]. The concentration of DNA in the reaction was adjusted to give a molar ratio of 1:3 vector to insert DNA, typical reactions contained 100ng of insert DNA. The ligation reaction was incubated for 16 hours at 4°C before transformation into *E. coli*.

2.5.7 Polymerase chain reaction (PCR)

Primers

Primers came in a 35% ammonia solution. 200µl was aliquoted into a eppendorf tube and 20µl 2M sodium acetate and 800µl 100% ethanol was added. The oligonucleotides were precipitated at room temperature for 10 minutes and then microfuged for 15 minutes. The pellet was washed in 70% ethanol and resuspended in 200µl of distilled water. Concentrations of primers were determined by measuring their absorbance at 260nm. Primers were diluted to a concentration of 50ng/µl for standard PCR applications, and 3.2pmol for automated sequencing reactions. Approximate annealing temperatures (T_m) for the primers were calculated using the equation of 4°C added for every purine and 2°C for every pyrimidine.

Standard PCR

The standard PCR protocol was based on that of Saiki *et al.*, (1985). Reaction conditions and the number of cycles varied according to the template DNA and primers used. All reactions were carried out in 20µl using 10-50ng of template DNA in a standard PCR buffer (50mM KCl; 10mM Tris-HCl pH8.4; 1.5mM $MgCl_2$) with 200µM dNTP's, 10ng of each primer, and 2.5 units of Taq polymerase. The sample was overlaid with an equal volume of light mineral oil to prevent evaporation.

PCR on *E. coli* Colonies

Single *E. coli* colonies were picked into 200µl Q water using a yellow tip. A 5µl aliquot of the cell suspension was spotted onto selective media for later reference. The cell suspension was boiled for 5 minutes to lyse the cells, and cellular debris removed by centrifugation at 13000rpm for 2 minutes. 5µl of this crude cellular extract was then used in a standard PCR reaction and subjected to 30 cycles of 1 minute 94°C, 1 minute annealing, and 3 minutes at 72°C.

PCR on Yeast Colonies

A small proportion of a yeast colony was swirled into a 50µl PCR reaction, with the primer concentration increased to 100ng. The reaction was carried out in a thermal cycler for 5 minutes at 94°C followed by 35 cycles of 1 minute at 94°C, 1.5 minutes annealing , and 3 minutes at 72°C.

2.5.8 Sequencing

Sequencing was carried out using the Perkin Elmer PRISM Ready Reaction DyeDeoxy Terminator cycle sequencing kit as per instructions. 0.2-1µg of template DNA and 0.8pmol of appropriate primer were added to the terminator premix provided with the kit. The final reaction volume was made up to 20µl with distilled water and overlaid with mineral oil. The cycling reaction consisted of 25 cycles of 96°C for 30 seconds denaturing; 50°C for 15 seconds annealing; 60°C for 4 minutes elongation. The reaction mix was then taken from under the oil and transferred to a clean eppendorf tube, to which 35µl of 95% ethanol was added. The reaction products were then allowed to precipitate for 10 minutes on ice and were subsequently microfuged for 30 minutes. The ethanol was removed and the pellet allowed to air-dry. The pellet was then stored at -20°C until loaded onto an ABI sequencing gel.

2.5.9 Primers used in this study

The primers used in this study are detailed below.

bak2 5` ATGAATGGCGAGAGACTGCTTGCT
bak3 5` CGCTTCCACAAACATTGCTCAAAA
teta 5` CGATCATGGCGACCACA
tetb 5` ACGATGCGTCCGGCGTA
δ 5` AGGGGAACTGAGAGCTCTA
γ 5` TCAATAAGTTATACCAT

2.6 Southern transfer and hybridisation of DNA

2.6.1 Southern Transfer

The method used to transfer DNA to a nylon membrane was essentially that of Southern (1975), with a depurination step before alkaline denaturation to facilitate the transfer of large DNA fragments (Wahl *et al.*, 1979).

After visualisation the gel was washed briefly in distilled water. The gel was washed twice in depurinating solution [0.25M HCl] for 15 minutes, then twice for 15 minutes in denaturing solution [0.5M NaOH, 1M NaCl], and finally twice for 30 minutes in neutralising solution [0.5M Tris-HCl pH7.4, 3M NaCl]. The gel was washed briefly in distilled water in between the different solutions. The gel was mounted on a glass sheet covered with a wick of 3MM paper (Whatman®) over a reservoir of 20 x SSC. The edges of the gel were covered with plastic film to prevent "short circuiting". Hybond N membrane, wetted in 2x SSC, was placed on the gel and covered with 5 sheets of 'Quick draw' blotting paper (Sigma). After overnight elution of DNA, the DNA was crosslinked onto the membrane by UV light with a dose of $70Jm^{-2}$. For elution of the DNA in one hour 10 sheets of 'Quick draw' blotting paper were used.

2.6.2 Preparation of hybridisation probes using random primers

Radioactive DNA probes for filter hybridisation were prepared using the random oligo-priming method of Feinberg and Vogelstein (1983). The labelled nucleotide was incorporated into the DNA in the presence of the other three unlabelled nucleotides.

The DNA probe was boiled for 3 minutes and stored on ice before use. The labelling reaction was carried out at 37°C for 2 hours by the addition of the following reagents in the stated order.

5µl	OLB Buffer (5 x concentration)
1µl	10mg/ml BSA (Enzyme grade)
Up to 15.5µl	DNA Fragment (25ng)
1µl	DNA Polymerase I Klenow fragment (2 units)
2.5µl	^{32}P a-dCTP (10µCi/ml)

The labelled probe was boiled for 3 minutes prior to use in hybridisation.

2.6.3 Filter Hybridisation

The nylon membrane was soaked in 6x SSC for 2 minutes then transferred to prehybridisation solution [6x SSC, 0.5% SDS, 5x Denhardt's] and incubated at 65°C for 2 hours. The prehybridisation solution was replaced with hybridisation solution [6x SSC, 0.5% SDS, 5x Denhardt's, 0.01M EDTA pH8.0] and the labelled probe added. The membrane was incubated at 65°C for 16 hours to allow hybridisation. After hybridisation the filter was washed for 5 minutes in 2x SSC; 0.5% SDS at room temperature, and then washed in 2x SSC; 0.1% SDS at room temperature for 15minutes. The membrane was finally washed 3 times in 0.1 x SSC; 0.5% SDS at 65°C over a period of 2-3 hours.

2.6.4 Detection of ^{32}P-labelled DNA Probes

The membrane was mounted on 3MM paper and wrapped in polythene film before being applied to X-ray film with intensifying screens and exposed at -70°C

2.7 Transformation of *E. coli* Strains

The preparation of competent cells and the transformation procedure was essentially that of Mandel and Higa (1970).

An overnight culture grown in LB media was diluted 50-fold into the same medium and grown to an OD_{600} of between 0.4 and 0.5. The culture was chilled on ice and the cells harvested at 4000rpm for 7 minutes at 4°C. The cells were washed in 1/2 volume of ice cold 100μM $CaCl_2$ harvested and resuspended in the same volume of ice cold 100μM $MgCl_2$ before incubating on ice for 20 minutes. The newly competent cells were then harvested, and for immediate use resuspended in 1/10 of the original culture volume of ice cold 0.1M $CaCl_2$. For long term storage, the cells were resuspended in 1/10th volume 100μM $CaCl_2$; 17.5% glycerol. 400μl aliquots were frozen in a dry ice/ethanol bath and stored at -80°C. Frozen competent cells were thawed on ice for approximately 30 minutes before use.

For each transformation reaction between 0.1 and 1μg of plasmid DNA in 10μl was added to 200μl of competent cells and incubated on ice for 1 hour. The cells were heat shocked at 42°C for 3 minutes then incubated on ice for a further 20 minutes before adding to 2ml of prewarmed LB media. The cells were incubated at 37°C for between 90 minutes to allow expression

of antibiotic resistance markers before plating to single colonies on selective media. Plates were incubated overnight at 37°C.

Transformation frequencies of between 10^5 and 10^7 per microgram of intact plasmid DNA were obtained using this protocol.

2.8 Transformation of *S. cerevisiae* Strains

The procedure used was based on the method of Gietz *et al.*, (1992). An overnight culture grown in liquid YEPD media to a concentration of 1-2 x 10^7 cells/ml was diluted to 2.5 x 10^6 cells/ml in fresh YEPD and regrown to 1 x 10^7 cells/ml. The cells were harvested by centrifugation at 3200rpm for 4 minutes then resuspended in 1.0ml of sterile water and transferred to 1.5ml Eppendorf tubes. The cells were harvested and washed in 1.0ml 1xTE/100µM LiAc (made fresh from 10x TE and 1M Lithium Acetate stocks); before being resuspended at a concentration of 2 x 10^9 cells/ml in 1xTE/100µM LiAc. 50µl of the yeast cell suspension was mixed with 1µg of DNA and 50µl of 1mg/ml single-stranded salmon sperm carrier DNA. 300µl of sterile 40%(w/v) PEG_{3350} solution (made fresh in 1xTE/100µM LiAc) was added and the suspension mixed thoroughly. The mixture was then incubated at 30°C for 30 mins, followed by a heatshock at 42°C for 15mins. The cells were then pelleted in a microcentrifuge for 1 minute, washed in 1ml of Q water, and finally resuspended in 1ml Q. 100µl was then plated out onto selective media and incubated at 30°C for 3-5 days.

2.9 Mutagenesis of *S. cerevisiae* Strains

The UV mutagenesis protocol used was essentially that of Lee *et al.*, (1988).

A 40ml culture of exponentially growing yeast cells was grown to $2x10^7$ cells/ml, the cells harvested, washed in an equal volume of sterile water, and finally resuspended in 20ml 0.9% Potassium Chloride. 100µl of the cell suspension was diluted and plated onto YEPD plates to check for viability after exposure to UV. The cell suspension was transferred to a lidless 9cm petri dish and exposed to a UV dose of $90Jm^{-2}$ (180 seconds at 0.5J/sec). During exposure the suspension was constantly agitated. Serial dilution's of the cell suspension were made and the cells plated onto YEPD plates to give single colonies. The plates were incubated at 30°C in the dark

to prevent photo-reactivation. Using this method UV exposure results in a 50% viability and an estimated mutation rate at a particular locus of $1.5x10^{-3}$ mutations/ cell (Duffy, 1996).

2.10 Conjugative Matings

E. coli-E. coli Matings

Overnight cultures in selective LB media of donor and recipient strains were diluted 50 fold into fresh media and regrown to A_{600}=0.5. 0.5ml of donor and recipient were combined and 0.5 ml of the mixture filtered onto a 25mm, 0.45µm pore cellular acetate filter. The filter was then placed onto the surface of a prewarmed LB plate and incubated for 1 hour at 37°C. At the end of the mating the cells were washed off the filter into 5ml PB+Nal_{25} and vortexed to interrupt mating, serial dilution's were made and 100µl plated onto media to titre transconjugants.

E. coli-Yeast Matings

The protocol employed for *E. coli*-yeast matings was developed in this work and will be explained in chapter 3. The standard method developed is detailed below.

Bacterial donor cells from overnight cultures were diluted 50 fold into fresh media and grown until reaching A_{600}=0.5, at which point 0.5ml is used for each mating. Recipient yeast cultures of exponentially growing cells were diluted to $2.5x10^6$ cells/ml and grown for 3 mass doubling's to $2x10^7$ cells/ml, the cells were harvested by centrifugation at 3200rpm for 4 minutes, and resuspended in 1/10 their original volume at ~$2x10^8$ cells/ml. 0.5ml of donor and recipient were combined onto a cellular acetate filter (25mm, 0.45µm). The filter was then placed on the surface of a prewarmed YEPD plate and incubated at 30°C for 1 hour. At the end of the mating the cells were washed off the filter into 1ml PB+nal_{25} and vortexed to interrupt mating. Serial dilution's of the mating mix were made and 100µl plated onto media selective for yeast transconjugants also containing 25µg/ml Naladixic acid.

2.11 Plasmids used in this study

The plasmids used in this study are described in Table 2.3 and the following figures.

Plasmid	Bacterial Sequences	Yeast Sequences	Figure	Reference
pAC87	*oriV*(pBR); Ap^r; Col-Ib-*oriT*.	2μm-*ORI/STB*; *LEU2*.	2.1	This Work.
pAC88	*oriV*(pBR); Ap^r; RP4-*oriT*.	2μm-*ORI/STB*; *LEU2*.	2.2	This Work.
pDB126	*oriV*(ColD); Cm^r; RP4-Tra1core(*traF-M*), Tra2(*trbB-M*).	None	2.3	Balzer *et al.*, 1994.
pLG221	ColIb-P9 *cib*::Tn*5*Km^r	None	-	Rees *et al.*, 1987.
pML123	*oriV*(colD); Cm^r;RP4-Tra2(*trbB-M*).	None	2.4	Lessl *et al.*, 1993
pOX38	56kb deletion variant F.	None	-	Guyer *et al.*, 1981
pSB2	*oriV*(pBR); Ap^r; F-*oriT*.	2μm-*ORI/STB*; *LEU2*.	2.5	This Work
pSB12	*oriV*(pBR); Ap^r;RP4-Tra1(*traA-M*), *upf54.4-54.6*.	*CEN4/ARS1; URA3*.	2.6	This Work
pSB13	*oriV*(pBR); Ap^r;RP4-Tra1core(*traF-M*).	*CEN4/ARS1; URA3*.	2.7	This Work
pSB20	*oriV*(pUC); Ap^r; RP4-*oriT*.	None	2.8	This Work
pUB307	Ap^S deletion RP1.	None	-	Bennett *et al.*, 1977
pVWDG 23310Δ0.1	*oriV*(pBR); RP4-*aphA*, Tra1(*traA-M*), *upf54.4-54.6*.	None	2.9	Lessl *et al.*, 1992a
YCp50	*oriV*(pBR); Ap^r; Tc^r.	*CEN4/ARS1*; *URA3*.	2.10	Rose et al., 1987
YEp13	*oriV*(pBR); Ap^r; Tc^r.	2μm-*ORI/STB*; *LEU2*.	2.11	Broach *et al.*, 1980
YEp213	*oriV*(pBR); Ap^r; Tc^r.	2μm-*ORI/STB*; *LEU2*.	2.12	Broach *et al.*, 1980

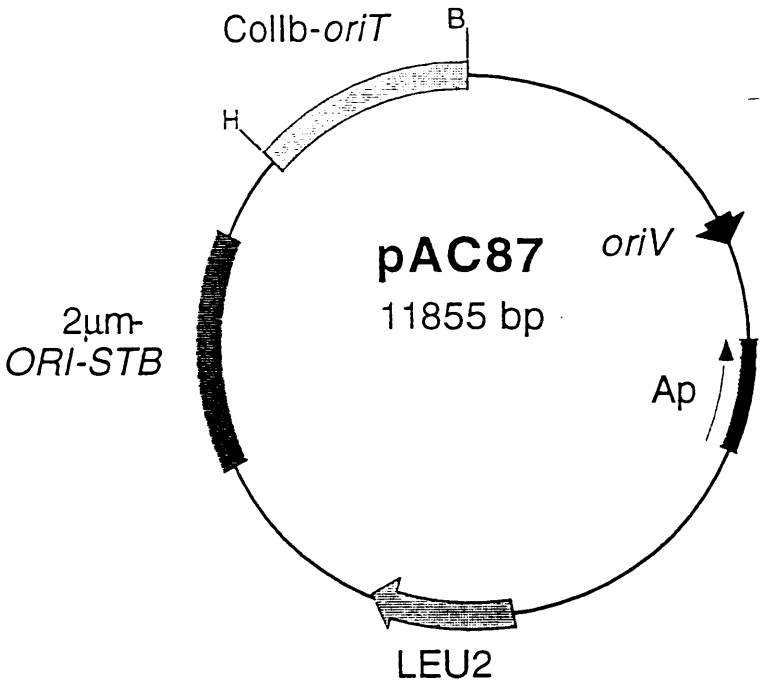


Figure 2.1 Plasmid pAC87.

Plasmid pAC87 was constructed by cloning the 1.55kb *Bam*HI/*Hin*dIII fragment of pNS11 (B.M. Wilkins personal communication) containing the ColIb *oriT* region into the corresponding sites in plasmid YEp13 (Figure 2.11). Hence, pAC87 can be maintained in *E. coli* by virtue of the origin of replication (*oriV*) from pBR322 and confers resistance to ampicillin (Ap). pAC87 is also maintained in yeast by the 2µm plasmid ARS and *cis* acting stability locus (*ORI-STB*), selection is conferred by the *LEU2* gene.
Restriction sites shown: B-*Bam*HI; H-*Hin*dIII

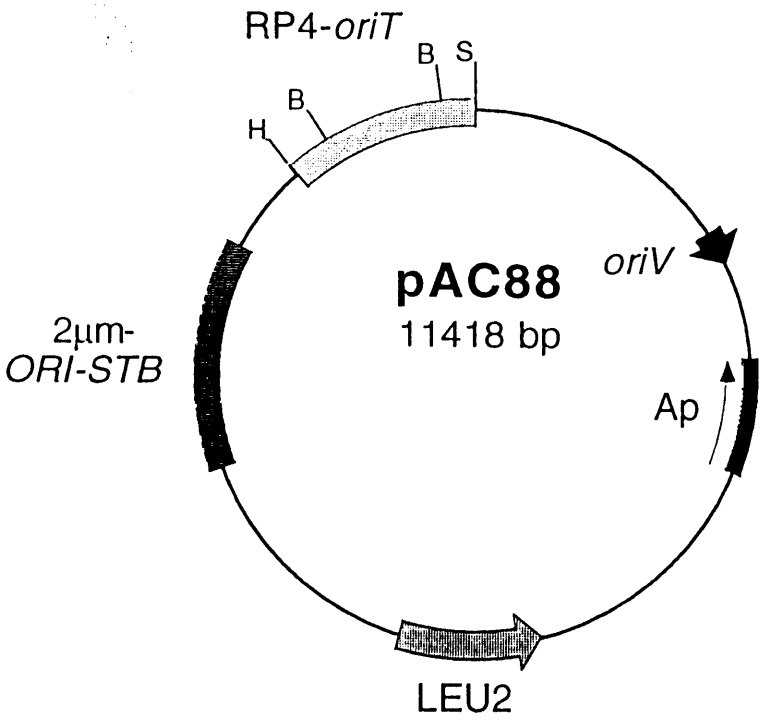
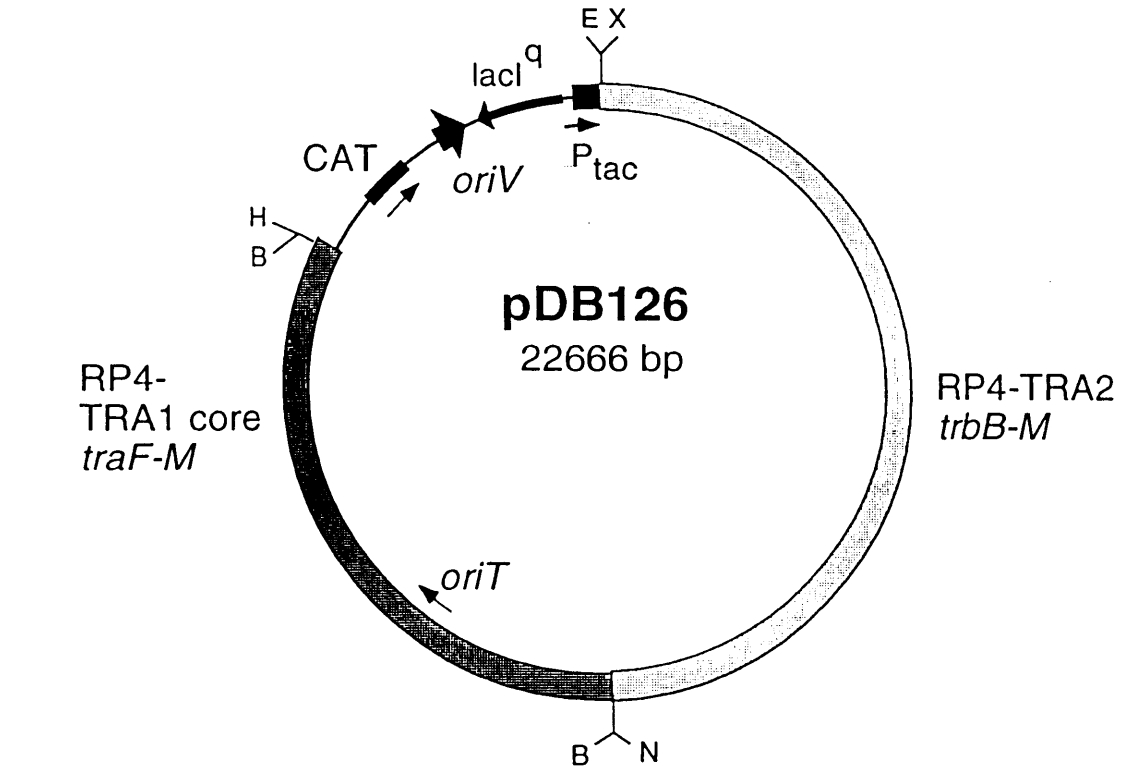


Figure 2.2 Plasmid pAC88.

Plasmid pAC88 was constructed by cloning the 1.3kb *Hin*dIII/*Sph*I fragment of pJF142 (Pansegrau *et al.*, 1988) containing the RP4 *oriT* region into the corresponding sites in plasmid YEp213 (Figure 2.12). Hence, pAC88 can be maintained in *E. coli* by virtue of the origin of replication (*oriV*) from pBR322 and confers ampicillin resistance (Ap). pAC88 is also maintained in yeast by the 2µm plasmid ARS and *cis* acting stability locus (*ORI-STB*), selection in yeast is conferred by the *LEU2* gene.
Restriction sites shown: B-*Bam*HI; H-*Hin*dIII; S-*Sph*I



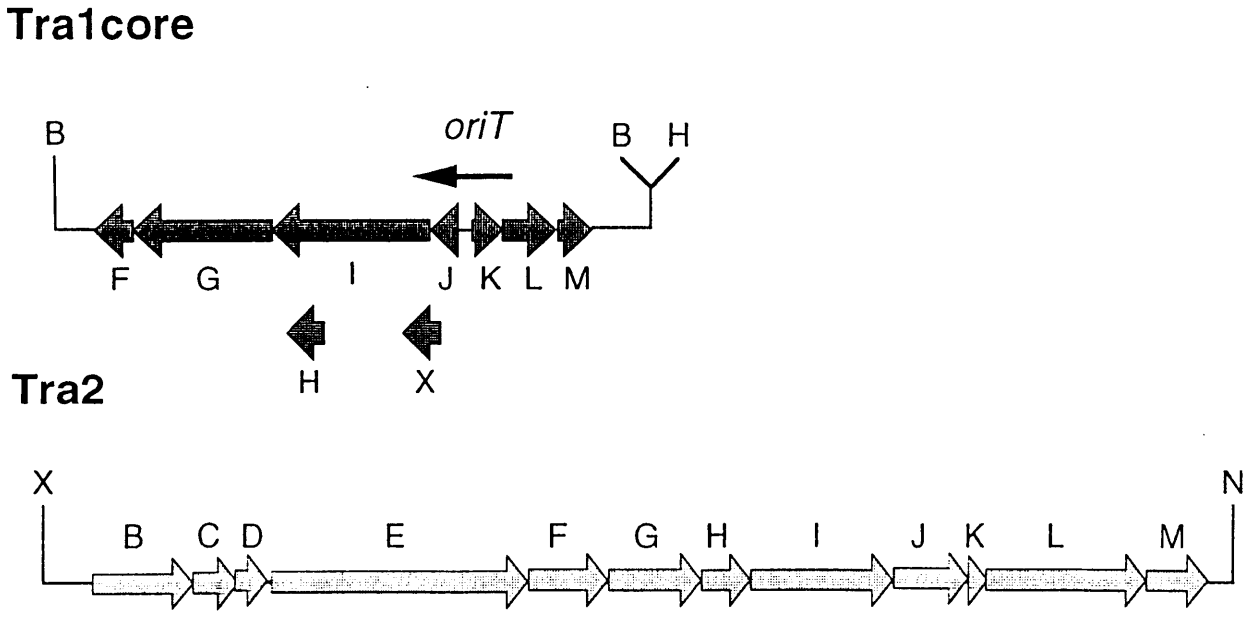


Figure 2.3 Plasmid pDB126 (Balzer *et al.*, 1994).

Plasmid pDB126 contains the origin of replication (*oriV*) from the ColD plasmid and is therefore compatible in *E. coli* with ColEI derived plasmids such as pBR322. Selection for pDB126 in *E. coli* is by the chloramphenicol acetyl transferase gene (CAT) conferring resistance to chloramphenicol. pDB126 also contains the Tra1 core region from RP4 (*trdF-M*) on a *BamH*I fragment, and the RP4 Tra2 core (*trbB-M*) fused to the leaky P_{tac} promotor to enable the operons expression. Hence, pDB126 is capable of mediating its own transfer between *E. coli* cells.

Restriction sites shown: B-*Bam*HI; E-*Eco*RI; H-*Hin*dIII; N-*Not*I; X-*Xba*I

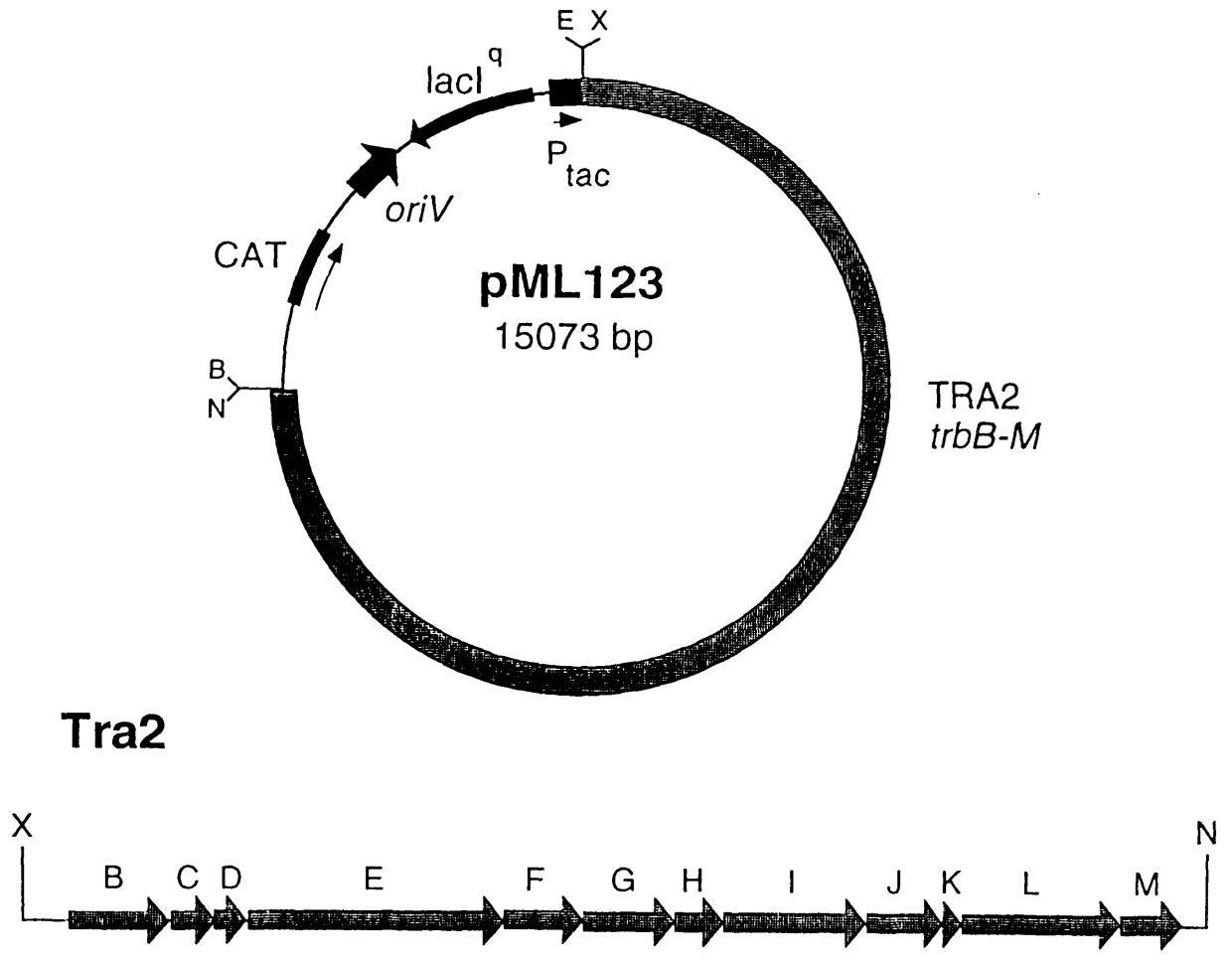


Figure 2.4 Plasmid pML123 (Lessl *et al.*, 1993).

pML123 carries the ColD based origin of replication (*oriV*), and the chloramphenicol acetyl transferase gene for replication and selection in *E. coli*. The Tra2 core region (*trbB-M*) of RP4 is carried on pML123 on an *Eco*RI/*Bam*HI fragment under the control of the leaky P_{tac} promotor.
Restriction sites shown: B-*Bam*HI; E-*Eco*RI; N-*Not*I; X-*Xba*I

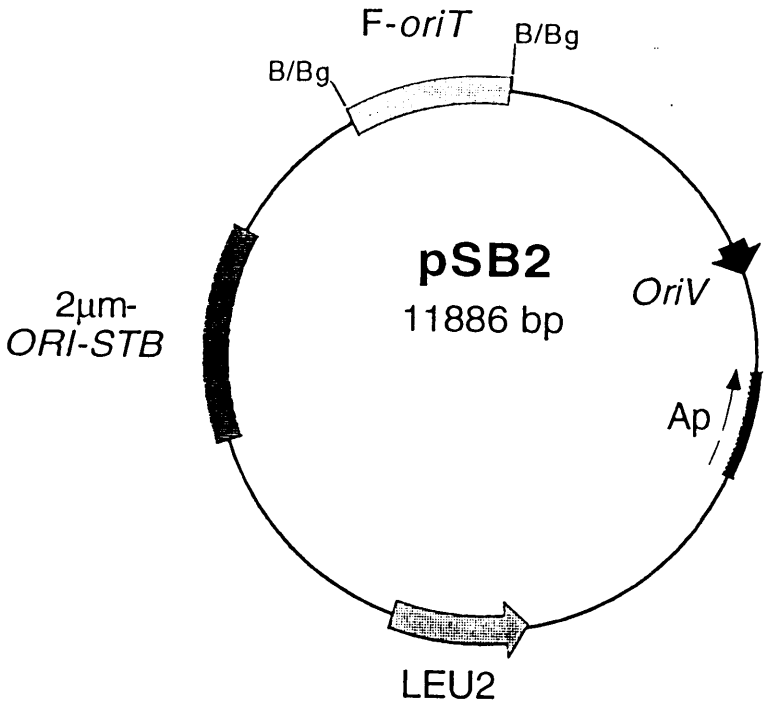


Figure 2.5 Plasmid pSB2.

pSB2 was constructed during this work by cloning the 1.1kb *Bgl*II fragment of pXRD606 (Deonier *et al.*, 1989), containing the F plasmid origin of transfer (F-*oriT*), into the unique *Bam*HI site of YEp213 (Figure 2.12). Replication and selection in *E. coli* is conferred by the pBR322 origin of replication (*oriV*) and ampicillin resistance (Ap). Maintenance in yeast is provided by the 2µm *ORI* and *STB* sequences and selection by leucine prototrophy of a leucine auxotroph. The position of the non regenerating *Bam*HI (B) and *Bgl*II (Bg) sites are shown.

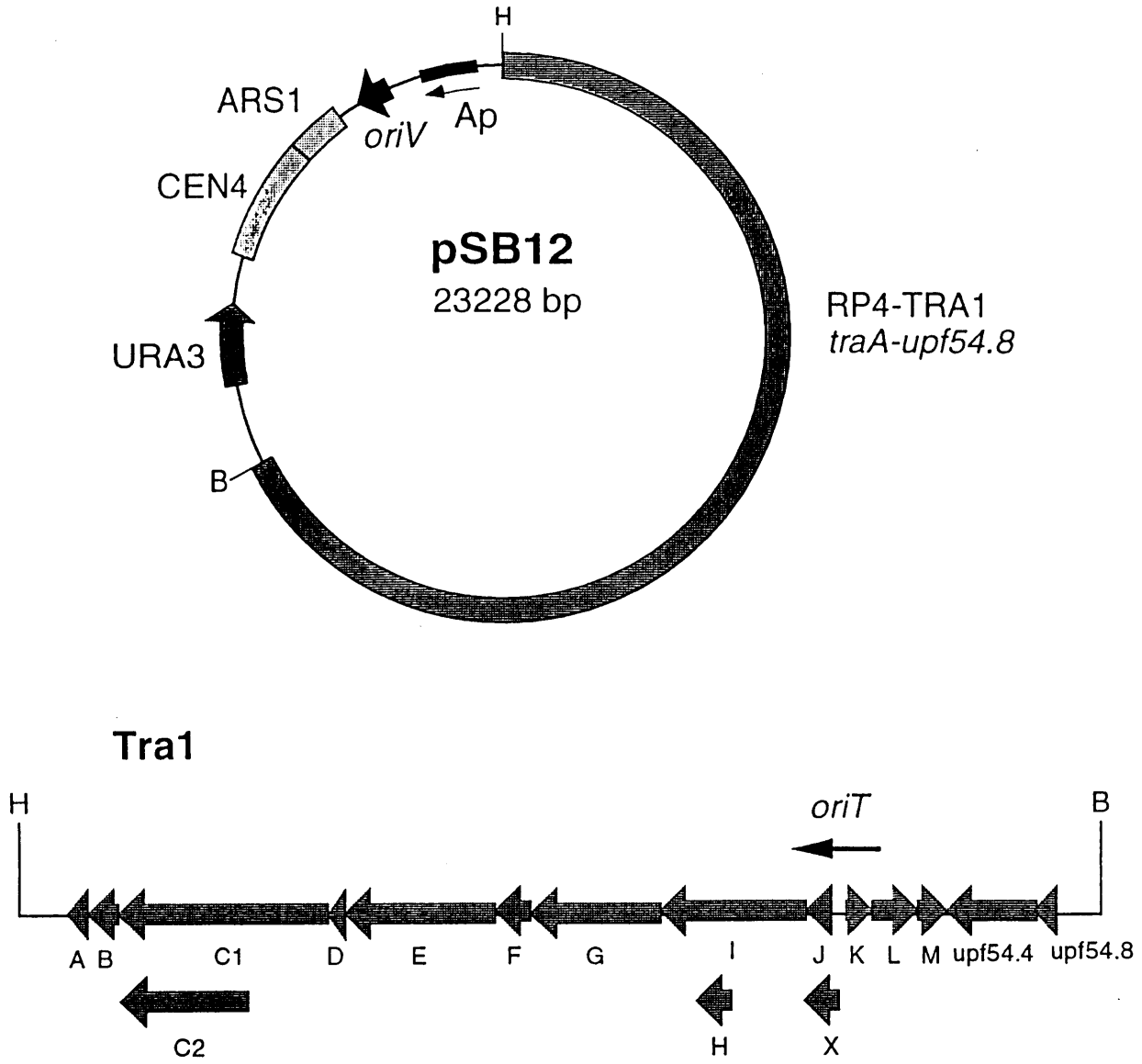


Figure 2.6 Plasmid pSB12.

pSB12 was constructed during this work by cloning the 15.6kb *Bam*HI /*Hin*dIII fragment of pVWDG23110Δ0.1 (Figure 2.9), containing the entire RP4 Tra1 region from *traA* to *upf54.6*, into the corresponding sites of YCp50 (Figure 2.10). Maintenance in *E. coli* is provided by the origin of replication (*oriV*) from pBR322 (ColE1 based) and selection by resistance to ampicillin. The *URA3* gene allows selection in yeast and the CEN4-ARS1 allows replication in yeast and maintains the plasmid at 1-2 copies per cell. pSB12 can be transferred between *E. coli* cells and *E. coli* and yeast cells if the RP4 Tra2 functions are provided in *trans* on the compatible plasmid pML123 (Figure 2.4).

Restriction sites shown: B-*Bam*HI; H-*Hin*dIII

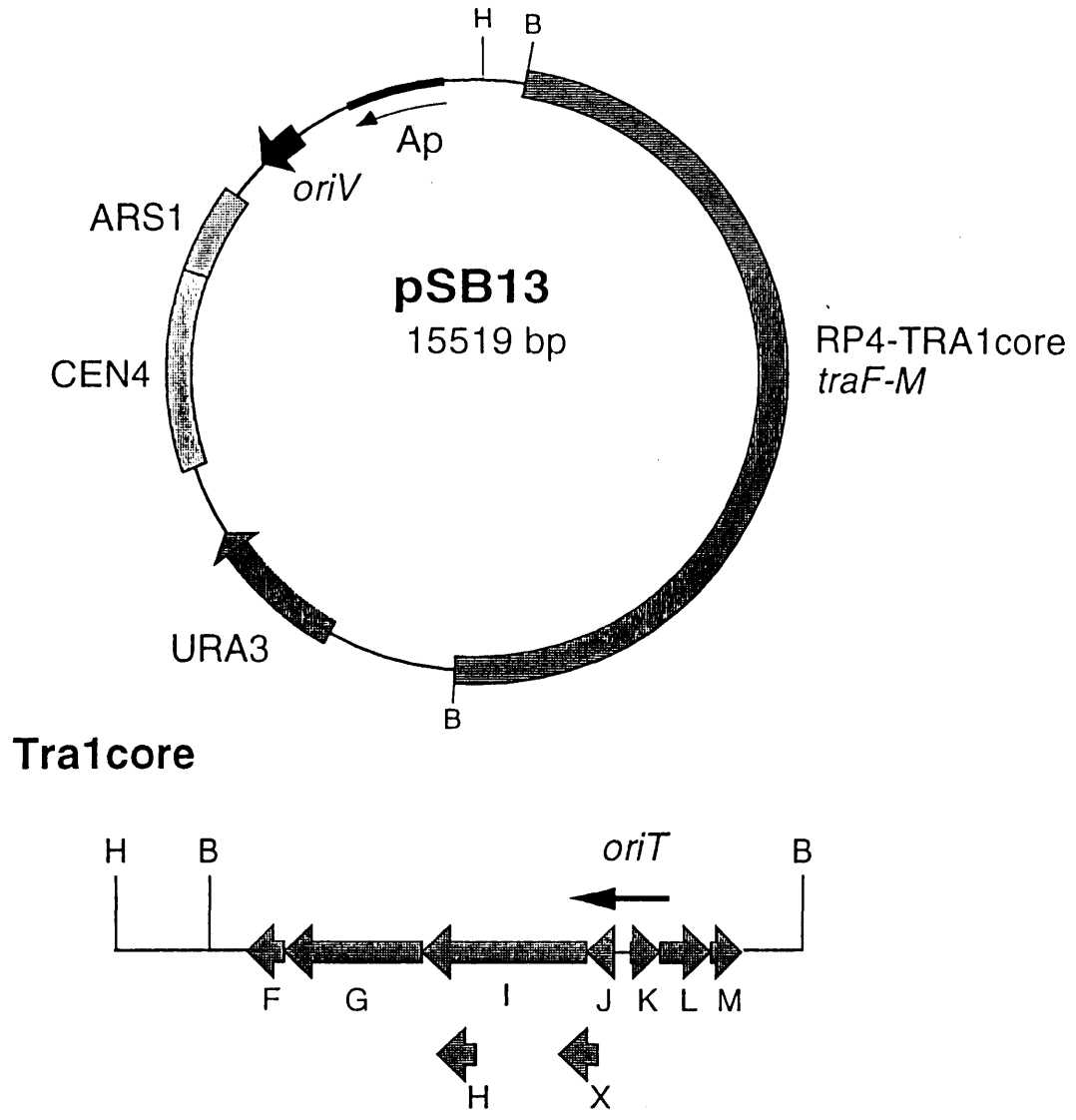


Figure 2.7 Plasmid pSB13.

pSB13 was constructed during this work by cloning the 7.6kb *Bam*HI fragment of pDB126 (Figure 2.3), containing the RP4 Tra1 core region from *traF* to *traM*, into the unique *Bam*HI site of YCp50 (Figure 2.10). Maintenance in *E. coli* is provided by the origin of replication (*oriV*) from pBR322 (ColE1 based) and selection by ampicillin resistance . The *URA3* gene allows selection in yeast and the CEN4-ARS1 allows replication in yeast and maintains the plasmid at 1-2 copies per cell.
Restriction sites shown: B-*Bam*HI; H-*Hin*dIII

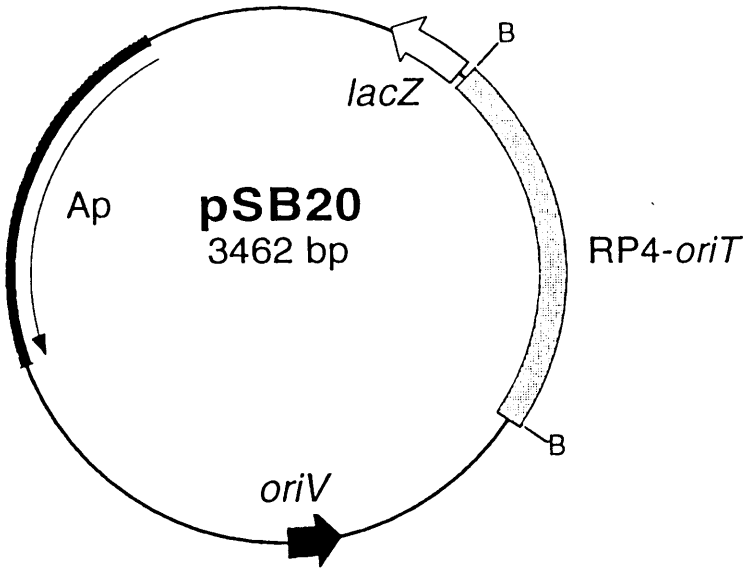


Figure 2.8 Plasmid pSB20.

pSB20 is a derivative of pUC19 containing the 776bp *Bam*HI fragment of pJF142 (Pansegrau *et al.*, 1988) encompassing the RP4 origin of transfer (*oriT*).
Restriction sites shown: B-*Bam*HI

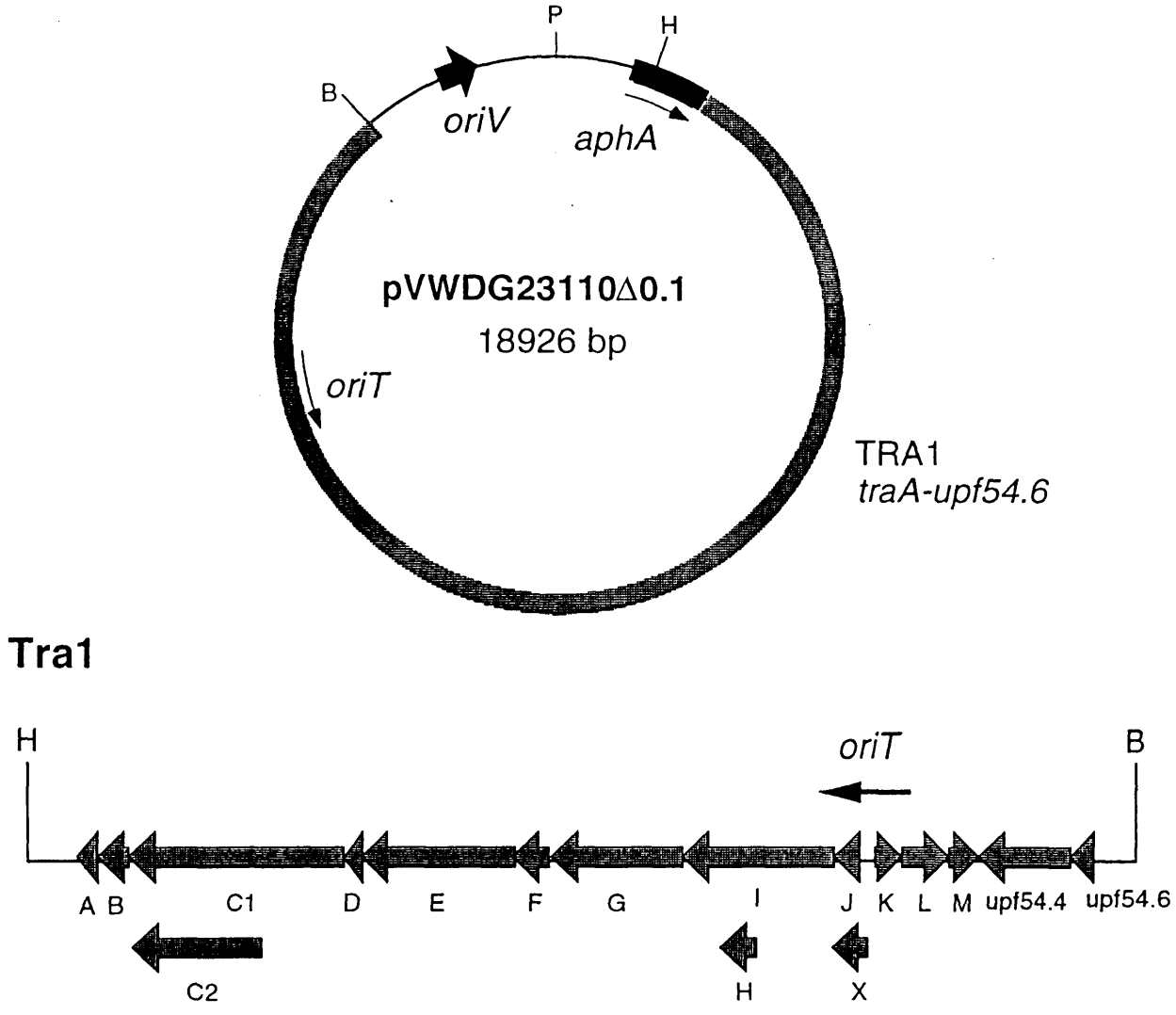


Figure 2.9 Plasmid pVWDG23310Δ0.1 (Lessl *et al.*, 1992a).

Plasmid pVWDG23310Δ0.1 contains the entire RP4 Tra1 region (*traA - upf54.6*) on a 15.6kb *Bam*HI / *Hin*dIII fragment cloned into the corresponding sites in pBR322. Selection in *E. coli* is conferred by the kanamycin resistance gene (*aphA*) also derived from RP4.
Restriction sites shown: B-*Bam*HI; H-*Hin*dIII; P-*Pst*I

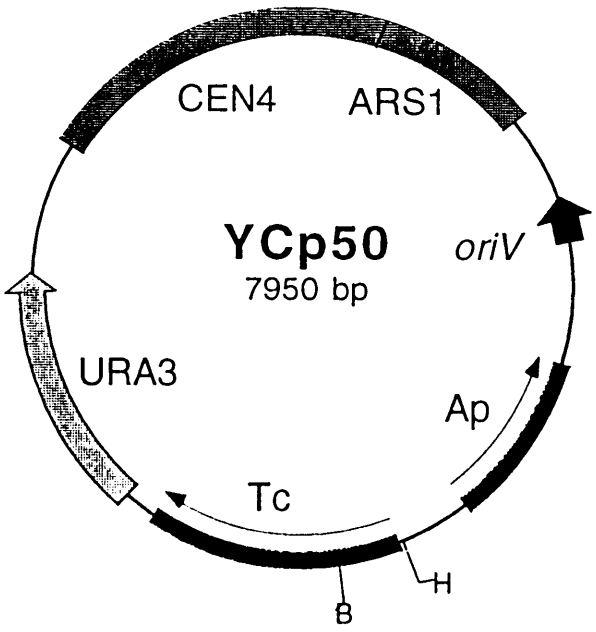


Figure 2.10 Plasmid YCp50.

Plasmid YCp50 can be maintained in both *E. coli* and yeast. Maintenance in *E. coli* is conferred by the origin of replication (*oriV*) of pBR322 and the ampicillin and tetracyline resistance determinants (Ap & Tc). Selection in yeast is determined by uracil prototropy by virtue of the *URA3* gene. The *CEN4-ARS1* region allows replication of the plasmid once per cell cycle and therefore maintains the plasmid at a low copy number. The genomic library used in this work had been constructed by cloning partial *Sau*3AI digests of wild type yeast genomic DNA into the unique *Bam*HI site of YCp50 (Rose *et al.*, 1987).

Restriction sites shown: B-*Bam*HI; H-*Hin*dIII

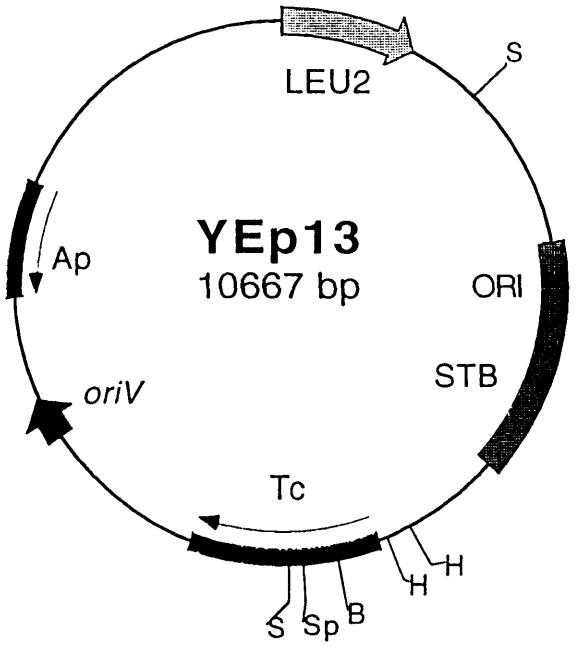


Figure 2.11 Plasmid YEp13 (Broach *et al.*, 1980).

Plasmid YEp13 confers resistance to ampicillin and tetracyclin (Ap & Tc) in *E. coli* and is replicated by virtue of the origin of replication from pBR322 (*oriV*). The *ORI* and *STB* sequences of the 2μm yeast plasmid allow replication of YEp13 in yeast. Selection in yeast is determined by Leucine prototrophy of a leucine auxotroph.
Restriction sites shown: B-*Bam*HI; H-*Hin*dIII; Sp-*Sph*I; S-*Sal*I

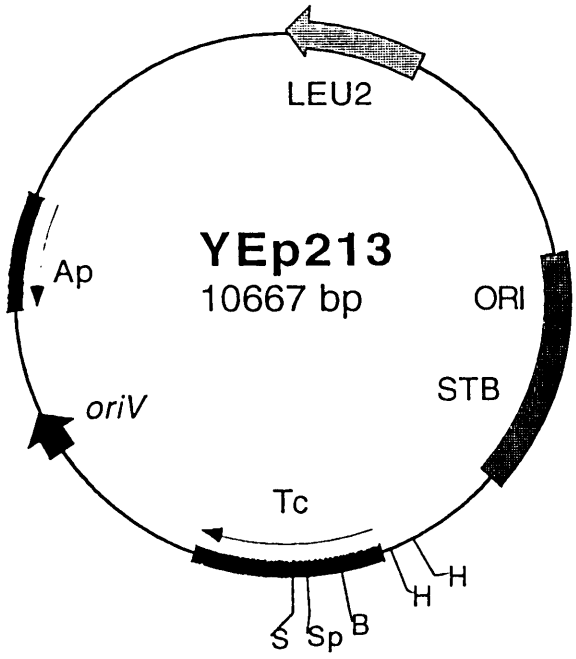


Figure 2.12 Plasmid YEp213 (Broach *et al.*, 1980).

Plasmid YEp213 is identical to YEp13 (Figure 2.11) except the orientation of the *LEU2* gene. The reversal of the *LEU2* gene removes the second *Sal*I site of YEp13, hence YEp213 has a unique *Sal*I site in the tetracyclin resistance gene.

Restriction sites shown: B-*Bam*HI; H-*Hin*dIII; Sp-*Sph*I; S-*Sal*I

Chapter Three

Development and Characterisation of Conjugative Plasmid Transfer to Yeast

INTRODUCTION

The majority of bacterial plasmids cannot be maintained in yeast. The one notable exception to this is the rolling circle plasmids of *Staphylococcus aureus* which can be maintained in *S. cerevisiae* (Goursot *et al.*, 1982). In order to assay for plasmid transfer to yeast an artificial system using shuttle vectors has been employed. Protocols for *E. coli* yeast conjugation have been developed by other workers in this field. However, each of these protocols relies on long periods of co-cultivation of donor and recipient cells. In the original description of transfer between *E. coli* and yeast, transfer was allowed to occur on the surface of agar plates for several days. Efficiencies are seen to range widely with this method and therefore cannot be compared with any confidence. Yoshida and co-workers (Nishikawa *et al.*, 1990) have limited transfer to a set time of 12 hours, with a minimum of 8 hours to detect transfer, using the antibiotic and potent inhibitor of conjugation nalidixic acid.

Plasmid transfer between bacterial cells is detectable almost instantly after the mixing of donor and recipient. Therefore it was our aim to develop and optimise a protocol for plasmid transfer between *E. coli* and yeast to determine over what time period and at what efficiency transfer could occur. To further optimise the protocol the physiological factors that affect yeast transconjugant formation will be investigated. Areas such as growth state, ploidy, and the stage of the cell cycle of the yeast recipient will be considered. One of our primary concerns was to develop a system whereby a large number of transconjugants were formed as this would allow the comparison of transfer efficiencies with more confidence. With the development and optimisation of a protocol for *E. coli* - yeast conjugation it is important to demonstrate that any transfer detected is due to a process analogous to bacterial conjugation. We also wished to compare the ability of different conjugative systems to mobilise plasmid DNA to yeast. In particular the systems we aimed to compare were the promiscuous broad host range IncP plasmid RP4 and the narrow host range IncF and IncI1 plasmids (F and ColIb respectively).

RESULTS

3.1 Construction of Mobilisable Shuttle Vectors for Transfer to Yeast.

As stated above bacterial plasmids cannot be maintained in yeast. Transfer has therefore been assayed by mobilising shuttle vectors between *E. coli* and yeast. In bacterial systems only the origin of transfer (*oriT*) is required in *cis* on a plasmid for mobilisation, as transfer functions can be provided in *trans* on a conjugative plasmid. Therefore, bacterial donors contained two plasmids; a conjugative plasmid to provide transfer functions in *trans*, and a shuttle vector carrying the relevant *oriT* region. As these two plasmids both contain an *oriT* region and therefore have regions of homology the *recA1* strain JO8 was chosen as a donor to remove the possibility of recombination occurring between the two plasmids.

Our initial shuttle vectors were based on the yeast episomal plasmid YEp13 and the closely related plasmid YEp213 (Table 2.3, Figure 2.12). These plasmids can be maintained in *E. coli* and yeast by the origin of vegetative replication (*oriV*) from pMB1, and the origin of replication and stability locus (*ORI/STB*) of the native 2μm plasmid of yeast respectively. Two 2μm-encoded proteins act *in trans* at the *STB* locus to ensure efficient maintenance of the plasmid. Therefore, for the stable maintenance of 2μm based plasmids it is essential that the recipient cell contains the native 2μm plasmid. Selection for these plasmids is conferred by ampicillin and tetracycline resistance in *E. coli* and leucine prototrophy of a leucine auxotroph in yeast.

Two mobilisable shuttle vectors had previously constructed in our laboratory, pAC87 and pAC88; carrying the ColIb and RP4 *oriT* respectively. These vectors were constructed by cloning the relevant oriT region into the tetracycline resistance gene of YEp13 for pAC87, and YEp213 for pAC88 (see figure 3.1). The first aim was to construct a new shuttle vector capable of being mobilised by the F plasmid, thus allowing the comparison of these conjugative plasmids to mediate transfer to yeast. To construct the F-*oriT* containing shuttle vector the 1.1kb *Bgl*II fragment from pXRD606 (Deonier *et al.*, 1989), containing the F*oriT* region, was ligated into the unique *Bam*HI site in the tetracycline resistance gene of YEp213. The ligation mix was transformed into BW96(pOX38), pOX38 is a deletion derivative of F. Instead of plating for transformants the transformed cells were grown in liquid NB media, conjugated with BW97N, and transconjugants selected for on Ap_{100} & Nal_{25}. This method directly selected for those recombinant plasmids that

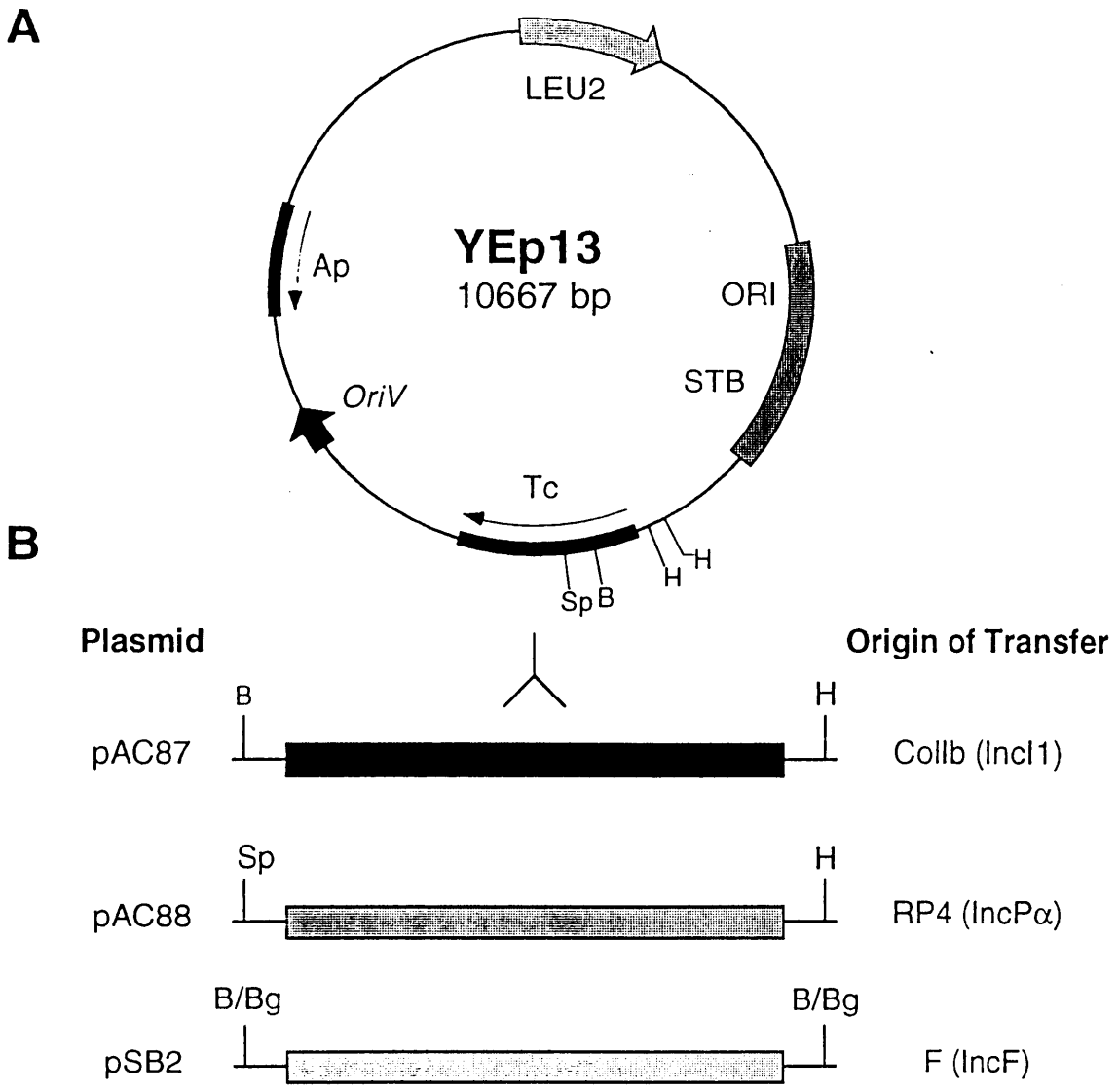


Figure 3.1. Construction of Shuttle Vectors for Transfer to Yeast.

Restriction enzymes shown are B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Sp, *Sph*I. Figure not drawn to scale

(A) YEp13 and YEp213 were used as the basis for our shuttle vectors. Both vectors are capable of autonomous replication in both *E. coli* and *S. cerevisiae* by *oriV*, and 2μm-*ORI/STB* sequences respectively. Selection is conferred by ampicillin resistance in *E. coli*, and leucine prototrophy of a leucine auxotroph in yeast.

(B) Mobilisable shuttle vectors were constructed by cloning the origin of transfer from ColIb, RP4, and F plasmids into the tetracyclin resistance gene of either YEp13 or YEp213. pAC87 was constructed by cloning a 1.55kb *Bam*HI/*Hin*dIII fragment from pNS11 into the corresponding sites in YEp13. pAC88 carries the RP4 *oriT* on a 1.3kb *Hin*dIII/*Sph*I fragment from pJF142 cloned into YEp213. The F *oriT* was cloned from pXRD606 (Deonier *et al.*, 1989) on a 1.1kb *Bgl*II into the unique *Bam*HI site of YEp213 to construct pSB2.

contain a functional F *oriT*. It also avoided the problem of orientation specific instability of plasmids containing the F *oriT* region in the presence of the F plasmid (Everett and Willets, 1987), as any recombinant plasmids containing the *oriT* in the unstable orientation would be lost. The resulting plasmid was called pSB2 (see figures 2.5 & 3.1. Table 2.3).

The ability of all three shuttle vectors to be mobilised was confirmed by carrying out bacterial conjugative matings (Table 3.1). The plasmids pAC87 and pAC88 were mobilised between *E. coli* cells at high efficiency by the conjugative plasmids pLG221 (IncI1) and pUB307 (IncPα) respectively. pOX38 mobilised pSB2 at the slightly lower efficiency of $5x10^{-1}$ transconjugants per recipient. All three shuttle vectors are maintained successfully in yeast as confirmed by their effective transformation of competent yeast cells (data not shown).

3.2 Initial Comparison of IncPα, F, and I1 Mediated Transfer to Yeast.

Initially, plasmid transfer to yeast was carried out by mixing donor and recipient cells, and plating onto yeast minimal media selective for yeast transconjugants, as described by Heinemann and Sprague (1989). In carrying out these matings we were not able to detect any yeast transconjugants formed by the IncI1 and IncF based systems. Only the IncPα system appeared capable of mediating plasmid transfer to yeast. This initial method was also found to yield inconsistent results, most likely as transfer could potentially occur over several days, hence efficiencies could not be compared with confidence. Therefore, it was our aim to develop a protocol where by transfer was limited to a set time. In developing our protocol for transfer the IncPα system was employed as it appeared to be more proficient at mediating plasmid transfer from bacteria to yeast.

3.3 Development and Optimisation of a Protocol for Plasmid Transfer to Yeast.

To prevent further transfer occurring after a set time period the bacteriostatic agent, and potent inhibitor of conjugation, nalidixic acid was used. When donor and recipient cells were plated onto media, selective for yeast transconjugants, containing 25μg/mL nalidixic acid (Nal_{25}) no transconjugants were formed, thus proving the ability of Nal_{25} to prevent conjugation. Nalidixic acid at higher concentrations (100μg/ml) is known to produce a transient cell cycle block at START, the main regulatory step of the yeast cell cycle (Singer and Johnston, 1979). To determine if Nal_{25} had any deleterious effects on yeast recipients we

Transfer System	Donor	Plasmid Selected	Transfer Efficiency
IncI1	JO8(pLG221,pAC87)	pLG221	$1x10^{0}$
		pAC87	$9.5x10^{-1}$
		Both	$1x10^{0}$
IncPα	JO8(pUB307,pAC88)	pUB307	$9.8x10^{-1}$
		pAC88	$9.8x10^{-1}$
		Both	$9.5x10^{-1}$
IncFI	BW96(pOX38,pSB2)	pOX38	$5.2x10^{-1}$
		pSB2	$5.1x10^{-1}$
		Both	$5.8x10^{-1}$

Table 3.1. Mobilisation of shuttle vectors between *E. coli*.

Bacterial matings were carried out between the donor strains described in the table and the recipient BW97N. Conjugative plasmids used were pLG221, pUB307, and pOX38 representing the Inc groups I1, Pα, and FI respectively. *E. coli* - Yeast shuttle vectors were pAC87, pAC88, and pSB2. All matings were carried out at 37°C on nutrient agar for 1 hour at a donor to recipient ratio of 1:1. Transfer of the shuttle vectors was selected for on Ap_{100} and Nal_{25}. pLG221, pUB307, and pOX38 were all selected for on Km_{50} and Nal_{25}. Transfer of both shuttle vector and the conjugative plasmid was selected for on Ap_{100}, Km_{50}, and Nal_{25}. Transfer efficiency is expressed as the number of transconjugants per recipient cell. The results indicated are the average of 2 experiments.

compared the growth of our recipient strain S150-2B, in both the presence and absence of Nal_{25}. In both cases the doubling time of S150-2B were found to be identical (106 minutes), hence Nal_{25} does not appear to affect growth. Colony formation was also unaffected by Nal_{25} as colony sizes in the presence of Nal_{25} were comparable to those in its absence; the viability of yeast to form colonies was also unaffected by Nal_{25}. These results confirm that Nal_{25} can be used effectively to prevent plasmid transfer between *E. coli* and yeast, and that it does not cause any obvious deleterious effects to yeast at the concentration used.

To determine over what time period transfer to yeast was possible, matings were carried out over varying time periods using the bacterial donor [JO8(pUB307,pAC88)] and the yeast recipient [S150-2B]. Donor and recipient cells were combined on cellulose acetate filters (0.45μm pore size) and the filters incubated on both rich yeast media (YEPD) and bacterial nutrient agar, at either 30°C or 37°C. Multiple matings were carried out in parallel and interrupted at different time points up to a maximum of 18 hours after mixing donor and recipient. The cells were then washed from the filters and plated onto media selective for yeast transconjugants, containing nalidixic acid to prevent any further rounds of plasmid transfer. Yeast transconjugant formation was only observed on matings carried out on YEPD and at 30°C. In contrast to previous reports transfer was detected after short mating periods. In fact, transfer was detected in as little as 10 minutes, as opposed to 8 hours previously reported as a minimum (Nishikawa *et al.*, 1990).

The time course for transfer to yeast is shown in Figure 3.2. It can be seen that there is an initial burst of transfer which peaks at about 30 minutes. This pattern is in agreement with observations of bacterial matings where there is a burst of conjugative mating immediately after the cells are mixed. After 30 minutes the transfer rate to yeast remains fairly constant, in fact there is a slight decrease. This decrease in transfer efficiency may be due to yeast cells which receive a plasmid early on in the conjugative mating subsequently losing the plasmid. As a standard we now carry out all *E. coli*-yeast matings for 1 hour.

The effect on transconjugant yield and transfer efficiency of altering the ratio of donor and recipient cells during mating was also investigated. Matings were carried out at donor to recipient ratios of 100:1, 10:1, 2:1, 1:1, 1:2, 1:10, and 1:100. It was found that decreasing the number of recipient cells, such that there was an excess of bacterial donors, resulted in higher transfer efficiencies (expressed as

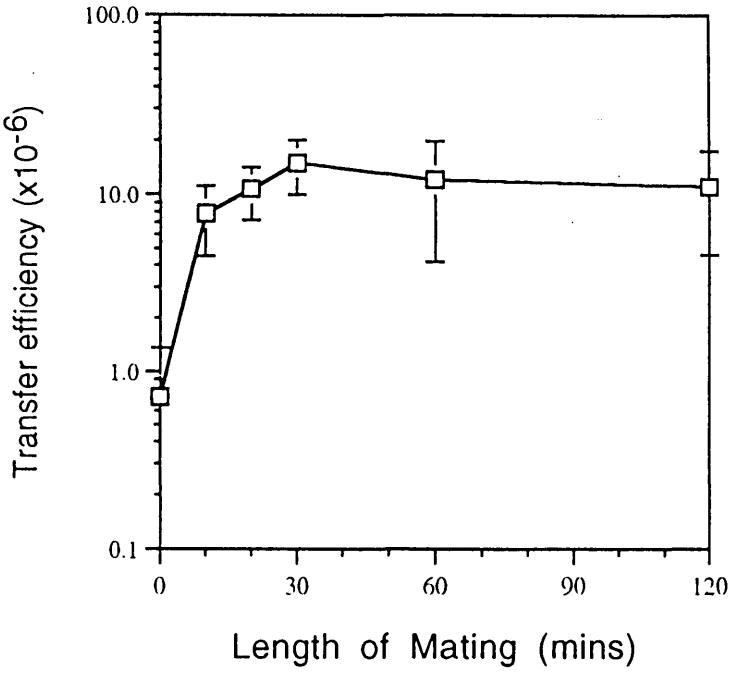


Figure 3.2. Time Course for Transfer to Yeast.

Equal concentrations of bacterial donors [JO8(pUB307,pAC88)] and yeast recipient cells [S150-2B] were combined on a cellular acetate filter (pore size 0.45μm). The filters were placed on the surface of YEPD plates and incubated at 30°C. Multiple matings were performed and interrupted at different time points. Transconjugants were selected for leucine prototrophy. Transfer efficiency is expressed as the number of transconjugants formed per recipient. Results shown are the average of 4 experiments. Error bars indicate the standard deviation from the mean in these experiments.

transconjugants formed per recipient cell) being obtained (Table 3.2). Our primary concern was to optimise the transfer protocol whereby a large number of transconjugants were formed. Hence, a donor to recipient ratio of 1:1 has been adopted as standard as this was found to yield the highest numbers of transconjugant cells. The benefit of having a larger number of transconjugants formed is that efficiencies of transfer can be compared with more confidence. These matings used 10^8 yeast recipient and donor bacterial cells, it was not possible to increase this number in order to obtain more transconjugants as higher numbers of cells appeared to have an inhibitory effect on transfer and resulted in lower transfer efficiencies.

In summary we have developed a protocol for assaying plasmid transfer to yeast. We have demonstrated that transfer can occur over a much shorter time period than previously reported. Our standard protocol adopted allows mating to continue for 1 hour at a donor to recipient ratio of 1:1. This typically results in $3.0x10^{-5}$ transconjugants formed per recipient (+/- $2.9x10^{-5}$ from an average of 60 matings, using the IncPα mating system).

3.4 Comparison of IncPα, F, and I1 Mediated Transfer to Yeast by the Optimised Protocol.

As mentioned in section 3.2 we were only able to detect transconjugants with the IncPα transfer system in initial matings. The shuttle vectors we have constructed are capable of being mobilised by IncPα, IncI1, and IncF plasmids (pAC88, pAC87, & pSB2 respectively); these three plasmids have distinct transfer systems, although the *nic* region in the *oriT* of IncPα and IncI1 plasmids are similar. We have compared the ability of the IncPα, IncI1 and IncF plasmids pUB307, pLG221 and pOX38 to mediate transfer to yeast using our standard protocol for RP4 transfer (Table 3.3). No transconjugants were obtained with the IncI1 and IncF transfer systems, indicating that if these plasmids are capable of mobilising plasmids to yeast they do so at a very low efficiency (less than $3x10^{-7}$). We attempted to increase the sensitivity of our protocol by increasing the number of cells used. However, this resulted in a lowering of efficiencies for the IncPα based system, and still did not produce transconjugants for the ColIb and F plasmid system. Increasing the time allowed for mating also failed to allow the detection of transconjugants. The ability of our shuttle vectors to transform yeast to leucine prototrophy was checked; all three shuttle vectors were capable of transforming yeast at comparable frequencies, hence the maintenance of the plasmid in yeast is

Donor : Recipient Ratio	Transconjugants /Filter	Transfer Efficiency
10:1	2900	5.8×10^{-4}
1:1	9570	1.9×10^{-4}
1:10	970	1.9×10^{-5}

Table 3.2. Effect of donor to recipient ratio on transconjugant formation and transfer efficiency.

Matings were carried out between the bacterial donor JO8(pUB307,pAC88) and the yeast recipient S150-2B at 30°C on rich media (YEPD) at various ratios of donor and recipient cells. The results shown are for 4 hour matings. Yeast transconjugants were selected for leucine prototrophy Transfer efficiency is expressed as LEU^+ transconjugants formed per recipient. Results with ratios of 100:1,2:1,1:2, and 1:100 gave a similar trend.

Donor	Inc Group	Transfer Efficiency
JO8 (pUB307,pAC88)	Pα	$5.7x10^{-5}$
JO8 (pOX38,pSB2)	FI	$<3x10^{-7}$
JO8 (pLG221,pAC87)	I1	$<3x10^{-7}$

Table 3.3. Comparision of RP4, F, & ColIb Mediated Transfer to Yeast.

The yeast recipient in all matings was S150-2B, all matings were carried out as outlined in Chapter two (Section 2.10). Yeast transconjugants were selected for leucine prototrophy. Transfer efficiency is expressed as transconjugants formed per recipient. The results shown are the average of 3 experiments.

not responsible for the lack of detectable transfer. The ability of the plasmids to be mobilised had already been confirmed in *E. coli* matings (Table 3.1). In conclusion, we have only been able to detect transconjugants with the IncPα based transfer system. This confirms the description of IncPα plasmids such as RP4 as promiscuous, and demonstrates that there are inherent differences in conjugative plasmid's ability to transfer to diverse organisms. This result is in contrast to the work of Heinemann and Sprague (1989) who were able to detect transfer to yeast mediated by the F plasmid.

3.5 Analysis of Yeast Transconjugants.

The formation of yeast colonies prototrophic for leucine after mating could be caused by reversion of the *leu2* mutation in the recipient strain. However, this is not likely since the standard recipient used, S150-2B, contains a double *leu2* mutation (*leu2-3, leu2-112*). To date in our work we have not detected any reversion of S150-2B to a LEU^+ phenotype, indicating that the reversion rate is below $1x10^{-7}$. To determine that transconjugant formation is due to the presence of the pAC88 shuttle vector in yeast we carried out Southern blot analysis on yeast transconjugants. Total DNA was obtained from 29 transconjugants from a mating between donor JO8(pUB307,pAC88) and the yeast strain S150-2B. The DNA was restricted to completion with *Xba*I, which linearises the pAC88 shuttle vector. After Southern transfer, hybridisation was carried out using a radioactively labelled RP4-*oriT* fragment from pSB20 (Table 2.3; Figure 2.8) as a probe. The resulting autoradiograph (Figure 3.3) clearly shows the presence of pAC88 DNA in the transconjugant cell lines, proving that plasmid transfer has occurred. Moreover, there has been no major rearrangements of the plasmid DNA during transfer as the band produced is the correct size for linearised pAC88.

The structure of transferred plasmid in the yeast recipient has also been investigated by rescuing these plasmids from yeast into *E. coli*. Restriction analysis of the plasmids rescued from yeast has confirmed the integrity of the plasmid in the yeast recipient (Figure 3.4). From a total of 17 plasmids analysed, no rearrangement or restriction fragment polymorphisms were detected. The major limitation with this approach is that if any changes had occurred which affected maintenance or selection in *E. coli* these plasmids would not have been rescued.

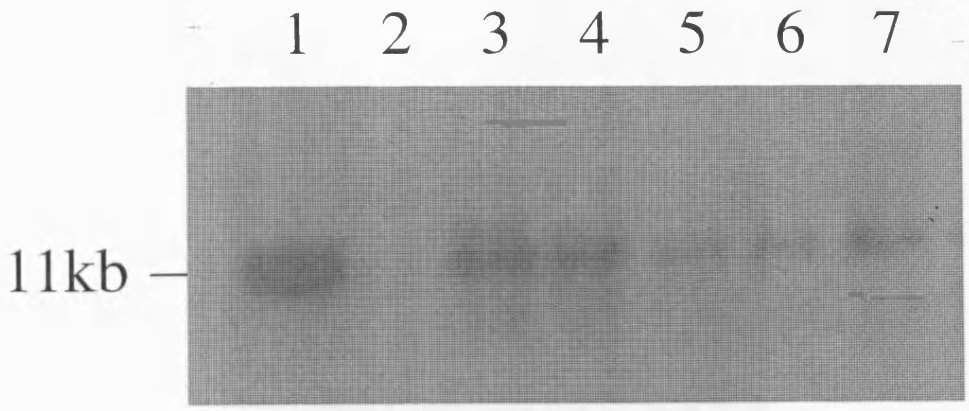


Figure 3.3. Southern Identifying Transferred Plasmid in Recipient Yeast Cells.

Total DNA was prepared from yeast as described in the materials and methods. Lanes 3-7 contain genomic DNA from transconjugant cell lines, lane 2 contains DNA from S150-2B, and lane 1 contains pAC88 DNA. All DNA samples were digested with *Xba*I, which linearises pAC88, producing a band at 11kb. Restricted DNA samples were subjected to electrophoresis and transferred to a nylon membrane. A 776bp *Bam*HI fragment from pSB20, containing the RP4 *oriT*, was radioactively labelled using P^{32} and used as a probe in Southern hybridisation. A total of 29 transconjugants have been tested, all show identical banding patterns.

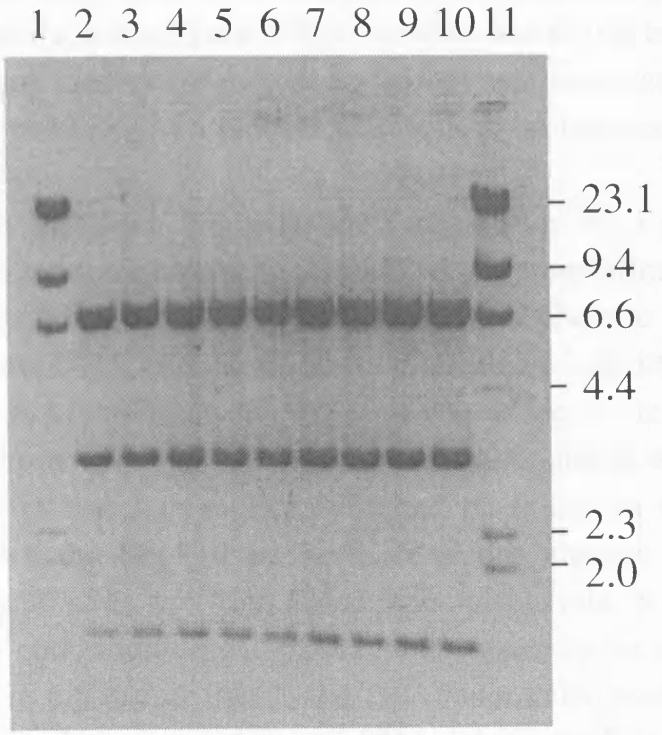


Figure 3.4. Restriction Analysis of Transferred Plasmids Rescued from Yeast.

Plasmid DNA was rescued from yeast by the method of Hoffman (1987), as described in Chapter 2. Lanes 2-9 contain plasmid DNA rescued from transconjugants, lane 10 contains pAC88 DNA. All DNA samples were restricted with *Hin*dIII and *Sal*I, before being subjected to electrophoresis. Lanes 1 and 11 contain λ *Hin*dIII markers. A total of 17 plasmid DNAs rescued from yeast transconjugants have been tested, all show identical banding patterns. The size of the λ *Hin*dIII markers is indicated in kb.

3.6 Physical and Genetic Characteristics of Plasmid Transfer to Yeast.

Previous workers have demonstrated that plasmid transfer to yeast displays the same physical and genetic characteristics of bacterial conjugation. However, as we have developed a revised protocol for transfer, and as this has shown transfer to be more efficient than previously reported, it was important to demonstrate that transfer was occurring by a process analogous to bacterial conjugation.

It is possible that transconjugants are formed from by a previously undescribed process of natural transformation, the DNA for transformation presumably being derived from either lysed donor cells, or from abortive conjugation delivering single stranded DNA into the media. To test this possibility two approaches have been used. Firstly 100units/mL DNase I was added to the mating cells. DNase I degrades both single and double stranded DNA, and is active in YEPD media. Addition of DNase I to mating cells had no affect on the transfer efficiency, indicating that the DNA is protected from this enzyme during transfer (Table 3.4A). Plasmid DNA has been added to recipient cells, in the conditions used for mating, at a concentration of 5μg/mL; this concentration is higher than if all the donor cells in the mating mix lysed (10^8 donor cells would produce ~0.02μg of pAC88 DNA). However addition of DNA did not result in LEU^+ yeast cells being formed. DNA has also been added to recipient cells in the presence of the *E. coli* strain used JO8, and JO8(pUB307), in both these cases no LEU^+ yeast cells were produced (Table 3.4B). Hence the production of transconjugants is not due to the misinterpretation of natural transformation.

Matings carried out between our donor strain JO8, containing a variety of plasmids, and yeast has demonstrated the absolute requirement for the *oriT* sequence in *cis* on the shuttle vector, and the requirement of a conjugative plasmid in the donor cell (Table3.4C). Physically separating donor and recipient cells by a filter has shown that cellular contacts are required (Table 3.4D), therefore, a diffusable factor is not responsible for plasmid transfer. Nalidixic acid, as mentioned previously, inhibits transfer occurring (Table3.4D). Finally, the transfer requirements in the donor cell show the same specificity as bacterial conjugation, in that other conjugative plasmids can only mobilise plasmids carrying the relevant *oriT* region (Table3.4E). In summary, plasmid transfer to yeast demonstrates all the physical and genetic characteristics of bacterial conjugative systems.

	Donor	Treatment	Transfer to Yeast
A	JO8 (pUB307, pAC88)	None	+
	JO8 (pUB307, pAC88)	+ DNaseI	+
B	JO8(pUB307, pAC88)	None	+
	JO8 (pUB307)	+ DNA	-
	JO8	+ DNA	-
	None	+ DNA	-
C	JO8 (pUB307, pAC88)	None	+
	JO8 (pUB307, YEp13)	None	-
	JO8 (pUB307)	None	-
	JO8 (pAC88)	None	-
	JO8	None	-
D	JO8 (pUB307, pAC88)	None	+
	JO8 (pUB307, pAC88)	Cells Separated	-
	JO8 (pUB307, pAC88)	+ NaI_{25}	-
E	JO8 (pUB307, pAC88)	None	+
	JO8 (pOX38, pAC88)	None	-
	JO8 (pLG221, pAC88)	None	-

Table 3.4. Transfer to Yeast Occurs by Conjugation.

Matings were carried out as outlined in the materials and methods using the yeast strain S150-2B as a recipient. Transconjugant yeast were selected for leucine prototrophy.

+ Indicates transconjugant formation at levels comparable to wild type.

- Indicates no transconjugants were detected.

(A) DNaseI was used at a final concentration of 100units/mL.

(B) pAC88 plasmid DNA was added to the media at 5μg/mL.

(C) YEp13 is a non mobilisable shuttle vector.

(D) Cells were separated by a 0.45μm pore cellular acetate filter, Nalidixic acid was used at 25μg/mL.

(E) pOX38 and pLG221 are derived from F and ColIb respectively.

3.7 Physiological Factors Affecting Yeast Transconjugant Formation.

We have previously shown that transfer to yeast is only detectable when donor and recipient cells are incubated on media permissive for yeast growth (see section 3.2). To determine if growth state affects the yeast cell's ability to receive a plasmid we compared the efficiency of transfer to growing cells, from exponential cultures and plates, as well as stationary phase S150-2B yeast cells (Table 3.5). It is clear from these matings that yeast cells are able to receive a plasmid no matter what their growth state before the mating. However, transfer is approximately 15 fold more efficient to exponentially growing yeast cells than either cells from plates or stationary phase cells. To further analyse the effect of growth state on plasmid transfer to yeast we carried out matings at intervals through yeast growth. Stationary phase yeast cells were inoculated into fresh rich media and incubated at 30°C with continuous shaking. At 1 hour intervals an aliquot of culture was taken, the cell concentration adjusted, and a standard mating with JO8(pUB307,pAC88) carried out. The results of this experiment are shown in figure 3.5. Transfer rates peak as the yeast cells exit lag phase, transfer then remains fairly constant during exponential growth, until dropping as the cells re-enter stationary phase. The values for transfer rates obtained are consistent with the previous observation that exponentially growing cells are 15 fold more efficient at receiving a plasmid than stationary phase cells. The growth state referred to relates to the yeast cell's state immediately prior to mating. Several factors could be affecting the transfer rate, such as the transferred plasmids' requirement to replicate, or the physical nature of the cell wall could limit transfer to stationary phase and vegetative yeast cells.

The work reported so far only uses the yeast strain S150-2B as a recipient. We have compared the transfer efficiency to other standard laboratory yeast strains (Table 3.6). Although this is a limited set of strains it appears that transfer to *Mata* and *Matα* cell lines are comparable. The most interesting result is that transfer to diploid cells is reduced 80 fold in comparison to haploid cells. This result is clearly specific to the ploidy of the recipient and not due to strain specific events, as the diploid formed from haploids known to mate efficiently (both DS8H and DSMC) exhibits this decrease. This suggests that haploid or diploid specific factors may play an important role in either the surface interactions or the uptake of the DNA. The molecular basis of this phenomenon, however, is as yet unclear.

Many physiological changes occur during the yeast cell cycle, we were therefore interested in determining if transfer occurs preferentially at specific times during

Growth State of Recipient	Transfer Efficiency
Exponential Culture	$3.3x10^{-5}$
Vegetative from plates	$2.3x10^{-6}$
Stationary Culture	$1.8x10^{-6}$

Table 3.5. Effect of Growth state on ability to receive a plasmid by conjugation.

All matings were carried out between the bacterial donor JO8(pUB307,pAC88) and S150-2B as a recipient, under the standard conditions of 30°C for 1 hour on rich media (YEPD). Exponentially growing and stationary phase cells were derived from liquid cultures, vegetatively growing cells were also obtained from colonies growing on YEPD agar plates at 30°C. In all matings 10^8 recipent yeast cells were used. Yeast transconjugants were selected for leucine prototrophy. Transfer efficiency is expressed as transconjugants per recipient. The results presented are the averages of at least two experiments.

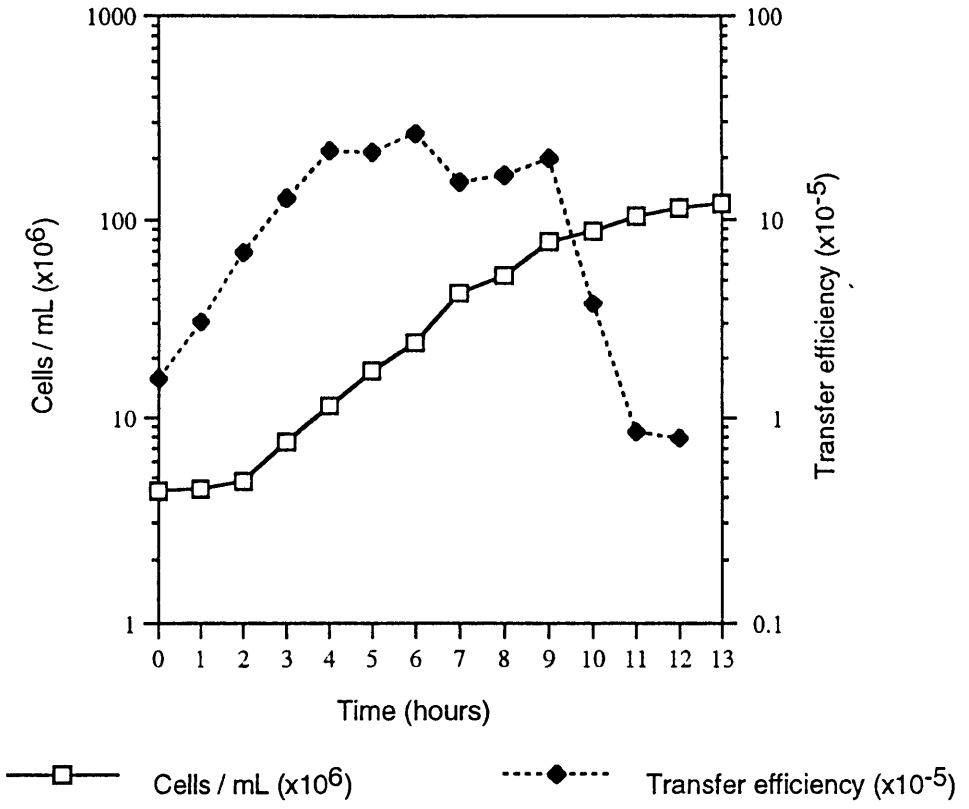


Figure 3.5. Plasmid Transfer to Yeast during Growth

A culture of S150-2B was started from stationary phase cells and allowed to grow in YEPD media until re-entering stationary phase. At 1 hour intervals during growth an aliquot of culture was taken, the cell titre assessed, and the cells concentrated to $2x10^8$ cells/mL. The concentrated cells were subjected to a standard mating with JO8 (pUB307, pAC88) as a donor. Transconjugants were selected for by leucine prototrophy. The results shown are representative of 3 independent experiments.

Mating Type	Strain	Transfer Efficiency
MATa	S150-2B	$3.0x10^{-5}$ (+/-$2.9x10^{-5}$)
MATα	8HB	$3.5x10^{-5}$ (+/-$3.0x10^{-5}$)
	MC16	$2.5x10^{-5}$ (+/-$2.0x10^{-5}$)
	RTY235	$2.4x10^{-5}$ (+/-$1.4x10^{-5}$)
MATa/α	842	$3.5x10^{-7}$ (+/-$2.0x10^{-7}$)
	DS8H	$4.9x10^{-7}$ (+/-$1.8x10^{-7}$)
	DSMC	$3.7x10^{-7}$ (+/-$2.3x10^{-7}$)

Table 3.6. Comparison of transfer efficiency to different yeast strains.

Matings were carried out between JO8(pUB307,pAC88) bacterial donors and the yeast strains detailed in the table. The conditions were as described in the materials and methods. All recipients were auxotrophic for leucine. Transfer efficiency is expressed as LEU^+ transconjugants formed per recipient. The results presented are the average of at least 3 experiments.

the cell cycle. To accomplish this we compared the transfer rate to synchronously growing yeast cells over fixed points of the cell cycle. Synchronous cultures of S150-2B were prepared using α factor, which blocks cells at the G_1/S phase boundary. These synchronously growing cells were mated with *E. coli* donors for 30 minutes at 0, 30, and 60 minutes after the removal of α factor, thereby covering the complete cell cycle from S phase through to the end to the next G_1. The results of these mating are shown in figure 3.6. Transfer can be seen to occur at all points in the cell cycle, there is a slight bias toward the onset of bud formation, however this increase is not significant. Further, it would not be possible to rule out prolonged effects of α factor being responsible for this slight increase. The onset of bud formation is characteristic of the start of S phase in the cell cycle. However, the requirement for the transferred plasmid to be replicated may not be an immediate concern; since studies where single stranded DNA has been transformed into *S. cerevisiae* have demonstrated that ssDNA can still be detected in the cell 18 hours after transformation (Simon and Moore, 1987).

DISCUSSION

This chapter details the successful development, optimisation and characterisation of a protocol for assaying plasmid transfer to yeast, based on the mobilisation of an *E. coli* - yeast shuttle vector. In contrast to previous reports, we were able to detect transfer after relatively short time periods. In fact, transfer can be detected in as little as ten minutes. As a standard we now carry out *E. coli* - yeast matings over a one hour period at a 1:1 donor to recipient ratio. This yields a large number of transconjugants thus allowing the confident comparison of transfer efficiencies. This standard protocol typically produces 3.5×10^{-5} transconjugants per recipient yeast cell. Plasmid transfer to yeast displays all the physical and genetic requirements of authentic bacterial conjugation.

One of the most interesting and debated topics in plasmid biology is that of promiscuity - due mostly to the evolutionary implications of plasmid transfer between distantly related organisms. Guiney (1982) demonstrated that the host range of a plasmid is limited by its replication functions, since then it has become increasingly obvious that many plasmids have a transfer range wider than their associated replication functions. However, this work clearly demonstrates that inherent differences exist in the ability of conjugative plasmids to transfer to diverse organisms, in that transfer to yeast was only detected by the IncPα plasmid RP4. No transfer was detectable by systems based on IncFI and IncI1 plasmids.

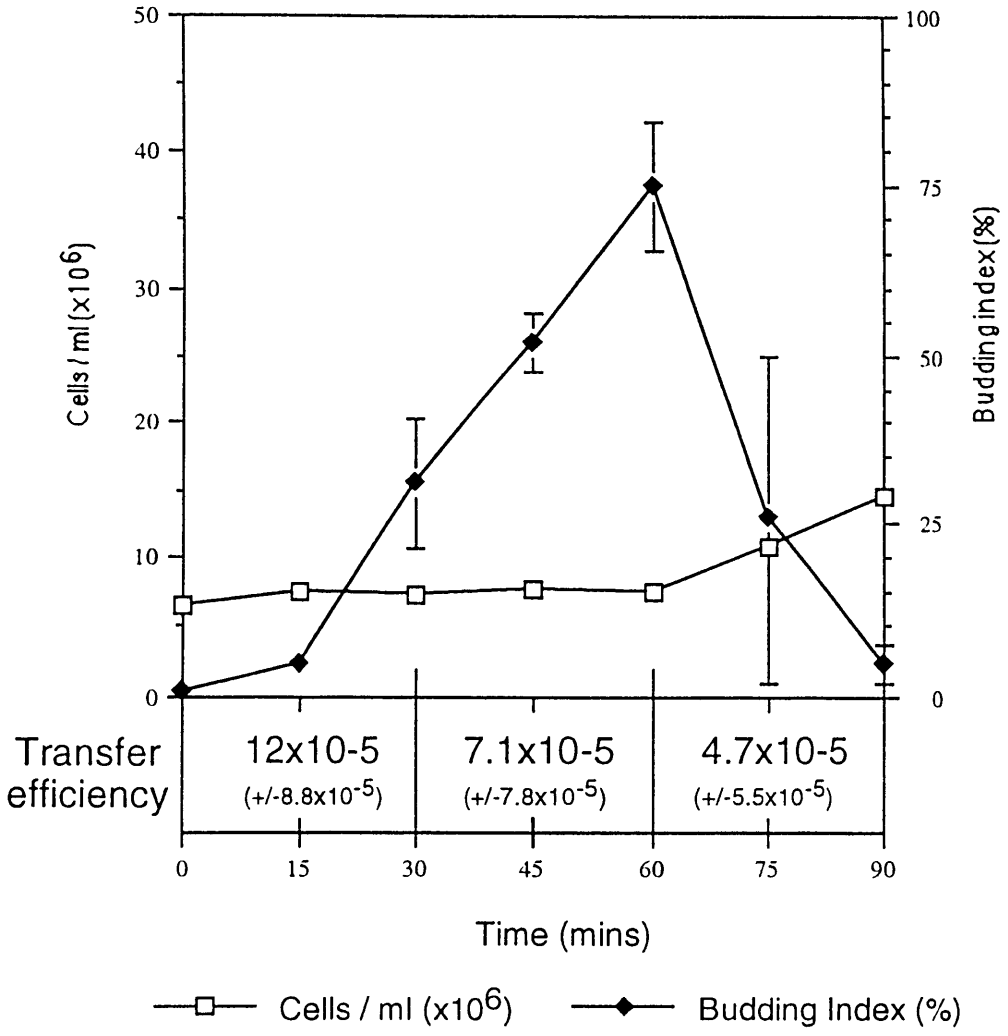


Figure 3.6. Plasmid Transfer to Yeast During Synchronous Growth.

Synchronous cultures of S150-2B were prepared by the addition of α factor to exponentially growing cells as described in Chapter 2. Synchronous growth was monitored by determining the percentage of total cells that are budding (budding index). During synchronous growth matings were performed for 30 minutes at 0, 30, & 60 minutes after the removal of α factor, thereby covering the entire cell cycle. The bacterial donor for matings was JO8(pUB307,pAC88). Transconjugants were selected for by leucine prototrophy. Transfer efficiencies are expressed as transconjugants formed per recipient, results shown are the average of 3 experiments.

The possibility that IncFI and IncI1 plasmids can mediate transfer to yeast cannot be ruled out but these findings suggest that transfer must occur at a very low frequency. In contrast to our observations, Heinemann and Sprague (1989) reported transfer to yeast mediated by the IncFI transfer system, in our work we have been unable to repeat this observation. The most likely reason for this discrepancy is in the choice of plasmid used to provide the F transfer system. In our work the F transfer system was provided by a deletion derivative of F, pOX38, this plasmid retains the F plasmid's replication functions and hence exists at a low copy number in donor cells. Heinemann and Sprague used the plasmid pRS2405 (Ray *et al.*, 1986) which consists of a 41 kb fragment of F containing the transfer functions cloned onto a multi copy pBR322 based replicon. The multi copy nature of this plasmid has been used to explain its higher transfer rate between bacteria, hence it is likely that this plasmid would also demonstrate elevated levels of transfer to yeast cells. As transfer mediated by this plasmid to yeast was very inefficient it is perhaps unsurprising that we failed to detect transfer to yeast in our system. In summary, although there is the potential for different plasmids to mediate transfer to yeast our results would suggest that plasmid promiscuity is not universal and that some plasmids, such as RP4, can truly be described as promiscuous.

Reports on plasmid transfer to yeast have described rearrangements of the transferred DNA in the recipient cell. However, through southern analysis of total DNA prepared from yeast transconjugants and restriction analysis of rescued plasmids we did not detect any rearrangement of plasmid DNA. The rearrangements detected in these earlier studies were probably caused by recombination events in the donor cell. These earlier studies used a recombination proficient donor strain, presumably in these cases recombination occurred between the conjugative plasmid and shuttle vector resulting in the rearrangements seen. Our analysis, and that of Nishikawa *et al.*, (1990) who also failed to detect plasmid rearrangements, used a recombination deficient donor strain. Hence, we can conclude that the transferred plasmid retains its integrity in the recipient yeast cell. An interesting question for future work would be to assess if plasmids rescued from yeast were still capable of being mobilised. If mobilisation was possible it would indicate that the nicked transferred DNA had been precisely religated.

Physiological studies have shown that exponentially growing cells are better suited as recipients in trans-kingdom conjugation, this may reflect the need for active DNA metabolism in the recipient, alternatively changes in the cell wall during

growth may affect the surface interactions between donor and recipient. If the plasmid DNA is delivered to the yeast cell surface, factors affecting the porosity of the yeast cell may also affect transfer. However, if DNA is delivered to the cell surface it must arrive in a form that is insensitive to DNaseI degradation. The fact that transfer occurs independently of the growth state and cell cycle of the recipient indicates that the receptor for mating pair formation must be constitutively expressed on the cell surface of the recipient. As transfer is independent of the stage of the cell cycle of the recipient it appears unlikely that there is an immediate requirement for the transfer plasmid to become replicated. This is perhaps not surprising as it has been shown that circular ssDNA can be detected in yeast up to 18 hours after transformation (Simon & Moore, 1987). Interestingly we observed that transfer to haploid strains was 40 fold more efficient than to diploids. The cell surface of haploid and diploid strains does differ, as apparent from cell morphology, bud site selection, and the presence of pheromone receptors. However, the molecular basis of the bias in transfer to haploids rather than diploids is unclear, obviously either haploid or diploid specific factors must play a role in modulating transfer.

Chapter Four

Genetic Analysis of the RP4 Mediated Promiscuous Transfer to Yeast

INTRODUCTION

The ability of different conjugative plasmids to mediate transfer to yeast was addressed in the last chapter. This analysis demonstrated that the IncPα plasmid RP4 was particularly proficient at mobilising plasmid DNA to yeast. The aim of the work described in this chapter was to elucidate the genetic basis of the promiscuity demonstrated by RP4. The main issue to be addressed was whether RP4 promiscuity was inherent in the plasmid's basic conjugation apparatus, or if ancillary genes outside the core transfer regions are involved. This analysis will use defined mutants in the *tra* genes to determine which Tra proteins are required for transfer to yeast.

RP4 is a small conjugative plasmid (60kb), its transfer genes are organised into two distinct regions Tra1 and Tra2. The genes in the Tra2 region (*trbB-L*) are all involved in the formation of mating pairs (Haase *et al.*, 1995). Of the Tra1 region (*traA-M*), only a core of five genes are required for interspecies *E. coli* matings (*traF,G,I,J,* and *K*); these genes are involved in the processing of plasmid DNA for transfer, except *traF* which is involved in the formation of mating pairs (Lessl *et al.*, 1993). Due to the organisation of RP4, it has been possible to separate the Tra1 and Tra2 regions on different compatible plasmids to form a bipartite plasmid system for transfer (Lessl *et al.*, 1992a); this has greatly facilitated the analysis of the RP4 transfer system.

The work presented in Chapter 3 demonstrated that a conjugative plasmid is required in the donor cell in order to mediate transfer to yeast. However, we have no evidence for the involvement of individual Tra proteins. To determine what transfer functions were required for transfer to yeast, we utilised the bipartite system for RP4 transfer, kindly made available by Dr E. Lanka. Initially this required the construction of new shuttle vectors carrying specific regions of the RP4 transfer system. Our first aim was to determine if both the Tra1 and Tra2 regions were required for transfer to yeast. This would demonstrate the requirement of both the mating pair formation system and the DNA processing enzymes for transfer to yeast. It has been postulated that in

interspecific matings extra plasmid encoded factors may be required, termed 'promiscuity determinants'. As discussed in the general introduction there is evidence that the *traC* and *upf54.4* genes of RP4, located in the Tral region, may be important for interspecific matings to certain *Psuedomonas* species (Krishnapillai, 1988). The *traC* gene encodes a primase that is transferred to the recipient (Merryweather *et al.*, 1986), and therefore may facilitate the establishment of the transferred strand in the recipient. It has also been proposed that other Tral genes, outside the core of five required for *E. coli* matings, may be important for interspecific matings. This hypothesis can be tested by comparing the ability of the Tral and Tral core regions to mobilise plasmid DNA to yeast. The Tra2 region of RP4 contains the genes for mating pair formation, the proteins produced from these genes are responsible for forming the cellular contacts between donor and recipient cells, they may also form the basis of the DNA transport pore. Lanka and co-workers have produced a set of plasmids based on pML123 (which carries the Tra2 region) that contain insertions in each of the *trb* genes (Lessl *et al.*, 1993; Haase *et al.*, 1995). Using these mutant plasmids it was shown that ten Tra2 genes are required for transfer between *E. coli* cells. The work described here utilises these mutants to determine if the same set of genes was also required for transfer to yeast.

RESULTS

4.1 Tra Regions of RP4 Required for Transfer to Yeast.

Two new shuttle vectors, based on YCp50 (Table 2.3, Figure 2.10), were constructed to determine if the whole Tral or just the Tral core is required for transfer to yeast. These shuttle vectors carried either the whole Tral region (pSB12) or the Tral core region (pSB13). pSB12 was constructed by cloning the whole Tral region (*traA-M*) on a 15.6 kb *Bam*HI-*Hin*dIII fragment from pVWDG23110Δ0.1 (Figure 2.9) into the corresponding sites in YCp50. A minimal Tral fragment containing *traF-M* from pDB126 (Figure 2.3) was cloned on a *Bam*HI fragment into the unique *Bam*HI site of YCp50 to construct pSB13 (see Figure 4.1). Restriction analysis confirmed that the orientation of the Tral region in both pSB12 and pSB13 was the same. YCp50 was chosen as the basis for these shuttle vectors due to the large size of pSB12 and pSB13; YCp50 is a centromeric based plasmid and therefore exists at a low copy number in the yeast cell, hence its use should lower the metabolic load of

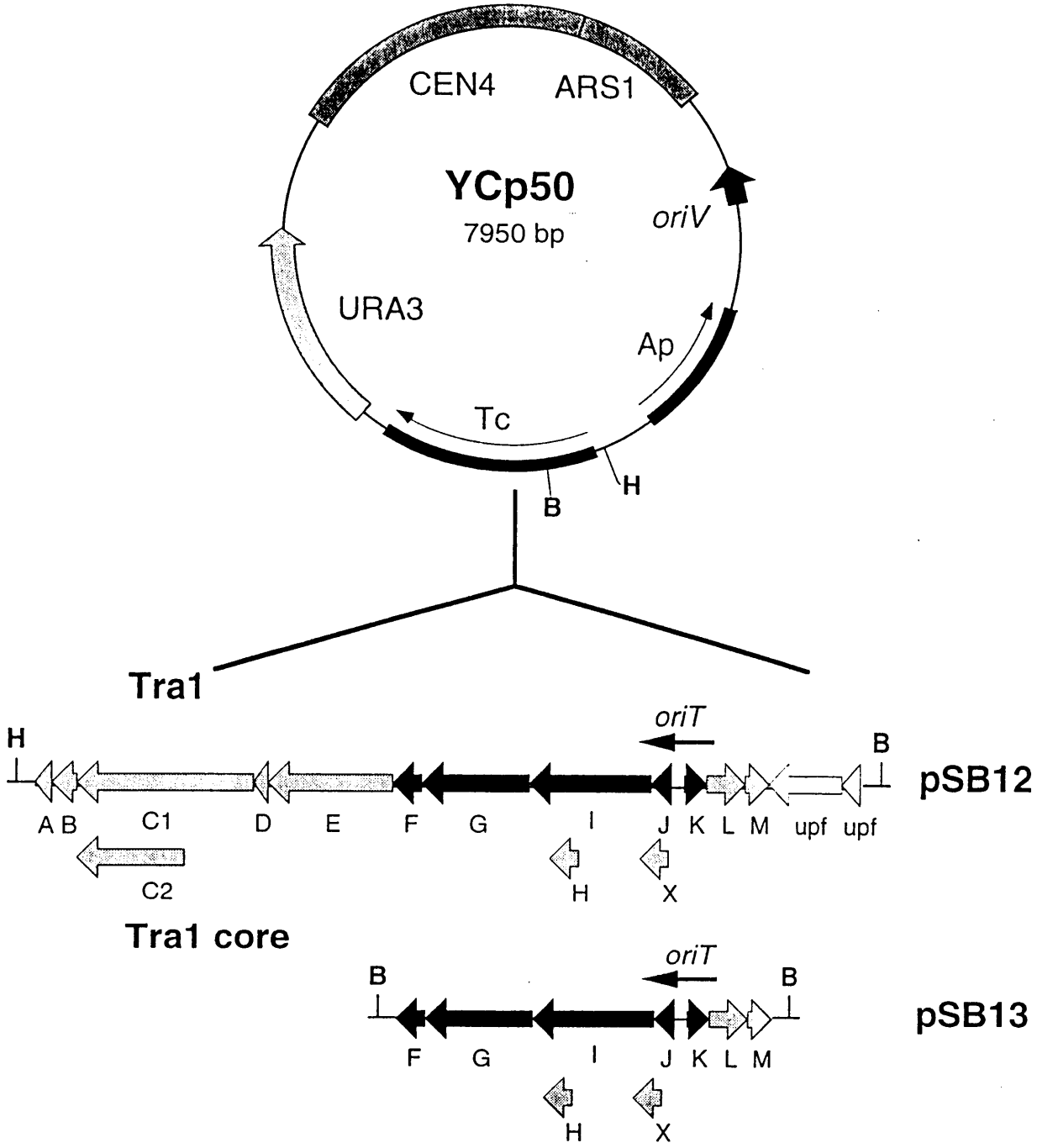


Figure 4.1. Construction of Tra1 and Tra1core containing Shuttle Vectors.

Both vectors are based on the yeast centromeric plasmid YCp50 (Rose *et al.*, 1987; Figure 2.10). pSB12 contains the whole of the Tra1 region (*traA-upf54.8*) cloned on a 15.6kb *Bam*HI/*Hin*dIII fragment from pVWDG23110Δ0.1 (Lessl *et al.*, 1992a; Figure 2.9). The 7.6kb *Bam*HI fragment from pDB126 (Balzer *et al.*, 1994; Figure 2.3), containing the Tra1 core region (*traF-K*) and *traL* and *traM*, was cloned into the unique *Bam*HI site of YCp50 to construct pSB13. The orientation of the insert in pSB13 was confirmed by restriction analysis. Both shuttle vectors can be mobilised between *E. coli* and yeast by providing the RP4 Tra2 functions in *trans* on the compatible plasmid pML123 (Lessl *et al.*, 1993; Figure 2.4). Transfer to yeast can be detected by uracil protrophy. Black boxes indicate genes essential for transfer between *E. coli*; Grey boxes indicate genes dispensable for transfer. Restriction enzymes shown are; B, *Bam*HI; H, *Hin*dIII. pVWDG23110Δ0.1, pDB126, and pML123 were gifts from E. Lanka.

maintaining pSB12 and pSB13. Transfer of both pSB12 and pSB13 to yeast can be selected by uracil prototrophy of a uracil auxotroph. Neither pSB12 or pSB13 could be transferred from *E. coli* to yeast without the Tra2 functions being provided in *trans* in the donor cell. The Tra2 functions were provided in the donor cell on the compatible plasmid pML123 (Figure 2.4; pML123 is a ColD based plasmid and therefore compatible in *E. coli* with the ColE1 based replicon of YCp50). Similarly no transfer to yeast was detectable from a donor cell carrying only the Tra2 functions (Table 4.1). Hence both Tra1 and Tra2 functions are required for transfer to yeast. It can also be seen from Table 4.1 that pSB12 and pSB13 were transferred to yeast at comparable efficiencies. Only the Tra1 core region contained on pSB13, in the presence of Tra2, is therefore required for transfer to yeast. These results indicate that transfer to yeast requires no extra plasmid encoded loci.

4.2 Requirement of Individual Tra2 Genes for Transfer to Yeast.

The Tra2 genes are required for the formation of cellular contacts between donor and recipient cells. Using a set of MURFI linker insertions in the Tra2 genes on pML123 it has been shown that ten *trb* genes are required for transfer between *E. coli* cells (Haase *et al.*, 1995). We wished to determine if the same genes in the Tra2 region were also required for transfer to yeast. The MURFI linker is a 14bp oligonucleotide containing an amber stop codon in each reading frame and a central *Xba*I site. Its insertion into each of the *trb* genes therefore results in a truncated protein being produced, the small size of the linker limits polar effects on the down stream genes (the position of insertion is shown in Figure 4.2A).

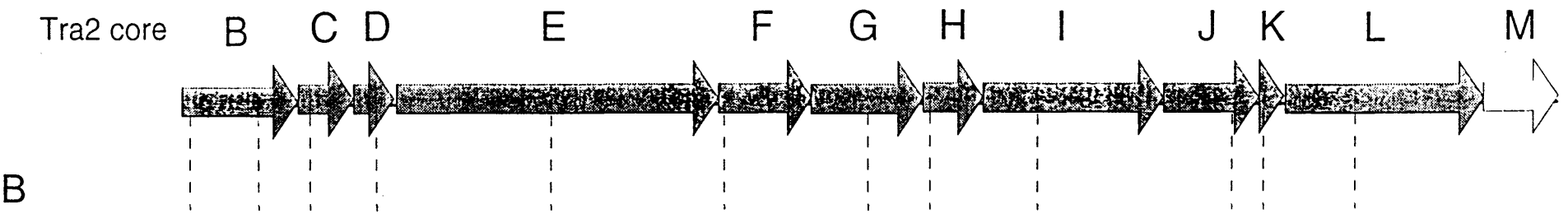
Donors containing pSB12 and the MURFI linker insertion *trb* mutants contained on pML123 were mated with *E. coli* and yeast recipients, to determine the individual *trb* gene requirements for transfer (Table 4.2 & Figure 4.2B). All the pML123*mtrb* mutants were defective for conjugation with yeast, except the *trbK9*, and *trbB204* mutants. In the case of *trbB204* the MURFI insertion lacks the final G residue, which removes the amber codon meant to terminate translation; this results in translation continuing in frame. Hence, *trbB204* contains 322 amino acids instead of 319 in the wild type gene product (a conversion of ^{204}IDVS to ^{204}IASLDYS). However, *trbB204* provides a functional gene product (Haase *et al.*, 1995). The *trbK* gene product is responsible for entry exclusion in bacterial donor cells, the process of

Donor	Transfer Regions	Transfer Efficiency
HB101 (pSB12)	Tra1	$<1.1x10^{-7}$
HB101 (pSB13)	Tra1core	$<1.1x10^{-7}$
HB101 (pML123, pAC88)	Tra2	$<1.1x10^{-7}$
HB101 (pSB12, pML123)	Tra1 / Tra2	$1.5x10^{-5}$ (+/-$0.4x10^{-5}$)
HB101 (pSB13, pML123)	Tra1core / Tra2	$1.5x10^{-5}$ (+/-$0.2x10^{-5}$)

Table 4.1. RP4 Transfer Regions Required for Transfer to Yeast.

E. coli - Yeast matings were carried out for 1 hour at 30°C on YEPD as described in Chapter two (Section 2.10) using S150-2B as the yeast recipient. The Tra regions contained in the bacterial donor cell are detailed. Yeast transconjugants were selected for the marker on the transferred plasmid, this was uracil prototrophy in all cases except HB101(pML123,pAC88) where transconjugants were selected for leucine prototrophy. Transfer efficiencies shown are the average of 3 experiments and are expressed as transconjugants formed per recipient.

A



B

mtrb Gene	B	B	C	D	E	F	G	H	I	J	K	L
Transfer												
E. coli	-	+	-	-	-	-	-	-	-	-	+	-
Yeast	-	+	-	-	-	-	-	-	-	-	+	-

Figure 4.2. Position and Phenotype of MURFI linker insertions into Tra2.

(A) The position of the MURFI linker insertions into each *trb* gene is shown by the dashed line. The MURFI linker is 14bp long with a central *Xba*I site and stop codon in each reading frame (Sequence: dCTAGTCTAGACTAG; the amber stop codons are underlined). MURFI linkers were inserted at unique restriction sites in each *trb* gene. No sites were available for *trbF* or *trbK*, hence suitable sites were created by oligonucleotide directed mutagenesis (Haase *et al.*, 1995).

(B) The phenotype of each mutant for transfer to *E. coli* and yeast is shown (see Table 4.2); + Indicates transconjugant formation; - Indicates no transconjugants were detected.

Donor	Transfer Efficiency	
	E.coli	*Yeast*
HB101 (pSB12, pML123)	$8x10^{-1}$	$1.4x10^{-5}$
HB101 (pSB12, pML123m*trb*B5)	$<1.5x10^{-7}$	$<1.1x10^{-7}$
HB101 (pSB12, pML123m*trb*B204)	$8x10^{-1}$	$9.4x10^{-6}$
HB101 (pSB12, pML123m*trb*C45)	$<1.8x10^{-7}$	$<1.0x10^{-7}$
HB101 (pSB12, pML123m*trb*D45)	$<1.9x10^{-7}$	$<9.5x10^{-8}$
HB101 (pSB12, pML123m*trb*E402)	$<3.0x10^{-7}$	$<1.5x10^{-7}$
HB101 (pSB12, pML123m*trb*F9)	$<1.9x10^{-7}$	$<1.2x10^{-7}$
HB101 (pSB12, pML123m*trb*G145)	$<1.4x10^{-7}$	$<1.2x10^{-7}$
HB101 (pSB12, pML123m*trb*H13)	$<1.4x10^{-7}$	$<1.3x10^{-7}$
HB101 (pSB12, pML123m*trb*I135)	$<1.4x10^{-7}$	$<1.3x10^{-7}$
HB101 (pSB12, pML123m*trb*J180)	$<1.7x10^{-7}$	$<1.0x10^{-7}$
HB101 (pSB12, pML123m*trb*K9)	$7x10^{-1}$	$8.6x10^{-6}$
HB101 (pSB12, pML123m*trb*L184)	$<1.7x10^{-7}$	$<1.2x10^{-7}$

Table 4.2. Tra2 Gene Requirements for Transfer to Yeast.

E. coli matings were carried out at 37°C on nutrient agar for 1 hour. *E. coli* - Yeast matings were performed for 1 hour at 30°C on YEPD media (see Chapter 2, Section 2.10). *E. coli* and yeast recipients were BW97N and S150-2B respectively. *E. coli* transconjugants were selected for ampicillin and nalidixic acid resistance, yeast transconjugants were selected for uracil prototrophy. Transfer efficiencies are expressed as transconjugants per recipient. *mtrbB5-mtrbL*184 indicate MURFI linker insertions into the specified gene, the number corresponds to the last amino acid produced in the truncated protein (Lessl *et al.*, 1993; Haase *et al.*, 1995). The transfer efficiencies shown are the average of 3 experiments.

preventing conjugation between cells harbouring similar plasmids. Hence, it is perhaps unsurprising that TrbKp is not required to potentiate conjugative plasmid transfer. Thus our work has shown that 10 genes in the Tra2 region (*trbB,C,D,E,F,G,H,I,J,* and *L*) are essential for transfer to yeast. Hence, the requirements for transfer to yeast are identical to those for transfer between *E. coli* cells.

The strain HB101 was used as a donor in these experiments which in retrospect was not the best choice as HB101 contains the *SupE44* allele, a suppressor of amber mutation. The *SupE44* allele however does not appear to have affected the results obtained. The *trb* gene products are structural proteins not enzymatic, therefore, a *SupE44* strain may not be able to produce enough protein to be active. Additionally, in 6 of the 11 mutants (*mtrbB5, C45, G145, H13, I135,* and *K9*) insertion of the MURFI linker would result in a frame shift mutation. Therefore, even if translation continued through the stop codon in these 6 mutants no active protein would be produced. In the cases of the 5 remaining mutants readthrough of the stop codon would be in frame and result in the addition of extra amino acids. However, in 3 of these mutants (*mtrbE402, J180,* and *L184*), where readthrough would be in frame, the pertinent stop codon is immediately followed by a pyrimidine which is known to reduce suppression. In the case of the *trbF* mutant, 2 stop codons immediately preceding each other were formed by the insertion of the MURFI linker, hence it seams unlikely that readthrough would occur through both of these codons. In summary, although HB101 may not have been the best choice for a donor in these experiments it does not appear to have affected the results as the mutants clearly fail to transfer plasmid DNA.

4.3 Possible NLS Sequences in Proteins known to Interact with the Transferred Plasmid DNA.

For stable inheritance and expression the transferred shuttle vector must reside in the nucleus of the yeast cell. This raises the question of how the transferred strand is targeted towards the nucleus. The VirD2 and VirE2 proteins of the Ti plasmid of *Agrobacterium* are transferred to plant cells; these proteins contain nuclear localisation signals (NLS) which target them and the transferred DNA strand to the plant cell nucleus (Tinland *et al.,* 1992, Citovsky *et al.,* 1992). VirE2 is a single stranded DNA binding protein whereas VirD2 is the relaxase. Interestingly, the VirD2 protein contains two NLS both of which have been

shown to work in yeast cells (Tinland *et al.*, 1992). VirD2 also shows significant homology to the TraI protein of RP4. Hence, we wished to determine if this homology stretched to the NLS; or if any of the other plasmid factors known to bind to the plasmid DNA contain potential NLS sequences which might potentiate plasmid transfer to yeast. Nuclear localisation sequences are typically short stretches of basic amino acids, usually lysine and arginine. Two consensus sequences for NLS have been proposed, a monoparte and a biparte signal (reviewed in Dingwall and Laskey, 1991), shown below:

Mono-	K-K/R-X-K/R
Bi-	K/R-K/R-$(X)_{10}$-[XXXXX 3/5 K or R]

The region of homology between the TraI and VirD2 does not include the two NLS in VirD2. However, analysis of the sequences of proteins known to interact with the plasmid DNA (Table 4.3) has revealed several possibilities for NLS signals. Potential monopartite signals were identified using the Gene Jockey molecular biology program for Apple Macintosh. However, no programmes were available that could detect a bipartite signal, hence the protein sequences were searched by eye to identify any potential bipartite signals. Although the homology between VirD2 and TraIp does not include the NLSs, TraI does contain a possible NLS. TraK contains one of the best matches with two monoparte signals overlapping each other (sequence ^{19}-KKRRR). The TraK protein binds to the plasmid *oriT* region over approximately 200bp in the leading region of transfer (Ziegelin *et al.*, 1992). It is, at present, unknown if TraI or TraK are transferred to the recipient cell during mating, but if they are they could be directed to the nucleus. The other notable possible NLS found was contained in the host single-stranded binding protein, which may bind to the transferred strand as it is transferred. The RP4 TraC protein contains two possible matches to the monoparte NLS, however, we have shown that this protein is not required for transfer to yeast. Interestingly no possible NLS were found on proteins encoded by the F plasmid, additionally, of the ColIb proteins, *nicB*, alone carried a single possible monoparte signal. The lack of NLS on F and ColIb proteins may help to explain why RP4 is more proficient at transferring to yeast.

Source	Gene	Function	Possible NLS
RP4	*traC*	Primase	48-KRyK 683-KKhR
	traF	Mpf	None
	traG	DNA transport	15-KKpK
	traH	Stabilise Relaxosome	None
	traI	Relaxase	303-RRRRdRlieaamRsnRlRR
	traJ	*oriT* recognition	None
	traK	Binds *oriT*	19-KKRRR 19-KKRRR
	traL	Unknown	None
	traM	Unknown	None
	ssb	Single stranded binding protein	None
F	*traD*	DNA transport	None
	traI	Relaxase	None
	traM	Mating signal	None
	traY	*oriT* recognition	None
	ssb	Single stranded binding protein	None
ColIb	*nicA*	*oriT* recognition	None
	nicB	Relaxase	8-KKRR
	ssb	Single stranded binding protein	None
	sog	Primase	None
Ti	*VirD2**	Relaxase	23-RKgR 416-KRpRedddgepseRKReR
	VirE2†	Single stranded binding protein	228-KlRpedRyiqteKygRR 296-KtKygsdteiKiKsK
Host	*ssb*	Single stranded binding protein	73-RKgsqvyiegqlRtRKw
	IHFα	Integration Host Factor (α subunit)	None
	IHFβ	Integration Host Factor (β subunit)	None

Table 4.3. Potential Nuclear Localisation Signals in Proteins Interacting with the Transferred Strand.

Basic residues are shown in uppercase, residues matching the consensus are underlined.

* The NLS sequences in *VirD2* have been shown experimentally to direct proteins to the nucleus of both plant and yeast cells.

† The atypical *VirE2* NLS sequences are known to direct proteins to the plant cell nucleus.

DISCUSSION

The phenomenon of plasmid promiscuity leads to the question of specialisation, do specific factors act as promiscuity determinants or is promiscuity inherent in the plasmid's transfer system? We have investigated these questions using derivatives of RP4. The particular aim was to determine if the whole Tra1 region, including the plasmid primase gene(*traC*), or just the Tra1 core region was required for mediating plasmid transfer to yeast. We have shown that the Tra1 core, in the presence of the Tra2 region, is sufficient for transfer from *E. coli* to yeast. Hence no additional plasmid encoded factors are required for promiscuous transfer. This also implies that the *traC* product is not required for priming on the transferred strand in the yeast recipient, hence, replication of the immigrant plasmid is reliant on proteins in the recipient. TraLp is not required for transfer in *E. coli*, whereas TraMp enhances transfer efficiency by 300 fold (Lessl *et al.*, 1993). It would be interesting to determine the effect of removing both *traL* and *traM* from our minimal Tra1 vector to determine if these genes play a role in mediating transfer to yeast. Further work on analysing the Tra1 requirements for transfer to yeast could also include an analysis of the importance of *traH*, which shows a modest effect on transfer efficiencies between bacterial species (Cole *et al.*, 1993).

The specificity of cellular contacts between conjugating cells must come into question when investigating promiscuous transfer. The RP4 Tra2 genes are involved in the formation of mating pairs. We have investigated the requirement of each of the individual Tra2 genes for transfer from *E. coli* to yeast. This investigation has demonstrated that 10 genes (*trbB,C,D,E,F,G,H,I,J* and *L*) are essential for transfer to yeast, the same set of genes that are required for transfer between *E. coli* cells (Haase *et al.*, 1995). A recent study determined the Tra2 requirements for transfer between *E. coli* and *Streptomyces lividans*, the requirements were identical as for transfer between *E. coli* except the product of the *trbF* gene was not required (Giebelhaus *et al.*, 1996). In our work on transfer to yeast we found *trbF* to be essential, however, mutations in this gene may have reduced transfer to below that detectable in our mating protocol. The nature of cellular contacts then remains an intriguing question, it is tempting to believe that contacts must be non specific. If so, the function of the Tra2 proteins may lie in the formation of the DNA transport pore and not the production of cellular contacts.

One of the most intriguing questions on plasmid transfer from *E. coli* to yeast is how the transferred DNA is transferred to the yeast cell nucleus, is it a passive process occurring by diffusion, or is it active requiring host or plasmid encoded factors? In Ti mediated transfer to plant cells' pilot proteins are transferred from the *Agrobacterium* donor to the plant cell, these proteins direct the DNA to the plant cell nucleus through nuclear localisation signals (NLS) in their amino acid sequence (Tinland *et al.*, 1992; Citovsky *et al.*, 1992). These same factors are also required for Ti plasmid mediated transfer to yeast (Bundock *et al.*, 1995). Several potential NLS sequences were identified in the amino acid sequence of proteins known to interact with the transferred DNA strand - of particular interest the RP4 relaxase TraIp contains a potential biparte signal. Classical models of conjugation place TraIp relaxase at the cellular junction between donor and recipient cell, however, there is no proof for this localisation. As the Ti plasmid relaxase is transferred to the plant cell, and given the homology between these systems, it is not unreasonable to postulate that the TraIp relaxase is also transferred to the recipient cell. If TraIp was transferred to the recipient, it may therefore target the transferred strand to the yeast nucleus. Interestingly, no potential NLS sequences were found in the ColIb or F plasmid encoded proteins associated with the plasmid DNA. Hence the fortuitous presence of a NLS sequence in TraIp may help to explain RP4 plasmid promiscuity to yeast.

Chapter Five

Isolation and Characterisation of Yeast Mutants with Altered Ability to Receive a Plasmid by Conjugation

INTRODUCTION

The work reported in the previous two chapters has concentrated on the bacterial factors required for plasmid transfer to yeast, and the physiological factors that affect the ability of yeast to receive a plasmid. The fact that plasmid transfer to yeast occurs raises many questions concerning both the surface interactions between bacteria and yeast, and the fate of the transferred strand. To address these questions we have used the classical approach of a mutant hunt in an attempt to identify yeast genes important for transfer to occur. Candidate genes liable to be identified could be involved in either the cellular contacts between *E. coli* and yeast, or the establishment of the transferred DNA strand in the yeast recipient. Factors affecting the surface interactions between mating cells would presumably be caused by changes in the yeast cell wall or plasma membrane. Establishment of the transferred strand in the recipient requires the passage of the single stranded DNA to the yeast nucleus and its replication. Hence, genes involved in DNA metabolism and movement may also be identified by this study.

Mutant isolation and characterisation in yeast is a relatively straight forward process due to the ease of genetic manipulation in yeast. We chose to use UV mutagenesis to produce our mutants as this approach had been used successfully in other projects in the laboratory. Once mutants have been isolated genetic analysis can be carried out to determine if the phenotype is dominant or recessive and whether a mutation in a single gene is responsible for the phenotype.

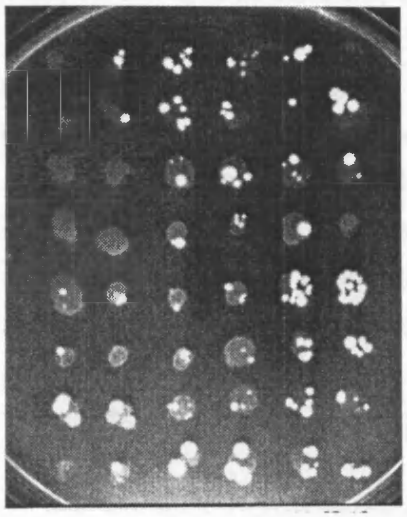
The work detailed in this chapter deals with the development of a plate screen to assay for transfer to yeast, the subsequent isolation of yeast mutants, and their genetic analysis.

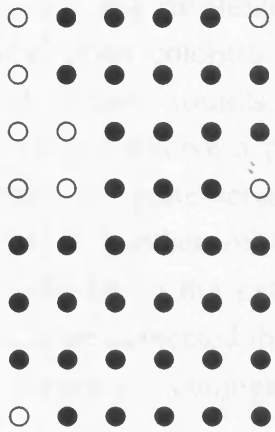
RESULTS

5.1 UV Mutagenesis and Isolation of Yeast Mutants.

UV mutagenesis was carried out on the recipient strain S150-2B as outlined in Chapter 2 (Section 2.9). $3.6x10^7$ S150-2B yeast cells were subjected to a UV dose of $90Jm^{-1}$ and plated out onto complete media (YEPD) and incubated at 30°C for 3 days to allow colonies to form. A sample of cells was also taken, diluted, and plated onto YEPD before UV irradiation in order for the viability of the cells after exposure to be calculated. The dose of $90Jm^{-1}$ used resulted in a 51% viability of the yeast cells, and has previously been successfully used in our laboratory with the strain S150-2B to identify yeast mutants (Duffy, 1996).

Successful plasmid transfer to yeast occurs infrequently ($3.0x10^{-5}$ transconjugants formed per recipient in one hour), hence, it was not possible to directly select for yeast cells that do not receive a plasmid by conjugation. Therefore, a plate screen was developed to assay for plasmid transfer to yeast from individual colonies arising after mutagenesis. The plate screen was based on the fact that transfer can occur directly on media selective for yeast transconjugants. The screen was carried out by preseeding plates selective for yeast transconjugants with bacterial donors carrying the RP4 mobilisable shuttle vector pAC88 (Figure 2.2) and the conjugative plasmid pUB307 (Table 2.3). Hence, selection for yeast transconjugants was conferred by leucine prototrophy. Yeast colonies from the UV irradiated population were picked into microtitre plates and spotted onto the preseeded plate, and a non selective minimal media plate for later reference. Minimal media was used for the reference plate as this allowed the screening out of yeast isolates containing mutations in additional auxotrophic markers or other genes that affect the ability of yeast to grow on minimal media. The plates were then incubated at 30°C for 5 days to allow transfer to occur and subsequent growth of transconjugants. Unfortunately, it was not possible to replace this method with a replica plating strategy due to the requirement of the plates to be damp from plating donors when the yeast cells were spotted. Where the yeast cells were spotted some background growth was apparent, probably resulting from nutrients provided by lysed bacterial donors. However, where plasmid transfer from *E. coli* to yeast has occurred yeast transconjugant colonies can be detected (see Figure 5.1). Yeast mutants unable to receive a plasmid by conjugation can therefore be identified as they do not form these transconjugant colonies. The





● Transfer - Wild Type

○ No Transfer - Mutant

Figure 5.1. Plate Screen To Isolate Yeast Mutants.

Mutagenesied yeast colonies were picked into 30µls of distilled water in microtitre plates. These cells were then spotted onto minimal media selective for leucine prototrophs to select for yeast transconjugants. The selective plates had previously been preseeded with 0.2ml of bacterial donors [JO8(pUB307,pAC88 A_{600}=0.5]. Yeast cells were also spotted onto nonselective media for later reference. The plates were incubated at 30°C for up to 5 days to allow transfer to occur and subsequent growth of transconjugant yeast cells. Where the yeast cells were spotted some background growth is apparent, however, where transfer occurs transconjugant colonies appear. Hence mutants unable to receive a plasmid by conjugation can be detected because these fail to produce transconjugant colonies. An example of the results obtained with this screen is shown above, the key illustrates the position of possible mutants.

reliability of this procedure to identify mutants was determined by using wild type cells in the screen and calculating the number of false negatives produced. The screen was found to be relatively reproducible and misclassified wild type strains as mutants at a rate of 20%. In total 2646 colonies from the UV irradiated population were picked and screened. After 4 rounds of rescreening 5 possible mutant strains were isolated that did not receive a plasmid in our plate screen, termed *con1-5*. Additionally from our plate screen one mutant was isolated that consistently produced a larger number of transconjugant colonies compared to the wild type strain S150-2B to the extent of almost confluent growth of the yeast cells. It was therefore suspected that this mutant, termed *exg1*, was better able to receive and maintain a conjugatively derived plasmid.

Standard *E. coli* yeast matings (Chapter 2, Section 2.10) were carried out between the donor bacterium JO8(pUB307,pAC88) and the potential yeast mutants to determine their phenotype (Table 5.1). None of the mutant strains isolated show a complete inability to receive a plasmid by conjugation. The mutants *con1* and *con4* demonstrated the most severe phenotype with a 35 fold reduction in their ability to receive a plasmid (from an average of four experiments). The other three mutants, *con2,3 and 5*, exhibited milder phenotypes ranging from a 5 to 8 fold reduction in transfer efficiency. Mutant *exg1* displayed an increase in transfer efficiency of 4 fold. The three mutants *con1*, *con4* and *exg1* were selected for further analysis due to their phenotypes. The actual transfer efficiencies obtained with these mutants are shown in Table 5.2.

A decrease in transfer efficiency could be caused by loss of the native 2μm plasmid from yeast, as our shuttle vectors require the 2μm for stable maintenance. The presence of the 2μm plasmid in our mutants was confirmed by carrying out colony PCR with primers specific for 2μm DNA (Figure 5.2). The primers were designed to amplify part of the 2μm B gene not present on our shuttle vector. Hence any PCR product obtained would indicate the presence of the native 2μm plasmid in our mutant strains. All the mutant strains were found to contain the 2μm plasmid, hence the mutant phenotype cannot be attributed to the lack of maintenance of the transferred plasmid.

Recipient	Transfer Phenotype
Wild type	100%
con1	3% (+/-1%)
con2	14% (+/-8%)
con3	13% (+/-9%)
con4	3% (+/-2%)
con5	20% (+/-1%)
exg1	357% (+/-1%)

Table 5.1. Phenotype of Possible Mutants.

Standard matings were carried out using the *E. coli* strain JO8(pUB307,pAC88) as a donor to the recipients detailed. Yeast transconjugants were selected for leucine prototrophy. The transfer phenotype is expressed relative to the wild type parent strain S150-2B. The results shown are the average of at least 3 matings.

Recipient	Transfer Efficiency (Transconjugants/Recipient)	Relative Phenotype
Wild Type	$1.5x10^{-5}$ (+/- $8.3x10^{-7}$)	100%
con1	$4.1x10^{-7}$ (+/- $7.8x10^{-8}$)	3% (+/-1%)
Wild Type	$4.2x10^{-5}$ (+/- $1.6x10^{-5}$)	100%
con4	$1.2x10^{-6}$ (+/- $7.9x10^{-7}$)	3% (+/-2%)
Wild Type	$2.6x10^{-5}$ (+/- $9.8x10^{-6}$)	100%
exg1	$9.3x10^{-5}$ (+/- $1.4x10^{-7}$)	357% (+/-1%)

Table 5.2. Transfer Phenotypes of Mutants *con1*, *con4*, and *exg1*.

Standard mating were performed using JO8(pUB307,pAC88) as a donor with the wild type strain S150-2B and the mutant isolates detailed. Yeast transconjugants were selected for leucine prototrophy, efficiencies are expressed as transconjugants formed per recipient. The phenotype relative to the wild type strain S150-2B is also shown. The data presented is the average of 4 experiments.

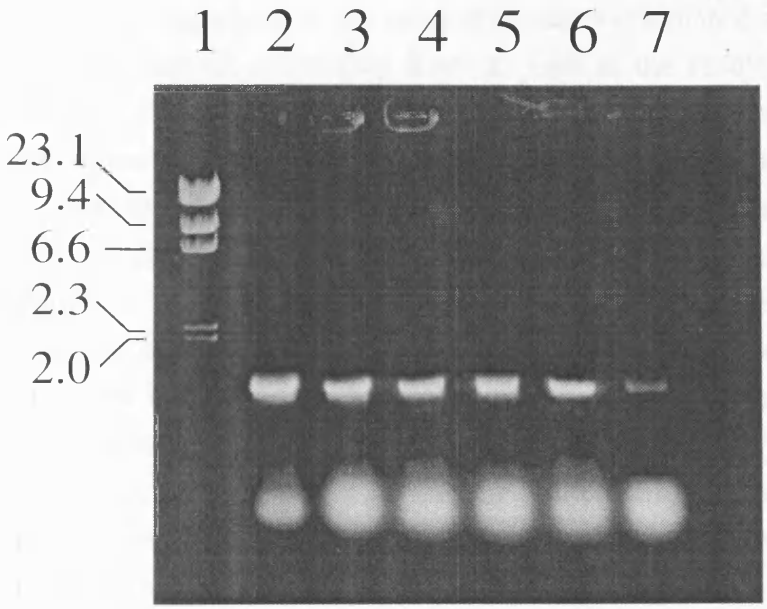


Figure 5.2. Colony PCR On Yeast Mutants To Determine The Presence Of The 2µm Plasmid.

Colony PCR was carried out as outlined in Chapter 2, using primers specific to the 2µm plasmid. These primers (*bak2* and *bak3*) should amplify a 1.2 kb fragment of the 2µm plasmid containing the B gene not present on our shuttle vector. Lane 1 contains λ /*Hin*dIII markers, lanes 3-7 contain the amplified product from *con1-5* mutant strains respectively. All mutants contain the 2µm plasmid.

5.2 Genetic Analysis of Yeast Mutants.

To test for dominance the mutant strains were mated with either 8HB or MC16, and the transfer efficiency from *E. coli* to the resulting heterozygous diploids (termed DCon1, DCon4, & DExg1) determined. The results of these matings are shown in Table 5.3. Interpretation of these results is difficult as transfer to a homozygous wild type diploid strain is itself inefficient ($\sim 4 \times 10^{-7}$ transconjugants per recipient formed in one hour; See Chapter 3 Section 3.5). However, it is clear that both *con1* and *con4* are recessive as the heterozygous diploids display a transfer frequency comparable to a wild type diploid strain, although DCon1 actually exhibits a slight increase in transfer efficiency. The *exg1* mutant exhibits dominant effects as the heterozygous diploid exhibits a 9 fold increase in transfer efficiency. Interestingly this phenotype appears more pronounced in the diploid state than in the haploid which only shows a 4 fold increase in transfer.

To determine if the phenotype observed in the mutant strains was due to a mutation in a single gene, tetrad analysis was carried out. The heterozygous diploids formed between the mutant and wild type strains were sporulated and the resulting asci dissected (Chapter 2, Section 2.35 & 2.37). The haploid progeny were scored for auxotrophic markers, mating type, and their transfer phenotype determined in standard matings. Auxotrophic markers were scored by patching each spore onto selective media; mating type was determined by analysing diploid formation with strains C1 and C2. A total of 10 tetrads were analysed for each of the three mutants. In all cases a 2:2 segregation of wild type to mutant was observed (see appendix 1). Hence it can be concluded that the mutant phenotype, in each case, is the result of a mutation in a single gene.

For DCon1 random spore analysis was also carried out, a total 96 haploid spores were isolated (as outlined in Chapter 2 Section 2.37). The phenotype of these haploid spores was then determined by carrying out our plate screen for transfer. The screen was carried out in triplicate to avoid the problem of false negatives. From these 96 spores a ratio of 49:47 wild type to mutant was obtained; clearly a 2:2 ratio and therefore further evidence of a single gene being involved in the phenotype of *con1*. All the haploid spores used in this analysis carried the *ade2* mutation, therefore, as we obtained a 2:2 segregation pattern for the mutant phenotype we can also conclude that *con1* is not linked to *ade2*.

Strain	Transfer Phenotype	Phenotype
DS8H	$6.0x10^{-7}$ (+/- $5.2x10^{-7}$)	-
DCon1	$1.8x10^{-6}$ (+/- $1.7x10^{-6}$)	Recessive
DSMC	$4.9x10^{-7}$ (+/- $1.8x10^{-7}$)	-
DCon4	$3.6x10^{-7}$ (+/- $1.7x10^{-7}$)	Recessive
DSMC	$4.9x10^{-7}$ (+/- $1.8x10^{-7}$)	-
DExg1	$4.4x10^{-6}$ (+/- $1.8x10^{-6}$)	Dominant

Table 5.3. Phenotype of Heterozygous Diploids.

Heterozygous diploids were formed from matings performed between the *con1* isolate and strain 8HB (DCon1), and the *con4* or *exg1* isolates with MC16 (Dcon4 & DExg1). *E. coli* yeast matings were carried out as standard using the *E. coli* donor JO8(pUB307,pAC88); yeast transconjugants were selected for leucine prototrophy, efficiencies are expressed as transconjugants formed per recipient. The results shown are the average of 5 matings for DS8H & DCon1, and 3 matings for DSMC, DCon4, & DExg1.

To determine if the mutant alleles *con1* and *con4* represented mutations in the same gene complementation group analysis was carried out. Mutant *exg1* was not investigated in this analysis as it had been shown to be dominant. Haploid strains displaying the mutant phenotype, identified through tetrad analysis of heterozygous diploids, were mated with the original mutant isolates of *con1* and *con4* in all pairwise combinations to form diploids. The transfer phenotype to these diploid strains was then determined in standard matings (Table 5.4). All heterozygous diploids formed between mutant and wild type strains exhibited a level of transfer comparable to the homozygous wild type diploid, confirming that the mutations are recessive. It was not possible to detect any transfer to the homozygous mutant diploids indicating that the phenotype of these mutants in a diploid background lowers transfer to below that detectable in our screen. The diploid formed carrying both *con1* and *con4*, however, was able to receive a plasmid by conjugation at wild type levels, indicating that complementation had occurred between the two mutant alleles. Hence *con1* and *con4* are in different complementation groups, and are therefore most likely mutations in different genes.

5.3 Phenotypes of *con1*, *con4*, and *exg1*.

To further characterise our mutants, and in the hope of identifying additional phenotypes to facilitate cloning of the wild type genes, a variety of phenotypic tests were carried out on the mutants. Growth of the mutant and wild type strains in complete media was not affected as they displayed comparable growth rates. Comparison of colony size of mutant and wild type strains after three days growth at 30°C on complete (YEPD) and minimal media also indicated that growth is not affected in the mutants. Qualitative experiments were also carried out to compare growth of the mutant strains at different temperatures. Mutant and wild type cells were spotted onto complete media (10^3 cells in 10µl), and the amount of growth at 24, 30, 37, and 42°C scored after three and five days growth. In all cases the mutants displayed a phenotype comparable to wild type cells.

As mentioned in the introduction to this chapter, a possible target of our screen would be mutations affecting the surface of the yeast cell. Many cell wall mutants isolated display an increased sensitivity to Calcoflour white, or high salt concentrations (Ray *et al.*, 1994). Known concentrations of mutant and wild type cells (from $5x10^4$ to $5x10^1$) were spotted onto YEPD media

	Transfer Efficiency (Transconjugants/Recipient)		
	Wild Type (S150-2B)	*con1*	*con4*
Wild Type (MC16)	4.9×10^{-7} ($+/- 1.8 \times 10^{-7}$)	1.1×10^{-6} ($+/- 2.8 \times 10^{-7}$)	3.6×10^{-7} ($+/- 1.7 \times 10^{-7}$)
con1 (H1)	-	$<1.0 \times 10^{-7}$	1.3×10^{-6} ($+/- 8.7 \times 10^{-7}$)
con4 (B4)	-	-	$<1.1 \times 10^{-7}$

Table 5.4. Complementation Group Analysis of *con1* and *con4*.

Diploids were formed from the haploid strains detailed in the checker box; H1 and B4 were derived from tetrad analysis on the original mutant strains (see appendix 1). *E. coli* yeast matings were carried out, under standard conditions, with the resulting diploid strains using JO8(pUB307,pAC88) as the bacterial donor. Yeast transconjugants were selected for leucine auxotrophy; the efficiencies shown are expressed as transconjugants per recipient, and are the average of 3 matings.

containing various concentrations of calcoflour white. Growth on these plates was then scored after 3 days incubation at 30°C (Figure 5.3). Mutants *con1* and *con4* were unable to form colonies when $5x10^2$ cells were spotted onto YEPD containing calcoflour white at 50μg/ml. However, this phenotype is very slight when compared to the sensitivity of wild type strains. As this phenotype was so slight it was not determined if it co-segregated with the altered transfer efficiency from *E. coli* to yeast apparent in these strains.

The effect of high salt concentration on growth of our mutants was determined in a similar manner used to determine the affect of calcoflour white. Concentrations of NaCl ranging from 0.5M to 1.0M were tested. However, none of the mutants displayed an altered sensitivity to salt concentration. The mutants were also tested for their ability to grow in various SDS concentrations. SDS as a detergent would denature proteins present in the cell wall. Both mutants *con1* and *con4* were able to grow in 0.02% SDS, where as wild type cells were not (Figure 5.4). However this ability to grow in SDS, although exhibiting a 2:2 ratio from tetrad analysis of the *con4* isolate, did not co-segregate with the *con4* mutant phenotype (see appendix 1). It was not determined if this phenotype co-segregated with mutant *con1* as the products of tetrad analysis were no longer available.

Another common phenotype of cell wall mutants is sensitivity to cell wall degrading enzymes, such as zymolyase. Zymolyase breaks down the β-(1,3)-glucan in the cell wall; eventually leading to cell lysis which can be followed by monitoring the drop in A_{600} on a spectrophotometer (see Chapter 2, Section 2.3.7). The zymolyase sensitivity of our mutants was compared to the wild type strain S150-2B (Figure 5.5). It is clear that *con1* mutant cells are more susceptible to cell wall degradation by zymolyase than cells from a wild type strain. From the products of 4 tetrads analysed this sensitivity appears to co-segregate with the mutant phenotype (see appendix 1). None of the other mutants displayed any increased sensitivity to zymolyase. We therefore have evidence that a *con1* mutant has altered cell surface properties.

DISCUSSION

The development of a plate screen to assay for plasmid transfer from *E. coli* to yeast has allowed the identification of several UV induced yeast mutants with an altered ability to receive and establish a plasmid by conjugation. These

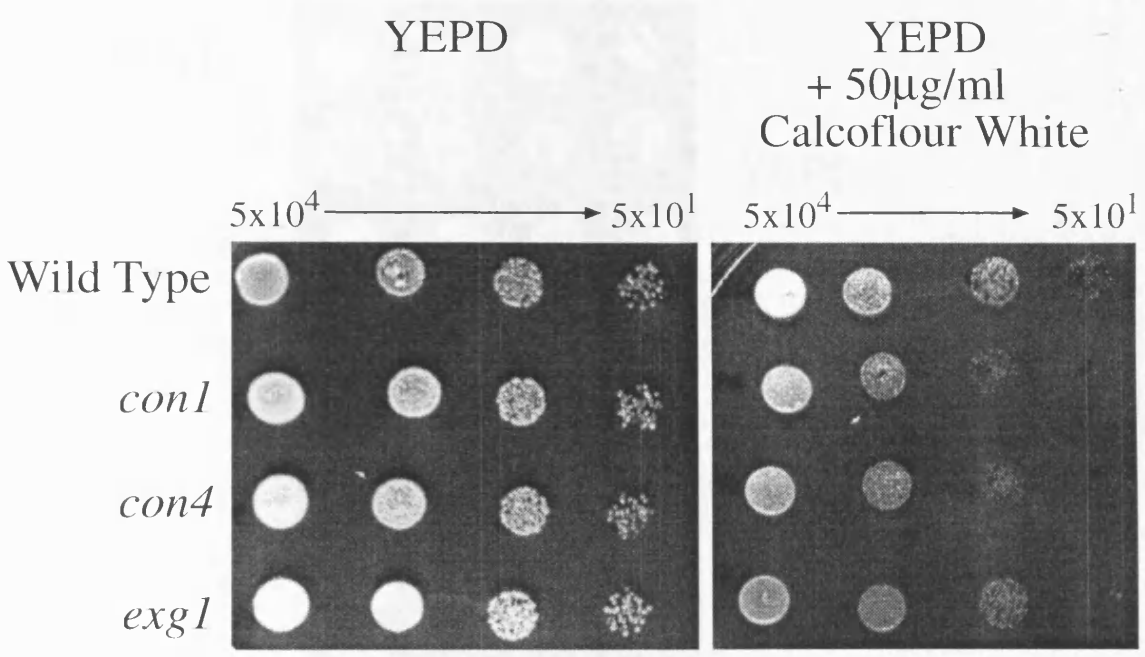


Figure 5.3. Calcoflour White Sensitivity of *con1,4*, and *exg1*.

A dilution series of yeast (containing from $5x10^4$ to $5x10^1$ cells) was spotted onto complete media containing calcoflour white at various concentrations. Calcoflour was made fresh as a stock solution of 1% w/v in distilled water. Plates were then incubated at 30°C and the growth after 3 days scored.

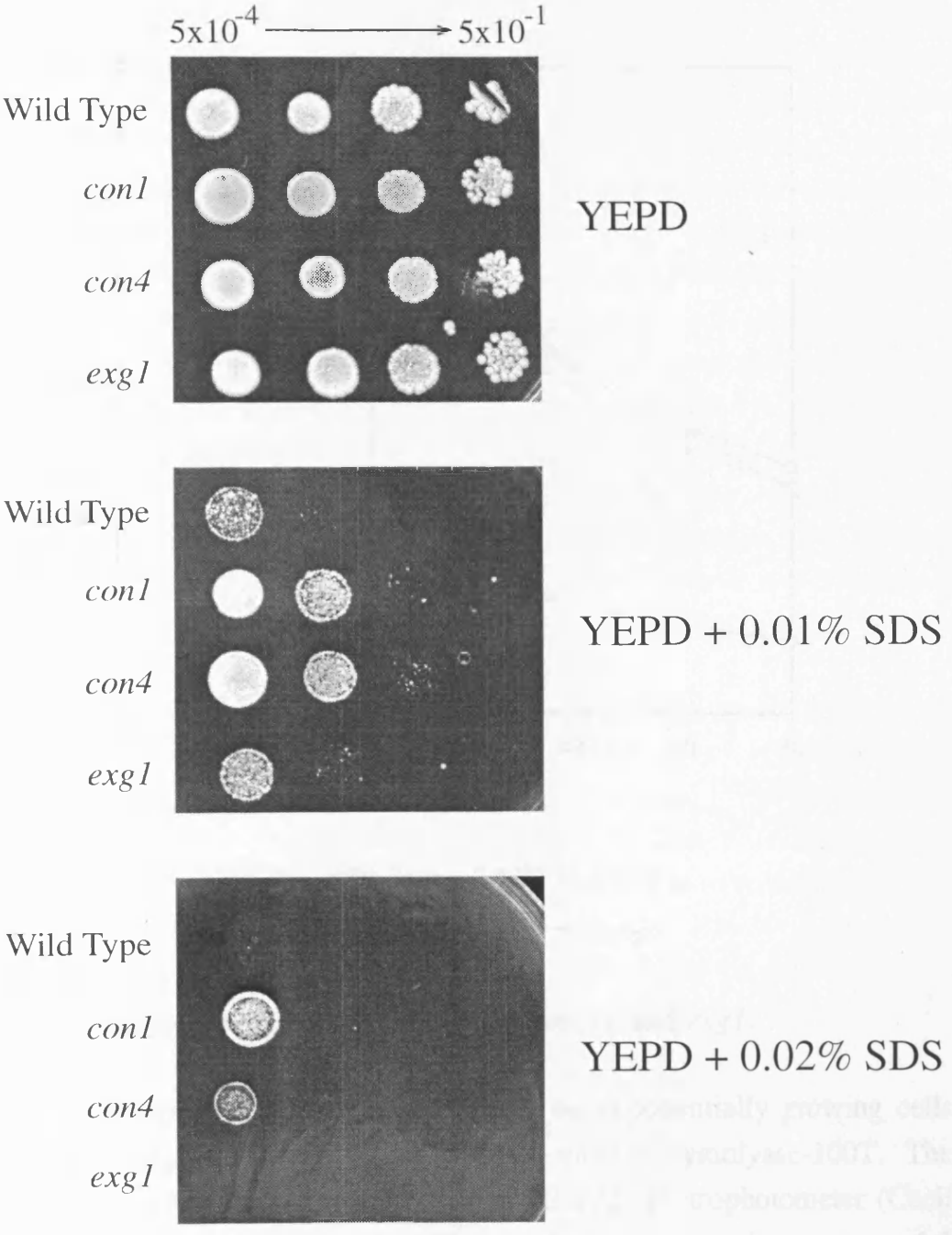


Figure 5.4. SDS Sensitivity of *con1,4*, and *exg1*.

A Dilution series of yeast (containing $5x10^4$ to $5x10^1$ cells) was spotted onto YEPD media containing SDS at various concentrations. Plates were then scored for growth after 3 days of incubation at 30°C.

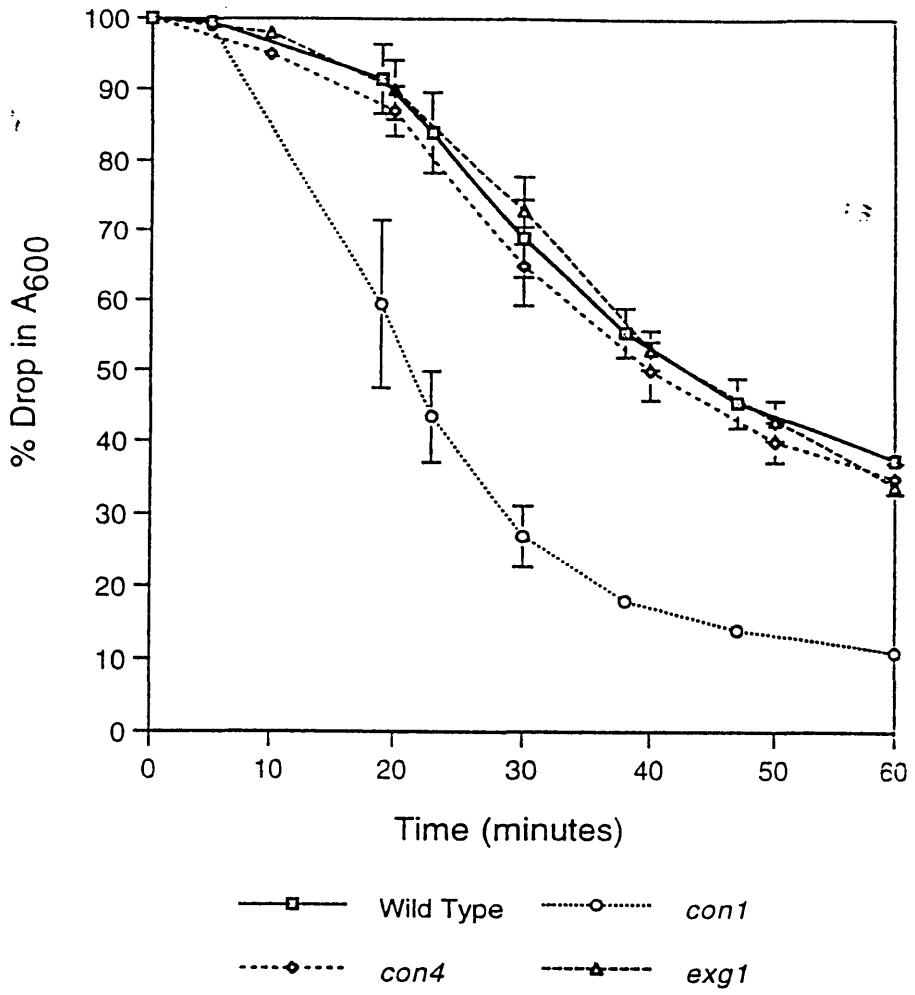


Figure 5.5. Zymolyase Sensitivity Assay for *con1,4*, and *exg1*.

Zymolyase sensitivity assays were carried out on exponentially growing cells as detailed in Chapter 2 (Section 2.38), using 5 units of zymolyase-100T. The drop in A_{600} as cells lyse was followed in a CE 272 spectrophotometer (Cecil Instruments) at 10 minute intervals. The results shown are the average of 3 experiments.

mutants could be affected in either the cellular interactions between conjugating cells or in the localisation and establishment of the transferred DNA strand. Our aim was to identify yeast mutants that were unable to receive a plasmid by conjugation. No mutants were isolated that were totally unable to receive a plasmid. Five mutants were isolated that displayed a reduction in their ability to receive a plasmid by conjugation. Their phenotype ranged from a mild 5 fold reduction in transfer efficiency to a 35 fold reduction compared to the parental wild type strain. Two of these mutants *con1* and *con4* where chosen for further study as they demonstrated the most severe phenotype, a 35 fold reduction compared to wild type. Genetic analysis has shown these mutants to be recessive and the defect caused by a mutation in a single gene. Complementation group analysis has demonstrated that the *con1* and *con4* phenotype is probably the result of a mutation in different genes. The fact that the mutations are recessive should allow the cloning of their respective wild type alleles by complementation with a yeast genomic library.

Whilst screening for mutants unable to receive a plasmid by conjugation we also isolated one mutant, *exg1*, that displayed an increase in transfer efficiency of 4 fold. A single gene was implicated in the *exg1* mutant phenotype, and this allele was also found to be dominant. Intriguingly, the *exg1* phenotype is more pronounced in a heterozygous diploid, a 9 fold increase as opposed to a 4 fold increase in a haploid strain, perhaps implying that *EXG1* is regulated differently in haploid and diploid cells or that its role is altered. However, due to the dominant nature of *exg1* and its modest phenotype it may prove difficult to isolate the wild type allele.

Potentially the mutants isolated may be defective in forming the cellular contacts with *E. coli* required for transfer to occur. This defect would presumably be the result of alterations to the yeast cell wall. Yeast cell wall mutants commonly display specific phenotypes such as sensitivity to certain agents like calcoflour white which affects cell wall structure, or the inability to grow under certain conditions such as high salt concentrations. Our mutants were analysed for such sensitivities as this would provided evidence for the nature of the mutation and hopefully identify an associated phenotype of the mutants which would facilitate cloning of the wild type alleles. No phenotypes were identified that would facilitate the cloning of the wild type alleles, although *con1* and *con4* displayed a some increased sensitivity to calcoflour white. The most interesting associated phenotype was displayed by the *con1*

mutant which clearly demonstrated an increased sensitivity to the cell wall degrading enzyme zymolyase. Hence it would appear that *con1* mutants have an altered cell surface and may therefore be defective in forming the cellular contacts required for conjugation.

The mutant phenotypes may also be caused by a defect in the process of DNA uptake and replication. Transformation efficiencies of the strains however were comparable to wild type strains. Therefore it may be unlikely that DNA uptake is affected in these mutants. However these transformations were only carried out with double stranded plasmid DNA and may not therefore reflect the uptake single stranded DNA accurately. Further insights into the point of action of these mutants should be obtained by cloning their wild type alleles.

Chapter Six

Cloning and Analysis of the Wild Type *CON1* Allele

INTRODUCTION

Six yeast mutants had been isolated from a UV mutagenisied population that display an altered ability to receive a plasmid by conjugation. The *con1* mutant was particularly interesting as it demonstrated sensitivity to the cell wall degrading enzyme zymolyase, therefore, providing evidence that this mutant has altered cell surface properties. As the *con1* mutant demonstrated the most severe decrease in transfer efficiency of our mutants and we had evidence to suggest it may be a cell wall mutant we concentrated our efforts on cloning the wild type *CON1* allele. The *con1* mutation is recessive therefore the wild type allele can be cloned by mutant rescue with a yeast genomic library. The release of the sequence of the entire *S. cerevisiae* genome has greatly facilitated the analysis of yeast genomic clones. A small amount of sequence data can be obtained from a rescuing clone and subsequently used in a computer search to identify the complete sequence of the yeast genomic DNA contained on the clone. This search will also reveal any potential open reading frames contained in that region. Further analysis, such as subcloning and transposon mutagenesis, can then be employed to identify the open reading frame responsible for rescuing the mutant phenotype.

RESULTS

6.1 Rescue of *con1* with a Yeast Genomic Library.

It was hoped that the *con1* mutant would exhibit additional mutant phenotypes that would provide an enrichment strategy for the wild type phenotype, and thus facilitate cloning of the *CON1* allele. However, no phenotypes were identified that could successfully be employed as an enrichment (Chapter 5, Section 5.3). Hence, to clone *CON1* we employed the plate screen for transfer initially developed to identify yeast mutants. In this instance we were looking for clones that rescued the inability of a *con1* mutant to receive a plasmid by conjugation. We used the yeast genomic library of Rose *et al.* (1987), based on the yeast centromeric plasmid YCp50 (Figure 2.10, Table 2.3). The library had been constructed by cloning partial *Sau*3AI genomic digests into the unique

*Bam*HI site in the tetracycline resistance gene of YCp50. This library was chosen due to its low copy number in yeast. Therefore, any rescuing clones identified are unlikely to be the result of multi-copy suppression of the mutant phenotype. Additionally, as YCp50 is selectable via uracil prototrophy the shuttle vector pAC88 (Figure 2.2 Table 2.3) can be used to assay for transfer to yeast. The reported size of genomic inserts for this library is 14kb, however, experience in our laboratory has shown the average size to be closer to 7kb.

The genomic library contained in YCp50 was transformed into the *con1* mutant strain. The resulting transformants were picked and analysed in our plate screen to determine their transfer phenotype, using JO8(pUB307, pAC88) as the bacterial donor. Mutant cells fail to form transconjugant colonies in this screen. Therefore, transformants containing a wild type *CON1* allele can be identified by their ability to receive a plasmid and subsequently form transconjugant colonies. 3012 transformants were analysed using our plate screen, after 3 rounds of rescreening 15 potential rescuing clones had been identified. *E. coli*-yeast matings were carried out with these 15 potential clones. Only two of the potential clones rescued the mutant phenotype in these standard matings. The plasmid DNA in these strains, termed pPC5 & pPC11, was transformed into *E. coli*, amplified and prepared, and then transformed into the *con1* mutant and wild type S150-2B yeast strains. Transformants were then used as recipients in *E. coli*-yeast matings to ensure that the clones still rescued the mutant phenotype. Clone pPC11 did not rescue the mutant phenotype when transformed into the *con1* mutant. Hence, it is possible that in the original isolate containing pPC11 the wild type transfer rate detected was due to the reversion of the *con1* mutation. However, clone pPC5 transformed into the *con1* strain still rescued the mutant phenotype (Table 6.1).

Clone pPC5 was also transformed into the yeast mutant *con4*, the resulting transformants were then subjected to standard matings with JO8(pUB307, pAC88) as the bacterial donor. The clone failed to rescue the mutant phenotype in *con4* (Table 6.1), thus supporting the conclusion from genetic analysis that *con1* and *con4* fall into different complementation groups.

6.2 Analysis of the *con1* Rescuing Clone (pPC5).

As the yeast genome sequencing project was completed by this time, it was decided to obtain a short amount of sequence data from each end of the

Recipient	Plasmid Carried	Transfer Efficiency	Level of Transfer
Wild Type	YCp50	$1.2x10^{-4}$ (+/- $4.7x10^{-5}$)	100%
con1	YCp50	$2.6x10^{-5}$ (+/- $2.1x10^{-5}$)	21% (+/- 17%)
con1	pPC5	$8.8x10^{-5}$ (+/- $3.6x10^{-5}$)	72% (+/- 30%)
con4	YCp50	$2.7x10^{-5}$ (+/- $0.9x10^{-5}$)	22% (+/- 8%)
con4	pPC5	$2.0x10^{-5}$ (+/- $0.6x10^{-5}$)	16% (+/- 5%)

Table 6.1. Rescue of Mutants *con1* and *con4* by Clone pPC5.

Recipient strains were grown in selective media to $5x10^6$ cells/ml and subjected to a standard mating as described in Chapter 2. The donor bacterium was JO8(pUB307, pAC88). Yeast transconjugants were selected for both leucine and uracil prototrophy to select for the incoming plasmid (pAC88), and the resident plasmid (either YCp50 or pPC5). Transfer efficiency is expressed as transconjugant yeast formed per recipient cell. The results shown are the average of 4 experiments.

genomic insert. It was important to obtain data from each end both to map the insert to the yeast genome, and to ensure the insert was derived from a continuous stretch of genomic DNA. Automated sequencing was carried out using primers flanking the *Bam*HI site of YCp50, orientated into the insert region. Approximately 500bp of sequence data was obtained, this data was used in a computer search against the sequence of the yeast genome using the FASTA program (Pearson & Lipman, 1988) (Figure 6.1). The homology identified demonstrated that the genomic insert in pPC5 originated from a 5.5kb region on the right hand arm of chromosome 8 (accession number U00030, from position 720 to 6180). This corresponded to the size of the insert in pPC5 determined by restriction mapping. The predicated restriction map from the sequence data also matched the restriction map generated from pPC5 (Figure 6.2), the main discrepancy being the orientation of the cluster of *Eco*RI, *Bgl*II and *Sph*I sites, which could not be resolved by restriction mapping.

The predicted open reading frames, of more than 100 amino acids in length, in clone pPC5 are shown in Figure 6.3. There is only one complete open reading frame in the clone, YHR185c, a tRNA for valine and the 3` end of reading frame YHR186c. The evidence for the presence of these ORF's is based on sequence data alone. As there is only one complete ORF in the insert in pPC5 it would appear likely that this ORF, YHR185c, is responsible for rescuing the *con1* mutant phenotype.

6.3 Tagged Tn Disruption of Clone pPC5.

To determine if indeed ORF YHR185c was responsible for rescuing the *con1* mutant phenotype a tagged transposon mutagenesis approach was employed (Sedgwick & Morgan, 1994). This approach has the benefit of being less time consuming than standard techniques for analysing genomic clones, such as subcloning. It also directly identifies the region within a clone containing the gene of interest. Briefly, the procedure is dependant on a transposition intermediate, the cointegrant, being formed between a conjugative plasmid carrying a transposon (in this case R388::Tn1000-*HIS3*), and the target plasmid (in our case pPC5). When the cointegrant is formed at the same time as bacterial mating this linked structure will be transferred to the recipient cell. The cointegrant is then resolved in the recipient to produce the original conjugative plasmid and the transposed target plasmid (Figure 6.4). Therefore, as the target plasmid is not naturally mobilisable, bacterial cells containing the

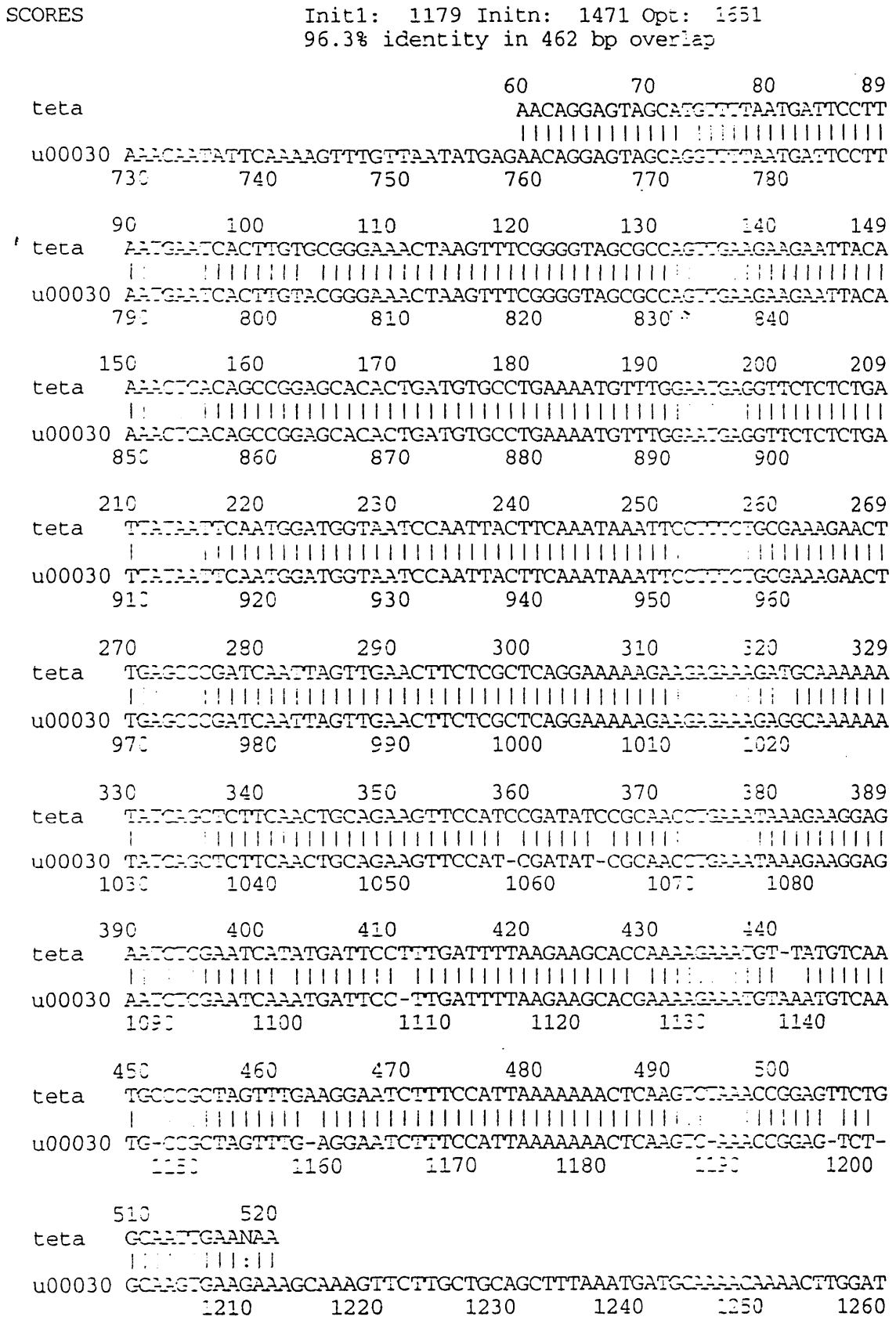
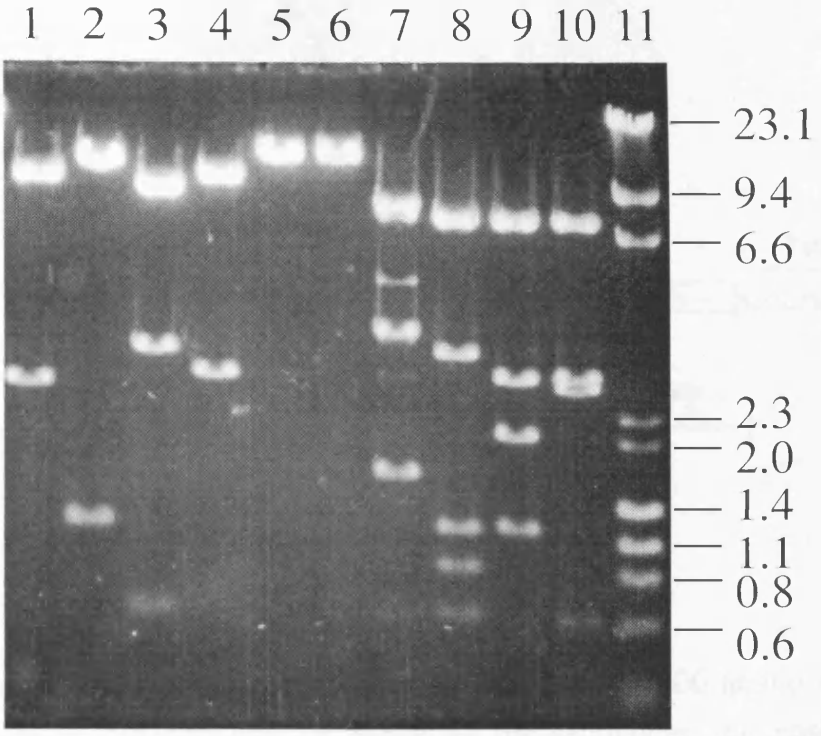


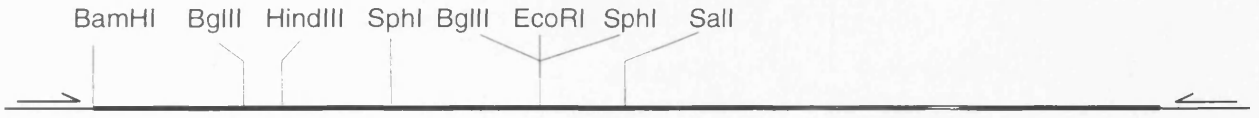
Figure 6.1. Homology Search with Sequence Data obtained from pPC5.

Sequencing reactions were carried out into the insert in pPC5 using Teta and Tetb primers. The sequence obtained was used in a fasta homology search (Pearson & Lipman, 1988) to the EMBL database. The results of the homology search using the sequence data generated from the Teta primer is shown above.

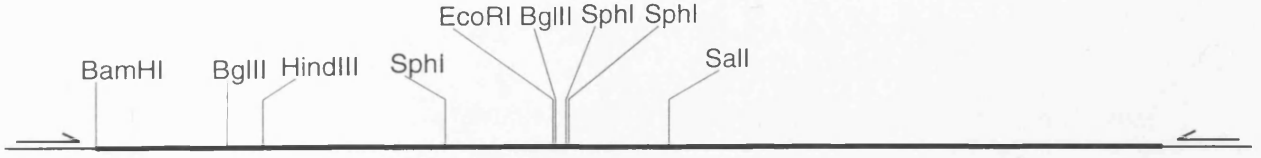
A



B



C



Enzymes that do not cut: XhoI, KpnI, NcoI.

1kb

Figure 6.2. Restriction Analysis of Clone pPC5.

A. Example of an agarose gel used in restriction mapping the genomic insert in pPC5.

Lane 1. *Eco*RI	Lane2. *Hin*dIII
Lane 3. *Sph*I	Lane 4. *Sal*I
Lane 5. *Kpn*I	Lane 6. *Xho*I
Lane 7. *Bgl*II	Lane 8. *Hin*dIII+*Sph*I
Lane 9. *Hin*dIII+*Sal*I	Lane 10. *Eco*RI+*Sal*I

Lane 11. λ *Hin*dIII + ϕX174*Hae*III Markers

B. Calculated restriction map of the insert in pPC5. The orientation of the clustered *BglII*, *Eco*RI, & *Sph*I sites was unclear.

C. Predicted restriction map of the pPC5 insert from sequence data.

Thin lines indicate vector sequences, the position of primers used for sequencing are represented by arrows.

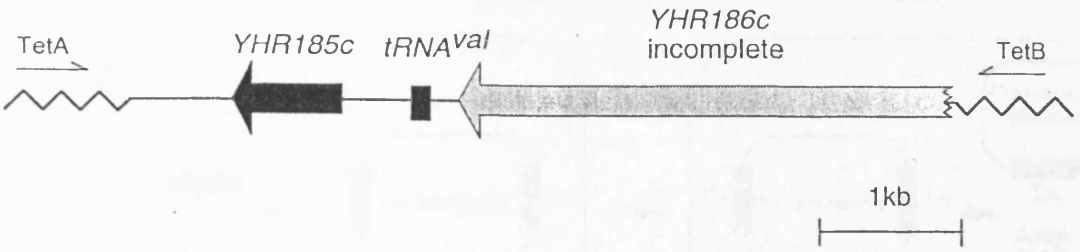


Figure 6.3. Predicted Open Reading Frames in Clone pPC5.

The position and orientation of open reading frames over 100 amino acids in length are shown. Zig zag lines indicate vector sequences, the position of primers used for sequencing into the insert is indicated. Only the 3` ¾ of ORF YHR186c is present on the insert.

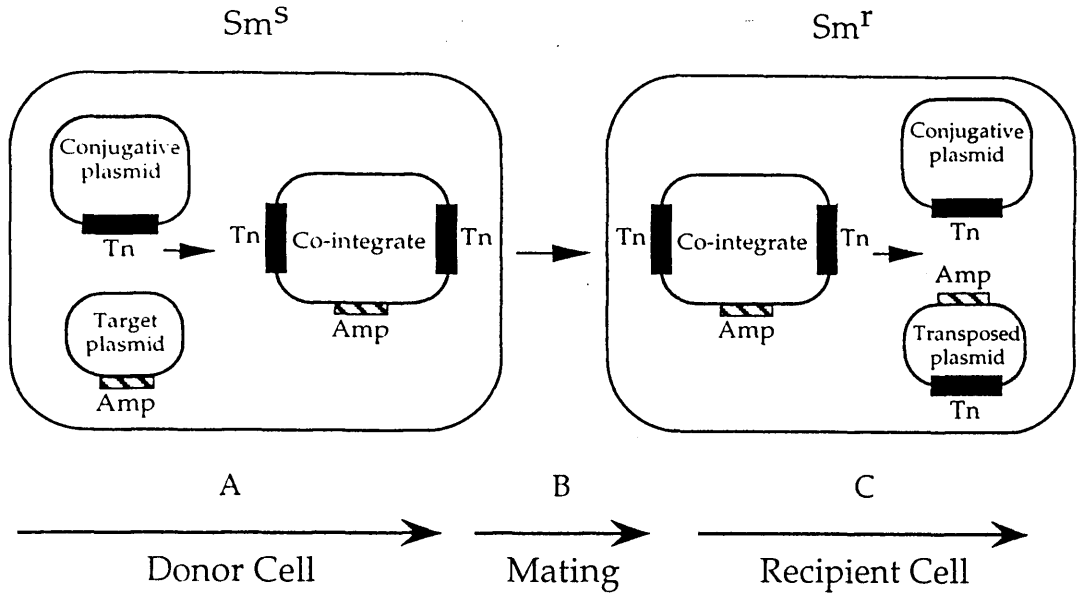


Figure 6.4. Tagged Tn1000 Transposition.
(Sedgwick & Morgan, 1994)

A. Transposition results in an intermediate, the cointegrant, being formed linking the conjugative and target plasmid.

B. Conjugative mating results in the transfer of the cointegrant from the streptomycin sensitive donor to the streptomycin resistant recipient cell.

C. The cointegrant is resolved in the recipient cell reforming the parent conjugative plasmid, and the newly transposed target plasmid.
The recipients containing the transposed target plasmid can be selected for by streptomycin and ampicillin resistance.

transposed target plasmid can be directly selected by plating on plates selective for bacterial recipients containing the target plasmid. To facilitate their use in *S. cerevisiae* transposon derivatives have been formed which carry yeast markers, allowing the selection of disrupted genes in yeast. This procedure was carried out successfully on pPC5, using a Tn1000 derivative carrying the *HIS3* gene. The *HIS3* derivative was chosen as this would allow the use of YCp50 and YEp13 based vectors in subsequent analysis. Clone pPC5 was transformed into DH1(R388::Tn-HIS3), and mated with MH1578. The recipient cells containing the transposed pPC5 were selected for ampicillin and streptomycin resistance.

As there was only one complete open reading frame on the genomic insert of pPC5 it was decided to identify individual transposed plasmids where the transposon had disrupted YHR185c. YHR185c is relatively close to the *Bam*HI (Teta primer site) end of the insert, hence, it was decided to use a PCR strategy to identify plasmids carrying the transposon in YHR185c. 30 recipient colonies containing transposed pPC5 were analysed by PCR using 3 primers; the TetA primer- into the genomic insert, and primers to the termini of the transposon (γ and δ, Sedgwick & Morgan, 1994). A PCR fragment between 740 and 1450bp would indicate transposon insertion into the YHR185c reading frame. From the 30 colony PCR's 1 plasmid was identified that contained the transposon in the correct position. This plasmid, pPC5-YHR::Tn, was prepared from *E. coli* and transformed into the *con1* mutant strain. The ability of pPC5-YHR::Tn to rescue the *con1* mutant phenotype was then assessed in standard *E. coli* yeast matings (Table 6.2). As a control a transposon insertion approximately 500bp upstream of YHR185c was also analysed. It can clearly be seen from Table 6.2 that the disrupted YHR185c allele failed to rescue the *con1* mutant phenotype. Hence, it can be concluded that YHR185c is responsible for rescuing our mutant, and is therefore most likely the wild type *CON1* allele.

The exact position and orientation of the transposon insertion in YHR185c was also deduced. To determine the orientation of the transposon PCR analysis was carried out using the Teta primer and either the primers to the γ or δ termini. The transposon was orientated with the δ terminus closest to the intact *Bam*HI site, i.e. orientated toward the Teta primer site. The position of the insertion was confirmed by restriction analysis with *Bgl*II. The insertion is approximately 70bp into the YHR185c open reading frame, hence only 10% of the wild type protein would be produced.

Recipient	Plasmid Carried	Transfer Efficiency	Level of Transfer
Wild Type	YCp50	$1.2x10^{-4}$ (+/- $4.7x10^{-5}$)	100%
con1	YCp50	$2.6x10^{-5}$ (+/- $2.1x10^{-5}$)	21% (+/- 17%)
con1	pPC5	$8.8x10^{-5}$ (+/- $3.6x10^{-5}$)	72% (+/- 30%)
con1	pPC5-YHR::Tn	$2.3x10^{-5}$ (+/- $0.8x10^{-5}$)	18% (+/- 6%)
con1	pPC5::Tn	$1.1x10^{-4}$ (+/- $4.7x10^{-5}$)	88% (+/- 38%)

Table 6.2. Rescue of *con1* by Disruptions in YHR185c.

Recipient strains were grown in selective media and mated with JO8(pUB307, pAC88), under standard conditions. The wild type yeast strain was S150-2B. Transconjugants were selected for leucine prototrophy and the resident plasmid. Transfer efficiency is expressed as transconjugants per recipient. pPC5-YHR::Tn contains the Tn insertion in YHR185c; pPC5::Tn contains the Tn insertion approximately 500bp upstream of the YHR185c ORF. The results shown are the average of 5 experiments.

6.4 Disruption of the Genomic Copy of YHR185c.

The use of the tagged transposon to identify YHR185c as rescuing *con1*, conveniently meant a suitable disruption cassette had already been created to disrupt the chromosomal copy of YHR185c. As discussed above the tagged transposon used in this study carried the *HIS3* allele as a marker. Chromosomal disruptions in *S. cerevisiae* are relatively easy to perform as recombination predominantly occurs in regions of homology. The ends of linear DNA are particularly recombinogenic. Hence to perform a disruption yeast is transformed with linear DNA carrying a marker gene flanked by DNA to target the cassette to a region of interest. The plasmid pPC5-YHR::Tn was restricted with *Bsr*GI and *Sna*BI to liberate a 7kb fragment containing the tagged transposon and surrounding insert sequences. This fragment was then transformed into the diploid strain 842 and the haploid strain S150-2B, and HIS^+ transformants selected (Figure 6.5). The analysis was carried out in the diploid strain in case YHR185c was an essential gene, or if its disruption severely reduced viability. HIS^+ transformants of 842 were sporulated and the resulting asci dissected to obtain the 4 products of meiosis. A 2:2 ratio for histidine requirement was obtained in the tetrads as would be expected if a single copy of YHR185c had been disrupted in 842 (See Figure 6.6). All 4 spores from 842(YHR::Tn-HIS3) were viable.

To ensure that the genomic disruption had been correctly targeted to YHR185c Southern analysis was carried out. Total DNA was prepared from the 4 spores as well as strains of 842 either homo- or heterozygous for YHR185c. The genomic DNA was restricted to completion with *Bsr*GI, and probed with a *Sph*I fragment internal to the *Bsr*GI fragment; the resulting autoradiograph is shown in Figure 6.6. The HIS^+ spores show a band shift of 6.1kb as would be expected by replacement of YHR185c with YHR185c::Tn-HIS3, thus confirming the successful disruption of YHR185c. Hence as the haploid spores containing YHR185c::Tn-HIS3 are viable it can be concluded that YHR185c is probably not an essential gene. However, to prove that YHR185c is non essential a null allele would need to be formed.

The transfer phenotype of the haploid spores from 842(YHR185c /YHR185c::Tn-HIS3), and S150-2B(YHR185c::Tn-HIS3) was determined in standard matings (Table 6.3). It is clear that there is a decrease in transfer efficiency to the disrupted strains. However, this decrease in efficiency is not

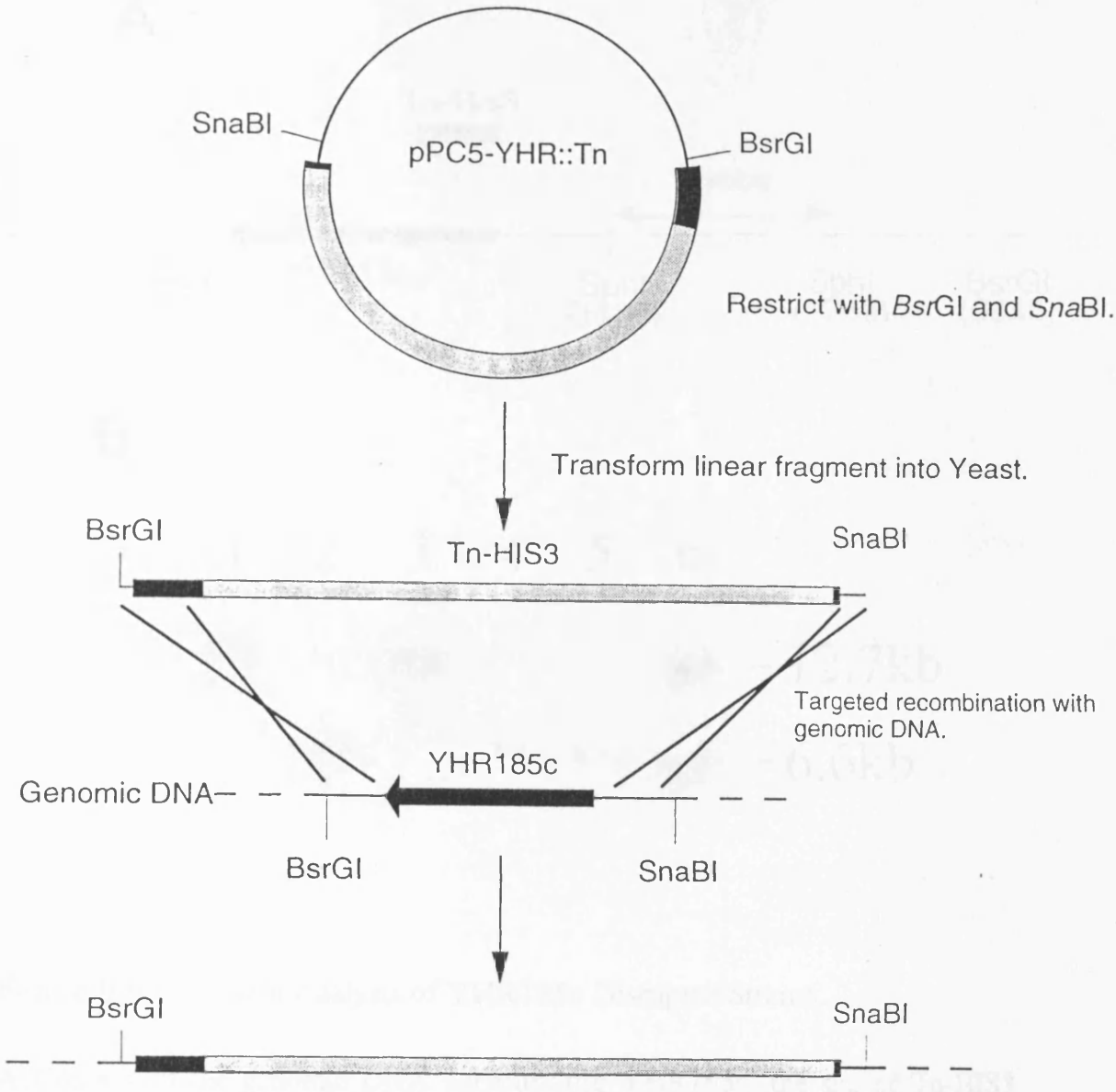


Figure 6.5. Strategy for Disrupting the Genomic Copy of YHR185c.

Plasmid pPC5-YHR::Tn was restricted with *Bsr*GI and *Sna*BI to liberate a linear fragment containing YHR185c (black) disrupted with Tn-HIS3 (grey). The linear fragment was transformed into yeast (842 and S150-2B) and HIS^+ transformants selected. It was expected that the sequences at the ends of the fragment would target the fragment to recombining with the genomic copy of YHR185c.

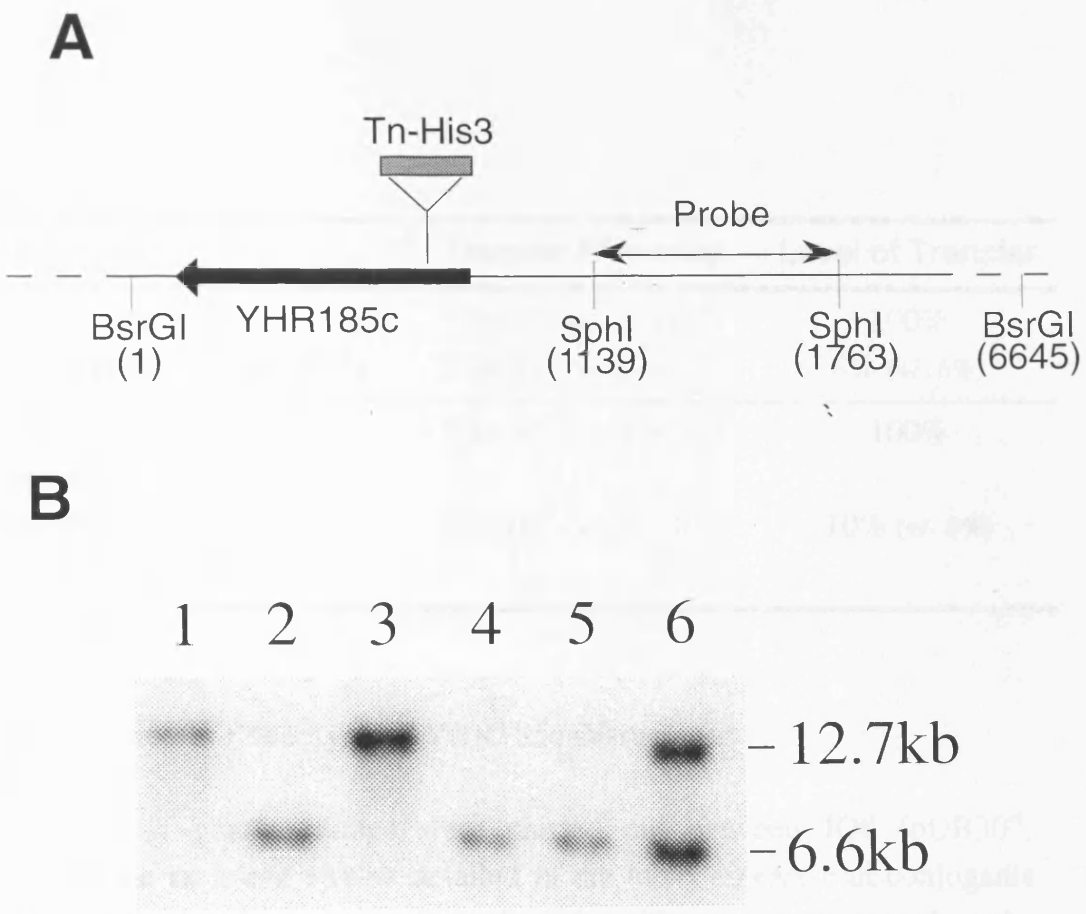


Figure 6.6. Southern Analysis of YHR185c Disrupted Strains.

A. Context of the genomic DNA surrounding YHR185c, the site of Tn-HIS3 insertion is illustrated. The 624bp *Sph*I fragment shown was used as a probe in Southern analysis. Not all *Sph*I sites in this region are shown. Figure not drawn to scale.

B. Autoradiograph confirming disruption of YHR185c.
Yeast total DNA was restricted to completeion with *Bsr*GI; after electrophoresis and Southern blotting the samples were probed by hybridisation using a radioactively labeled *Sph*I fragment adjacent to the YHR185c open reading frame (See above).

Lane 1. A1: Matα (HIS^+) Lane 2. A2: Mata (HIS^-)
Lane 3. A3: Matα (HIS^+) Lane 4. A4: Mata (HIS^-)
Lane 5. 842
Lane 6. 842 (YHR185c / YHR185c::Tn-HIS3)

A1-A4 are the products of tetrad analysis of 842 (YHR185c / YHR185c::Tn-HIS3).

Recipient Strain	Transfer Efficiency	Level of Transfer
S150-2B	$6.9x10^{-5}$ (+/- $2.1x10^{-5}$)	100%
S150-2B (YHR185c::Tn-HIS3)	$5.2x10^{-6}$ (+/- $0.4x10^{-5}$)	8% (+/- 6%)
842 (YHR185c) -Haploid Spores	$5.1x10^{-5}$ (+/- $3.7x10^{-6}$)	100%
842 (Yhr185c::Tn-HIS3) -Haploid Spores	$5.2x10^{-6}$ (+/- $3.1x10^{-6}$)	10% (+/- 6%)

Table 6.3. Transfer Phenotype of YHR185c Disruptants.

Standard *E. coli* yeast matings were carried out between JO8 (pUB307, pAC88) and the recipient strains detailed in the table. Yeast transconjugants were selected for leucine prototrophy. Transfer efficiency is expressed as the number of transconjugants per recipient. The level of transfer compared to wild type is also detailed. Results shown are the average of at least 3 experiments.

as severe as in the original mutant, a fall of 10 fold compared to 35 fold in the original *con1* mutant. The zymolyase sensitivity of the disruptants has also been addressed. The S150(YHR185c::Tn-HIS3) disruptant clearly demonstrates an increased sensitivity to zymolyase (Figure 6.7). However, interestingly none of the haploid spores from the 842 disruptant showed any increased sensitivity to zymolyase. It is therefore likely that other strain specific factors are involved in the zymolyase sensitivity of a YHR185c mutant.

As YHR185c was isolated from a low copy yeast library it is highly likely that it is the wild type *CON1* allele. However, it is still possible that YHR185c could be suppressing the *con1* phenotype. Hence it was important to determine if YHR185c is allelic to *con1*. To achieve this a diploid was formed between the original *con1* mutant and a haploid spore displaying the mutant phenotype from 842(YHR185c/YHR185c::Tn-HIS3). The ability of this diploid to receive a plasmid was determined and compared to a wild type diploid strain and diploid strains homo- and heterozygous for the original *con1* mutation (Table 6.4). Transfer to the *con1*/YHR185c::Tn-HIS3 diploid was reduced to a level below that detectable in our mating assay, comparable to the effect of a *con1* homozygous diploid. Hence, it can be concluded that *con1* is allelic to the open reading frame YHR185c.

6.5 Sequence Analysis of *CON1* (YHR185c).

DNA Sequence Analysis of *CON1*.

Inspection of the region 5` of the *CON1* open reading frame identified 2 possible TATA boxes at -24 and -116 from the translation start codon. Although the -24 sequence (TATAAA) is a more typical TATA box it is very close to the translation start site, hence the -116 sequence (TATAAC) is favoured as the possible TATA element (Figure 6.8).

The 600bp 5` of the YHR185c translation start site was inspected using the MatInspector program (Quandt *et al.*, 1994) to search for possible transcription factor binding sites. This program uses a matrix for each binding site containing the probability of a base being at a set position, the probabilities being derived from experimental data. At present the database only contains a small number of entries for fungi. However, 3 possible binding sites were

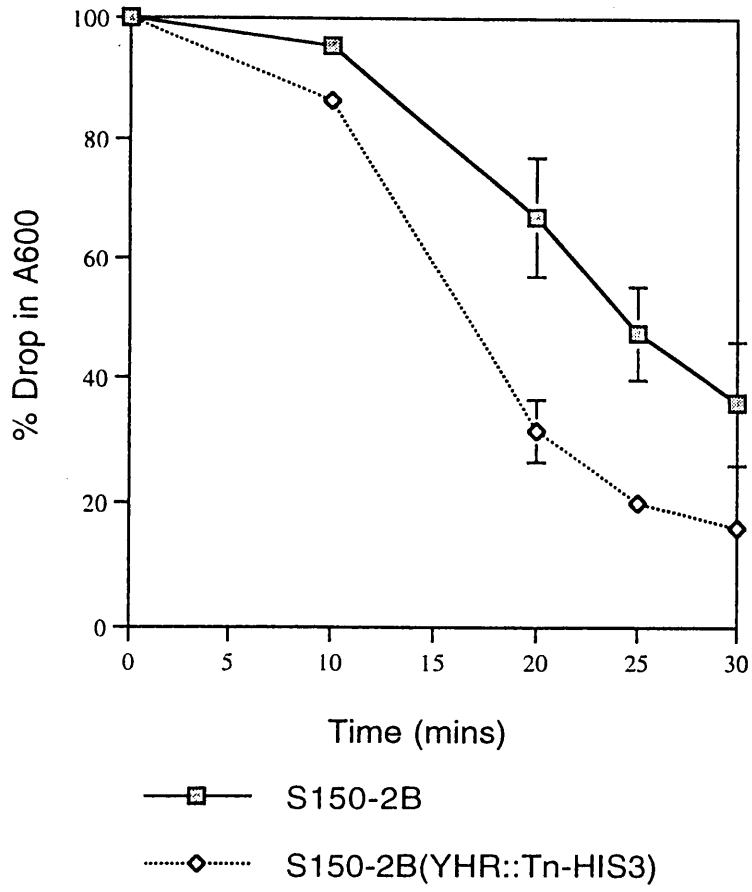


Figure 6.7. Zymolyase Sensitivity of YHR185c Disruptants.

Zymolyase sensitivity assays were carried out as detailed in chapter 2, using 5 units of zymolyase-100T. The drop in A600 was measured on a CE272 spectrophotometer (Cecil Instruments) at 5-10 minute intervals. The results shown are the average of 3 experiments.

Diploid Recipient	Transfer efficiency
CON1 / CON1	$9.4x10^{-7}$ ($^{+}$/-$6.2x10^{-7}$)
CON1 / con1	$1.0x10^{-6}$ ($^{+}$/- $2.3x10^{-7}$)
con1 / con1	$<1x10^{-7}$
con1 / YHR185c::Tn-HIS3	$<1x10^{-7}$

Table 6.4. Allelism of *con1* to YHR185c.

Standard *E. coli*-yeast matings were carried out between JO8 (pUB307, pAC88) and the diploid recipients detailed in the table. Transfer was detected by leucine prototrophy and expressed as transconjugants formed per recipient. The results shown are the average of 3 experiments.

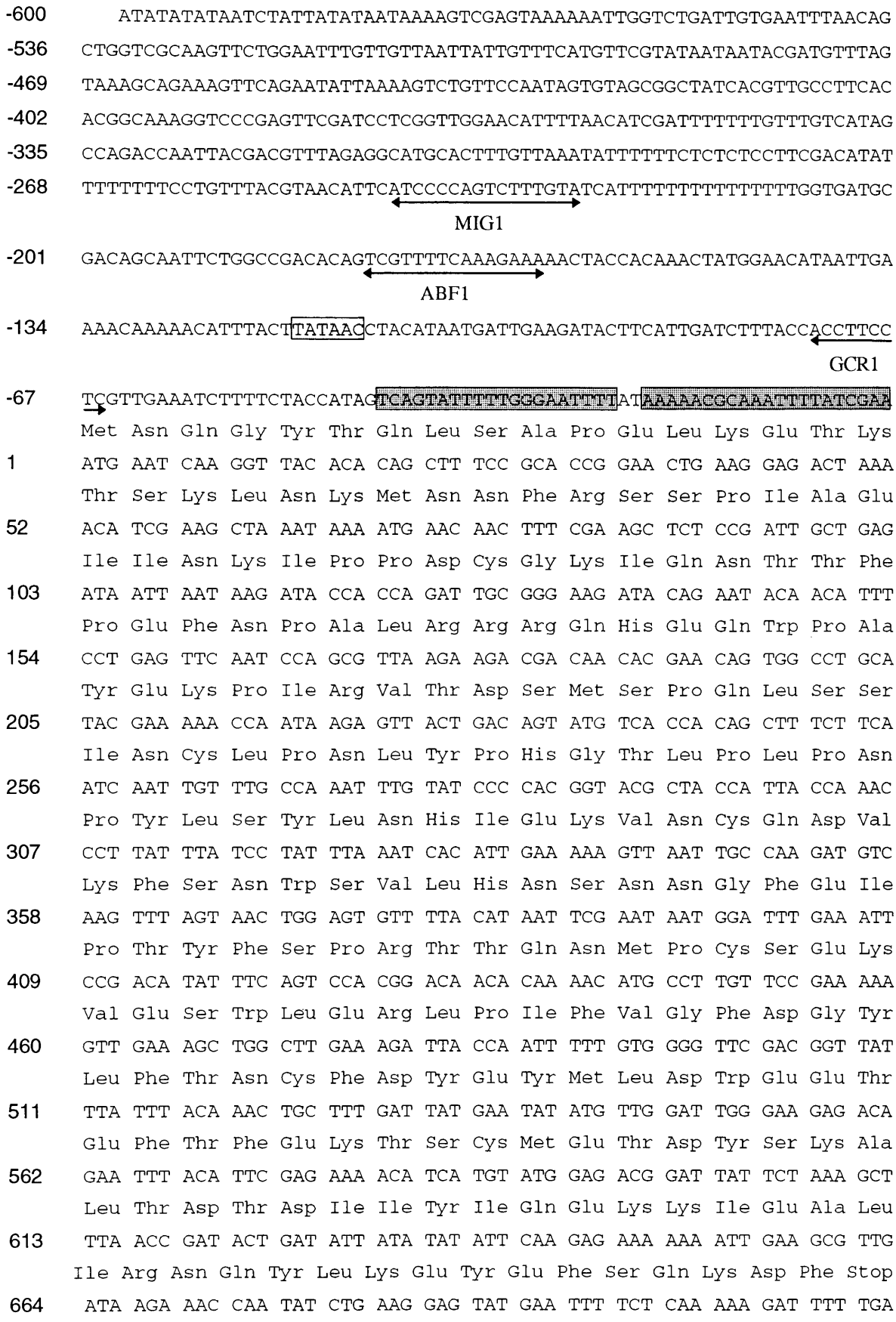

-600 ATATATATAATCTATTATATAATAAAAGTCGAGTAAAAAATTGGTCTGATTGTGAATTTAACAG
-536 CTGGTCGCAAGTTCTGGAATTTGTTGTTAATTATTGTTTCATGTTCGTATAATAATACGATGTTTAG
-469 TAAAGCAGAAAGTTCAGAATATTAAAAGTCTGTTCCAATAGTGTAGCGGCTATCACGTTGCCTTCAC
-402 ACGGCAAAGGTCCCGAGTTCGATCCTCGGTTGGAACATTTTAACATCGATTTTTTTGTTTGTCATAG
-335 CCAGACCAATTACGACGTTTAGAGGCATGCACTTTGTTAAATATTTTTTCTCTCTCCTTCGACATAT
-268 TTTTTTTCCTGTTTACGTAACATTCATCCCCAGTCTTTGTATCATTTTTTTTTTTTTTTGGTGATGC
MIG1
-201 GACAGCAATTCTGGCCGACACAGTCGTTTTCAAAGAAAAACTACCACAAACTATGGAACATAATTGA
ABF1
-134 AAACAAAAACATTTACTTATAACCTACATAATGATTGAAGATACTTCATTGATCTTTACCACCTTCC
GCR1
-67 TCGTTGAAATCTTTTCTACCATAGTCAGTATTTTTTGGCAATTTTTATAAAAACGCAAATTTTATCGAA
Met Asn Gln Gly Tyr Thr Gln Leu Ser Ala Pro Glu Leu Lys Glu Thr Lys
1 ATG AAT CAA GGT TAC ACA CAG CTT TCC GCA CCG GAA CTG AAG GAG ACT AAA
Thr Ser Lys Leu Asn Lys Met Asn Asn Phe Arg Ser Ser Pro Ile Ala Glu
52 ACA TCG AAG CTA AAT AAA ATG AAC AAC TTT CGA AGC TCT CCG ATT GCT GAG
Ile Ile Asn Lys Ile Pro Pro Asp Cys Gly Lys Ile Gln Asn Thr Thr Phe
103 ATA ATT AAT AAG ATA CCA CCA GAT TGC GGG AAG ATA CAG AAT ACA ACA TTT
Pro Glu Phe Asn Pro Ala Leu Arg Arg Arg Gln His Glu Gln Trp Pro Ala
154 CCT GAG TTC AAT CCA GCG TTA AGA AGA CGA CAA CAC GAA CAG TGG CCT GCA
Tyr Glu Lys Pro Ile Arg Val Thr Asp Ser Met Ser Pro Gln Leu Ser Ser
205 TAC GAA AAA CCA ATA AGA GTT ACT GAC AGT ATG TCA CCA CAG CTT TCT TCA
Ile Asn Cys Leu Pro Asn Leu Tyr Pro His Gly Thr Leu Pro Leu Pro Asn
256 ATC AAT TGT TTG CCA AAT TTG TAT CCC CAC GGT ACG CTA CCA TTA CCA AAC
Pro Tyr Leu Ser Tyr Leu Asn His Ile Glu Lys Val Asn Cys Gln Asp Val
307 CCT TAT TTA TCC TAT TTA AAT CAC ATT GAA AAA GTT AAT TGC CAA GAT GTC
Lys Phe Ser Asn Trp Ser Val Leu His Asn Ser Asn Asn Gly Phe Glu Ile
358 AAG TTT AGT AAC TGG AGT GTT TTA CAT AAT TCG AAT AAT GGA TTT GAA ATT
Pro Thr Tyr Phe Ser Pro Arg Thr Thr Gln Asn Met Pro Cys Ser Glu Lys
409 CCG ACA TAT TTC AGT CCA CGG ACA ACA CAA AAC ATG CCT TGT TCC GAA AAA
Val Glu Ser Trp Leu Glu Arg Leu Pro Ile Phe Val Gly Phe Asp Gly Tyr
460 GTT GAA AGC TGG CTT GAA AGA TTA CCA ATT TTT GTG GGG TTC GAC GGT TAT
Leu Phe Thr Asn Cys Phe Asp Tyr Glu Tyr Met Leu Asp Trp Glu Glu Thr
511 TTA TTT ACA AAC TGC TTT GAT TAT GAA TAT ATG TTG GAT TGG GAA GAG ACA
Glu Phe Thr Phe Glu Lys Thr Ser Cys Met Glu Thr Asp Tyr Ser Lys Ala
562 GAA TTT ACA TTC GAG AAA ACA TCA TGT ATG GAG ACG GAT TAT TCT AAA GCT
Leu Thr Asp Thr Asp Ile Ile Tyr Ile Gln Glu Lys Lys Ile Glu Ala Leu
613 TTA ACC GAT ACT GAT ATT ATA TAT ATT CAA GAG AAA AAA ATT GAA GCG TTG
Ile Arg Asn Gln Tyr Leu Lys Glu Tyr Glu Phe Ser Gln Lys Asp Phe Stop
664 ATA AGA AAC CAA TAT CTG AAG GAG TAT GAA TTT TCT CAA AAA GAT TTT TGA

Figure 6.8. YHR185c Open Reading Frame and Upstream Sequence.

The sequence of the YHR185c open reading frame is shown with its corresponding protein sequence, and the 600bp 5` of the translation start site. Areas of interest dealt with in the text are highlighted. The potential TATA element is identified in the light grey square. Potential transcription factor binding sites are underlined, and the switch from T rich to A rich sequence characteristic of transcription initiation sites is highlighted by the dark grey squares.

identified that exactly matched the matrix core and were over 85% similar to the whole matrix, these were sites for MIG1, GCR1, and BAF1 binding.

MIG1 is involved in glucose repression, its site in *CON1* matched the published consensus exactly (Lundin *et al.*, 1994; (G/C),(C/T),G,G,G,G). However, *in vitro* studies have shown binding of MIG1 to require an A/T rich region immediately 5` to the binding site, with more than one G or C residue in the first 5bp upstream abolishing binding. In the case of *CON1* there is a G residue at -2 and a C residue at -5. Hence it is probably unlikely that MIG1 binds to the *CON1* upstream region, however, *in vivo* binding studies for MIG1 have yet to be carried out.

The GCR1 transcription factor is required for the high level expression of glycolitic genes. The GCR1 site in *CON1* contained one mismatch to the 16bp consensus sequence (Huie *et al.*, 1992), however, binding of GCR1 has been shown with up to 4 mismatches to the consensus. Hence, it is possible that GCR1 may bind in the region upstream of *CON1* and may modulate its expression.

The role of the BAF1 (or ABF1) transcription factor is as yet unclear, it is implicated in both DNA replication as well as transcriptional activation, and may be a general transcription factor. The possible BAF1 site upstream of *CON1* matches exactly the basic consensus sequence (TCN_7ACG), however, it contains 2 mismatches to the more detailed consensus (Halfer *et al.*, 1989; $TCRYYN_4ACG$).

Analysis to determine possible transcription factor binding sites was also carried out using the transfac database (Wingender, 1994), however, the results using this approach were mostly inconclusive. Several matches to the 5bp consensus for heat shock factor binding were found. However, for HSF binding 3-6 sites need to be found running concurrently on the DNA (Fernandes *et al.*, 1994), this was not the case upstream of *CON1*, hence, these sites were discounted. MATa1 sites were also found upstream of *CON1*. However, these were also discounted as they did not exhibit the correct spacing and orientation required for MATa1-MATα2 or MATα2-MCM binding, involved in repression of haploid specific and a-specific genes respectively (Goutte *et al.*, 1994).

Transcription initiation in yeast occurs in the initiation region (IR). Several factors have been reported to determine the position of the IR, including the position of the TATA element and the sequence of the IR itself. However, homologies between IR's are limited to a Pyrimidine-A consensus; transcription starting from the A residue. Although there is no specific sequence, a trend in IR regions has been noted (Maicas & Friesen, 1990); this involves the switch from a T rich region to a A rich region, transcription characteristically starting approximately 10 residues after the T rich region. A similar switch in base composition can be seen in the upstream region of *CON1*. From residues -43 to -23 the composition is 55%T/ 20% A switching to 24%T/ 52%A in the region -21 to +2. This would place the transcription start at approximately -13 (Sequence -14 CA), which matches the weak consensus Y-A. Transcription from this site would be initiated 100bp downstream of the proposed TATA element and produce a short leader sequence in the mRNA of approximately 13 nucleotides; both of these values are acceptable when compared to other known transcription start sites (typically 30-120 nucleotides) and the length of leader sequences (reported between 11 and 500 nucleotides).

The leader sequences of yeast mRNA's also exhibit specific sequence context (reviewed in Donahue & Cigan, 1990). The region -25 to -1 is usually A rich and G depleted. In *CON1* this region is composed of 48% A and only 8% G, thus fitting data from other known leader sequences. This region is also devoid of possible stem loops which would inhibit ribosome binding. No specific sequence is required in the mRNA for efficient translation except the AUG start codon. However, a consensus has emerged around the start codon (A/Y,A,A/U,A,AUG), the region in *CON1* only contains one mismatch to this consensus at the -3 position, a G replaces the A residue. Interestingly, the -3 position is the most conserved with 75% of genes analysed having an A residue at this position. However, studies have shown that changing this residue has little effect on translation, the most pronounced effect being an A to U substitution resulting in a 2 fold decrease in translation.

The coding region of *CON1* was used in FASTA (Pearson & Lipman, 1988) homology searches against the EMBL database, and the fungi subset of EMBL. The results of these searches were inconclusive, producing only low scoring homologies over short stretches of the coding region. This is not surprising due to codon usage at the 3rd base, searches using the protein sequence are

therefore more likely to produce informative results. The FASTA results though inconclusive do indicate that *CON1* is unique in the yeast genome.

It is possible to predict the level of expression of a protein by calculating the codon adaptation index (CAI; Li and Luo, 1996). This uses codon bias tables to determine the best set of codons for producing a specified protein and compares them to the actual codons used. A CAI between 0.4 and 0.6 is characteristic of a highly expressed genes, where as incorrectly assigned open reading frames from the genome project usually score lower than 0.1. *CON1* has a CAI of 0.13, which is indicative of a poorly to moderately expressed protein.

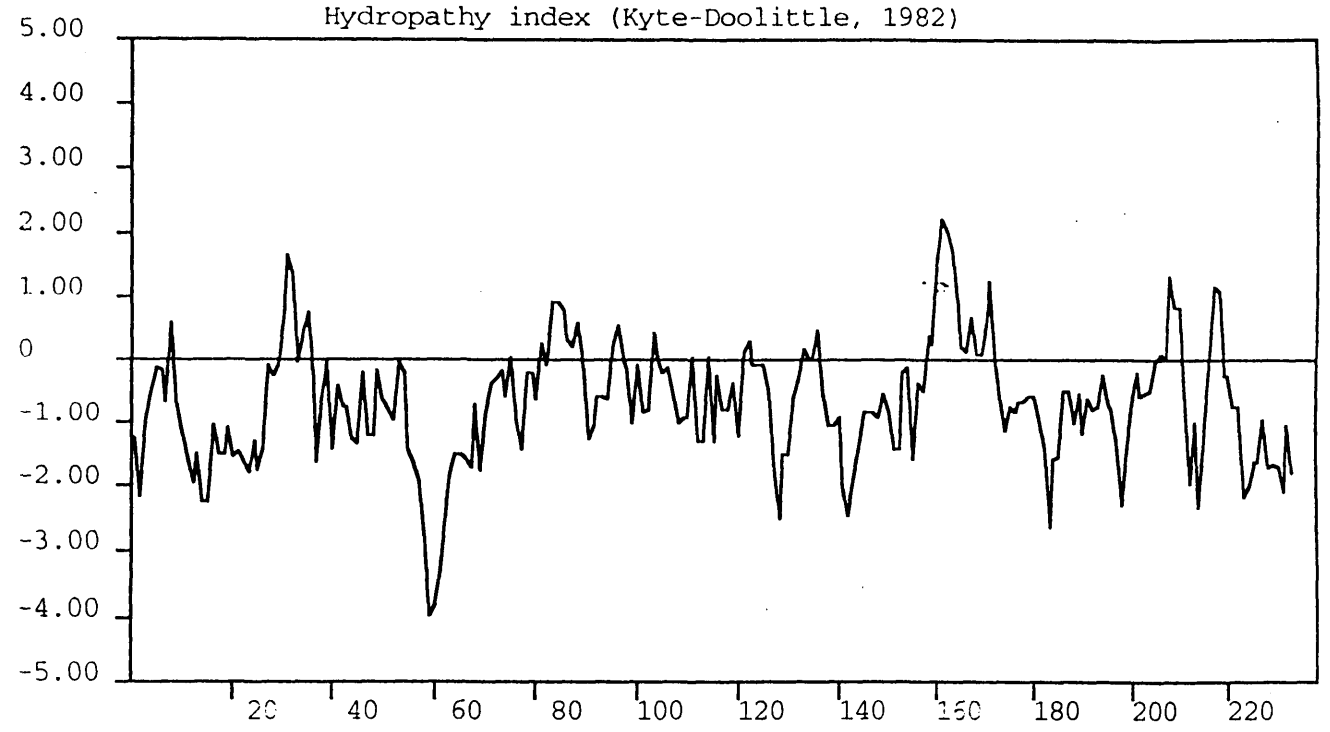
Amino Acid Sequence Analysis of CON1p

Analysis of the amino acid composition of CON1p was carried out using the gene jockey program. The hydropathy index, determined by the method of Kyte and Doolittle (1982), shows the protein to be strongly hydrophilic (Figure 6.9A). Analysis of the net charge along the length of the protein (Klein *et al.*, 1984) highlighted regions of strong net positive charge in the N terminal region of the protein, and areas of strong negative charge in the C` terminus (Figure 6.9B). The amino acid sequence was also examined for possible phosphorlyation or glycosylation sites. This identified 7 possible PKA phoshorylation sites, 1 possible CDC28 phoshorylation site, and 2 N-glycosylation sites.

Homology searches using the CON1p sequence were also carried out using the FASTA, BLAST, and BLITZ programs (Pearson & Lipman, 1988; Altschul *et al.*, 1990; Smith & Waterman, 1981) against the swissprot database. The results of these searches were mostly unrewarding, with the majority of hits being low scoring and only over short stretches of amino acids. Hence it can be concluded that CON1p is unique in the database.

Of the limited homologies detected to *CON1*, a number of them identified viral proteins that have been implicated in RNA synthesis. For example, homologies were found to the L protein of 2 members of the paramyxovirdiae; the best homology being 28.2% identity, 73% similarity over 78 amino acids to the human parainfluenza 2 virus. The L protein is the large structural protein of the virus, which is proposed to be the active RNA-directed RNA polymerase for

A



B

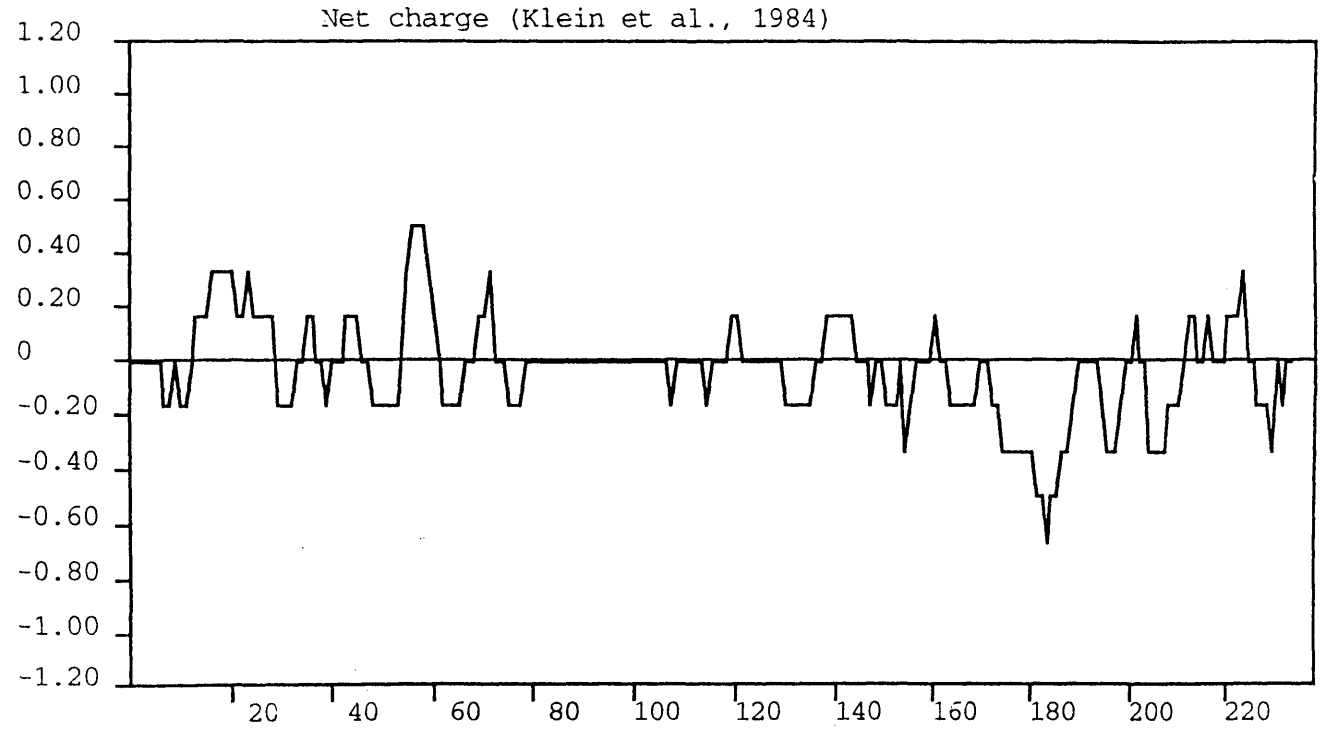


Figure 6.9. Protein Composition Analysis of CON1p.

(A) Hydropathy Index. (Kyte & Doolittle, 1982)

(B) Net Charge. (Klein *et al.*, 1984)

viral RNA synthesis (Schmaljohn & Dahrymple, 1983). The area of homology fell into the same region in both L proteins identified and in YHR185c, which would suggest a functional homology. Unfortunately, however, no data is available on the domain structure of the L protein. Homologies were also found to the P2B core protein of 3 picornaviruses, specifically Theilers's murine encephalomyelitis virus. Homologies to P2B were comparable at 29% identity, 76% similarity over a 49 amino acid overlap, which would account for the almost the entire C terminal half of P2B. The function of P2B is at present unknown, however, it has been implicated in a number of roles including viral host range, and most interestingly viral RNA synthesis (Bernstein *et al.*, 1986).

Weaker homologies were also found to DNA polymerases, the majority of which were bacterial in origin and belonged to the B family of polymerases, homology was also seen to a mitochondrial polymerase from *Neurospora crassa*. However, these homologies did not fall into the 6 conserved regions found in B family polymerases.

Apart from the homologies to proteins associated with nucleic acids the only other interesting result from homology searches was a 16% identity, 58% similarity over 74 amino acids to the β glucan synthesis associated protein KRE6p. Homology searches were also carried out against the prosite and sbase databases, these databases contain functional protein domain data, however, no matches were found to CON1p.

DISCUSSION

This chapter describes the successful cloning of *CON1*, a gene required for yeast to efficiently receive a plasmid from *E. coli* by trans-kingdom conjugation. The open reading frame is allelic to the previously uncharacterised ORF YHR185c located on the right arm of chromosome 8 and identified through the genome sequencing project. *CON1* is a 0.7kb ORF unique in the yeast genome encoding a predicted protein of 237 amino acids. Inspection of the sequence upstream of *CON1* reveals a potential TATA box at position -116 from the ATG start codon. This region shows other characteristics typical of a gene such as a switch from a T rich to A rich region reminiscent of transcription initiation regions. Binding sites for the general transcription factors GCR1 and BAF1 are also present. The region immediately prior to the start codon is also A rich and G depleted typical of

mRNA leader sequences. In summary this region displays all the characteristics typical of a transcribed gene.

Insertion of a 6kb bacterial transposon tagged with a yeast *HIS3* gene at 70 bp into the *CON1* ORF is viable. As *CON1* is unique in the genome this would suggest that *CON1* is not an essential gene. However, a defined deletion mutant would be required to confirm this. It is unlikely that the first 10% of the protein (~23 amino acids) before the insertion would be functional, however, the remainder of the gene down stream of the transposon may fortuitously become translated and form a partially active protein. If this was the case it would help explain why the disrupted allele has a less severe phenotype than the original *con1* isolate.

Homology searches using the CON1p amino acid sequence identified homology to proteins implicated in viral RNA synthesis. Hence CON1p may posses an intrinsic ability to bind nucleic acids, perhaps binding the transferred DNA strand and protecting it from degradation. Alternatively CON1p may be involved in the actual priming of the transferred strand for replication. CON1p also displayed limited homology to the KRE6p, a protein involved in β-glucan synthesis the main component of the yeast cell wall. CON1p may alternatively act then at the level of the cell wall, perhaps affecting its biosynthesis or structure, a role supported by the zymolyase sensitivity of a *con1* mutant. However, as other strain specific factors are involved in the zymolyase sensitivity of a *con1* mutant the role of CON1p at the cell wall level is confused.

Chapter Seven

General Discussion

Horizontal transfer processes have been important in the evolution of prokaryotes, as exemplified by the spread of drug resistance in the last 50 years. Three main routes exist for the horizontal transfer of DNA, tranformation, transduction and conjugation. Of these processes conjugation is believed to be the major route for DNA transfer occurring between bacteria. However, the process of conjugation is not limited to transferring DNA between related prokaryotes. Indeed transfer is possible to many diverse organisms and can even cross the kingdom barrier transferring DNA from prokaryote to eukaryote. Two such trans-kingdom transfer events have been reported; the specialised transfer of the T-DNA from the Ti plasmid of *Agrobacterium tumefaciens* to plant cells (see Kado, 1993), and transfer from *Escherichia coli* to the budding yeast *Saccharomyces cerevisiae* (Heinemann & Sprague, 1989) the subject of this thesis. Unlike transfer of the Ti plasmid, DNA transfer from *E. coli* to yeast is unlikely to be the result of a specialised system, as there is no apparent common niche for these two organisms. Therefore, conjugative transfer may provide a general route for the dissemination of DNA between organisms, and may have broader evolutionary implications. In prokaryotes there are obvious examples of the impact of horizontal transfer, such as the spread of antibiotic resistance. Evidence for trans kingdom transfer however is scarce. Although (as reviewed in the introduction to this thesis), there are a growing number of cases where horizontal transfer events are being invoked to explain discrepancies in phylogenetic trees (Syvaman, 1994; Smith *et al.*, 1992).

DNA transfer from *E. coli* to yeast can be regarded as the product of three main events. Firstly cellular contacts must be formed between the two cells, the nature of these interactions are unknown but presumably they occur between the conjugative pilus and the yeast cell wall. Once the cellular contacts have been formed DNA transfer occurs, delivering a single strand of DNA to the yeast cell. In doing so the transferred DNA must bypass both the yeast cell wall and plasma membrane to enter the yeast cytoplasm. After transfer the ssDNA molecule must then become established in the yeast cell. Establishment presumably involves movement of the DNA into the yeast nucleus, and its replication to form a double stranded circular molecule. Uptake into the

nucleus is assumed as this would be the normal location for replication and expression of the transferred DNA. However, replication outside of the nucleus, in the cytoplasm or mitochondria cannot be ruled out. The aim of this work was to determine both the bacterial and yeast genetic factors required for transfer to better our understanding of this phenomenon.

Paramount to determining the genetic factors required for plasmid transfer from bacteria to yeast was the development and optimisation of a transfer protocol to assay for yeast transconjugant formation. We successfully developed a protocol for plasmid transfer using an *E. coli* - yeast shuttle vector to monitor transfer levels to yeast. Successful plasmid transfer to yeast was possible in as little as 10 minutes using this protocol, an improvement on the previously reported minimum of 8 hours (Nishikawa *et al.*, 1990). This protocol typically results in 1 in every 29000 yeast cells receiving and maintaining a transferred plasmid after a 1 hour incubation.

Historically, plasmids were classified according to their ability to replicate in different organisms. However, it has become apparent that many conjugative plasmids have a transfer range wider than their associated replication functions (Guiney, 1982; Tardiff & Grant, 1983). The ability of bacterial plasmids to mediate transfer to yeast, where they cannot be maintained, is consistent with this observation. Our work also clearly demonstrates that there are inherent differences in the ability of conjugative plasmids to transfer promiscuously, as only the IncPα plasmid RP4 could mediate transfer to yeast at detectable levels. Transfer to yeast could not be detected by plasmids of the IncFI and IncI1 groups. Previous workers have detected transfer to yeast mediated by the IncFI plasmids (Heinemann & Sprague, 1989). However, in our work we were unable to repeat this observation. This discrepancy was probably caused by differences in the plasmid used to provide the F transfer functions. Heinemann and Sprague supplied the F transfer functions on a multicopy vector, known to result in elevated transfer levels (Ray *et al.*, 1988). Hence, it is likely that this plasmid would also result in an elevated transfer level to yeast. The plasmid used in our work more accurately resembled native IncF plasmids as it contained both the replication and transfer systems of the native F plasmid. As the efficiency of transfer reported by Heinemann and Sprague was extremely low it is perhaps unsurprising that we failed to detect such transfer in our work. In summary, if transfer to yeast by the IncFI and IncI1 plasmids is possible it must occur at a very low rate, below that detectable in our work. Therefore,

although there is the potential for all conjugative plasmids to mediate transfer to yeast some plasmids, such as RP4, can truly be described as promiscuous.

It has been hypothesised that plasmid encoded factors, additional to those essential for transfer between *E. coli* cells, may be required for promiscuous transfer (Lessl *et al.*, 1993). To address this hypothesis we determined the genetic requirements for RP4 mediated transfer to yeast cells. The main conclusion from this work was that the factors required for transfer from *E. coli* to yeast cells were identical to those essential for transfer between *E. coli* cells. Therefore, only the genes for DNA processing and mating pair formation are required for transfer to yeast cells. Similar results have recently been found for RP4 mediated transfer to *Streptomyces lividans* (Giebelhaus *et al.*, 1996). Therefore, it appears that the promiscuous nature of RP4 is inherent in the plasmids transfer machinery, and no extra plasmid encoded factors are required for promiscuity. These results also indicate that the set of genes required to form cellular contacts between *E. coli* cells must also be involved in forming contacts between *E. coli* and yeast.

The actual molecular basis of RP4 promiscuity remains unclear. A compelling theory would be that the mating pair formation system of RP4 is non-specific, such that contacts are easily made between widely different organisms. This theory is appealing as it is unlikely that a specific receptor would be universally present on the surface of diverse organisms. Other conjugative plasmids, such as the IncI1 and IncFI groups, may require more specific contacts to be formed between cells. This hypothesis, that the cellular contacts are the basis of promiscuity, could be tested by comparing the ability of IncPα and IncI1 plasmids to mobilise an IncQ plasmid to yeast. IncQ plasmids encode their own DNA processing functions but require the mating pair formation system of other plasmids to potentiate DNA transfer. An IncQ-based *E. coli* - yeast transfer system could be produced by cloning sequences for selection and maintenance in yeast onto a IncQ plasmid. The ability of this plasmid to be mobilised to yeast by IncPα and IncI1 plasmids could then be compared. Any differences in the transfer rate to yeast should reflect on the mating pair formation system of the two plasmids, and thus provide an insight into the relative importance of this process for promiscuous transfer.

Establishment of the transferred strand in the yeast recipient requires its replication and movement to the yeast cell nucleus. One of the most intriguing

aspects of *E. coli*-yeast transfer is how these events are accomplished. In Ti plasmid-mediated transfer to plant cells the relaxase, VirD2p, remains covalently bound to the transferred DNA strand and is co-transferred into the plant cell. In the plant cell VirD2p acts as a pilot protein to mediate movement of the DNA into the plant cell nucleus by virtue of two nuclear localisation signals (NLS) in its amino acid sequence (Tinland *et al.*, 1992; Citovsky *et al.*,1992). Recently it has been demonstrated that this NLS sequence is also required for Ti plasmid mediated transfer to yeast cells (Bundock *et al.*, 1995). The amino acid sequence of the RP4 relaxase, TraIp, contains a good match to a bipartite NLS sequence. Classical models of conjugation place TraIp at the junction between mating cells. However, there is no proof for this localisation. Given the similarity between the Ti plasmid *vir* system and the RP4 transfer system it is not unreasonable to suggest the relaxase may be transferred to the recipient cell. Therefore, if TraIp is co-transferred to the yeast recipient it could mediate uptake of the transferred strand into the yeast nucleus. Interestingly, transfer proteins encoded by the F plasmid did not contain any possible NLS sequences, and only the NicBp of ColIb contained a possible match to a monopartite NLS. Therefore, as well as helping to explain establishment of the transferred strand in the recipient, the presence of an NLS sequence on TraIp may also help explain the promiscuous nature of RP4 transfer to yeast.

The first step in testing the significance of the potential NLS sequence of TraIp would be to determine if the NLS is active in yeast. Initially this would involve protein localisation studies of TraIp expressed in yeast, perhaps using an epitope tagged TraIp or by raising specific antibodies to TraIp and carrying out *in situ* hybridisation studies. It would then be necessary to demonstrate that the NLS sequence is required for transfer to yeast. This would require the construction of a mutant TraIp that does not contain the NLS sequence but retains its relaxase function, and is therefore transfer proficient. It has proved possible to construct such mutants for the VirD2 protein of the Ti plasmid. It would also be rewarding to demonstrate that TraIp is indeed transferred to the yeast recipient. This may not be possible as it would require technology capable of detecting a single protein molecule in a cell. However, if the potential NLS is active, and required for transfer to yeast but not *E. coli* transfer of TraIp into the recipient could be inferred.

To determine the yeast factors required for successful transfer to occur we isolated yeast mutants with an altered ability to receive a plasmid by conjugation. The aim being to clone yeast genes required for transfer. Analysis of these genes should hopefully provide an insight into the events in the yeast cell; either at the level of establishment of the transferred strand, or in the surface interactions allowing transfer to occur. Six yeast mutants were isolated from a UV mutagenisied population using a plate screen developed to assay for transfer to yeast. Of these mutants three were chosen for further study as they exhibited the most extreme phenotypes. Two of these mutants, *con1* and *con4*, exhibited a decrease in transfer efficiency of approximately thirty five fold (3% of the wild type level). Both mutations were recessive, the result of a mutation in a single gene, and fell into different complementation groups. The third mutant, *exg1*, displayed a consistent increase in transfer efficiency of four fold. This mutation was also due to a single gene defect and was dominant. Potentially the mutants isolated may be affected in the formation of the cellular contacts required for transfer, presumably as a result of alterations to the yeast cell wall. Therefore, the mutants were tested for a variety of phenotypes that are commonly associated with cell wall mutants. Interestingly, the *con1* mutant displayed an increased sensitivity to the cell wall degrading enzyme zymolyase, a phenotype indicative of cell wall mutants. Therefore, this mutant has altered cell surface properties, which may be affecting the cellular interactions between mating cells.

The wild type *CON1* allele was cloned by mutant rescue with a yeast genomic library. *CON1* is allelic to the previously uncharacterised ORF YHR185c, located on the right arm of chromosome eight encoding a predicated protein of 237 amino acids. The context of the coding and upstream sequence is consistent with *CON1* being a transcribed gene. A defined mutant allele of *CON1* was formed by inserting a 6kb bacterial transposon into the coding region. This defined allele displayed the mutant phenotype, further proof that *CON1* is a functional gene. As the defined mutant was viable this would suggest that *CON1* is a non essential gene. However, to prove this a null allele would need to be produced. Homology searches were carried out using the predicted CON1p amino acid sequence in the hope of discerning clues as to its cellular function. However, CON1p appears to be unique in the available sequence databases, as such homology searches were largely inconclusive. Limited homology (25-30% identity, 70-80% similarity) was found to viral proteins implicated in RNA synthesis. Hence, CON1p may be involved in the

establishment of the transferred DNA strand in the recipient, perhaps in the replication of the single stranded plasmid DNA, or by binding to the DNA and protecting it from degradation. Phenotypic analysis of the original *con1* mutant identified the mutant as being sensitive to zymolyase and therefore likely to have altered cell wall properties. This is supported by the finding of limited homology between *CON1* and *KRE6*, a golgi protein involved in cell wall biogenesis (Roemer & Bussey, 1991). However, a defined mutant allele of *CON1* did not display an increased sensitivity to zymolyase in all laboratory strains tested. Therefore, it is not possible to conclude that CON1p acts at the level of the cell wall. In order to more fully understand the role of CON1p it would be interesting to determine its cellular location, and identify proteins that interact with CON1p using the yeast two hybrid system. It would also be interesting to study the expression of *CON1* using Northern analysis. Particularly to assess if the level of expression changes in relation to the ploidy of the yeast strain as we know that transfer levels are dependent on the ploidy of the recipient.

There is no apparent common niche for *E. coli* and *S. cerevisiae* in nature. Therefore, DNA transfer between these two organisms is unlikely to be the result of a specialised process. Other pro- and eukaryotes however would come into contact in nature, most likely in the soil or intestinal tracts of animals. Hence, conjugation has the potential to be a general system for the movement of DNA between different organisms. Evidence to support the wide spread horizontal transfer of DNA comes from phylogenetic studies where horizontal transfer events have been proposed to explain discrepancies in phylogenetic trees (Syvaman, 1994; Smith *et al.*, 1992).

The work reported in this thesis used *S. cerevisiae* as a recipient in trans-kingdom conjugation. However, plasmid transfer to other yeast species is possible (Sikorski *et al.*, 1990; Hayman & Bolen, 1993; Inomata *et al.*, 1994). Of further interest to yeast genetics would be to assess if plasmid transfer is possible to the human pathogen *Candida albicans*, a dimorphic yeast notoriously resistant to transformation. If transfer to *C. albicans* is possible, the use of this process could greatly facilitate its analysis. As mentioned above theoretically the scope of trans-kingdom conjugation need not be limited to yeast species. Hence, conjugation could provide a general mechanism for introducing DNA into organisms. During successful transfer from *E. coli* to *S. cerevisiae* the immigrant plasmid retains its integrity. However, as the

metabolism of the immigrant DNA is reliant on functions in the recipient cell the integrity of the transferred DNA would have to be checked when matings were performed with different species. Caution must also be taken to address the ethical issues of transferring DNA between organisms, especially on assessing the risk of such experiments.

DNA transfer in nature, between such diverse organisms as *E. coli* and yeast, would be typically unproductive due to the lack of sequences for replication in the new recipient. Stable maintenance of the transferred DNA would require its integration into the host genome. In the laboratory integrative transfer has been detected by plasmids mobilised to yeast by conjugation (Nishikawa *et al.*, 1992). The plasmids used in these studies however contained homology to yeast DNA and thus integrated into the yeast genome by homologous recombination. DNA transferred in nature though would be unlikely to contain significant homology to yeast sequences, therefore, integration would have to occur by illegitimate recombination. Integration in yeast occurs predominantly by homologous recombination, however, illegitimate recombination is possible (Schiestl & Petes, 1991; Gjuracic & Zgaga, 1996). In fact it has recently been shown that Ti plasmid transfer to yeast cells can result in the integration of DNA into the yeast genome by illegitimate recombination (Bundock *et al.*, 1996). Therefore, it is possible that foreign DNA transferred to an organism by conjugation could become integrated into that organisms genome. It is also pertinent to note that integration is reliant on the new recipient's recombination pathway. As mentioned above, in yeast integration occurs predominantly by homology, however, many other organisms do not display a requirement for homology and integration occurs predominantly by illegitimate recombination.

If productive transfer did occur in nature one further question remains, what would be the result of such transfer? Transfer could result in the movement of entire genes into a new organism. The problem would then occur of how these genes could become expressed. Expression would have to be fortuitous perhaps by integration into a transcriptionally active area or by the chance formation of promotor signals. Perhaps more likely is that short coding regions could be inserted into existing genes thereby moving or producing new protein domains and delivering novel functions to existing proteins. The movement of DNA could also be mutagenic by insertional mutagenesis or altering the expression of genes. The expression of genes could be disrupted by insertion events into yeast promotors. Alternatively, it has been shown that bacterial

sequences can increase the transcription of yeast genes (Sidhu & Bollon, 1990). Finally it has been shown that transformation of yeast with single stranded DNA can result in genomic rearrangements. The potentially lethal effects of such rearrangements have been proposed to explain why intact yeast cells are resistant to transformation with single stranded DNA (Gjuracic & Zgaga, 1996).

In summary, we have developed and optimised an efficient protocol to study trans-kingdom transfer from *E. coli* to yeast. Subsequently, two main areas of research have been pursued. Firstly the basis of plasmid promiscuity by the IncPα plasmid RP4 was investigated. This analysis revealed that promiscuity was inherent in the plasmids transfer system. The actual basis of promiscuity remains unclear. Potentially, the basis of promiscuity may lie in the nature of cellular contacts between cells, or stem from the presence of 'pilot' proteins which aid the establishment of the transferred plasmid DNA. The second main line of investigation has been to determine the yeast factors required for transfer. Five yeast mutants (*con1-5*) defective in their ability to receive and establish a conjugatively derived plasmid were isolated. *CON1* was shown to be allelic to the ORF YHR185c. The function of this ORF remains unclear, it may act on the formation of cellular contacts between cells, or in the establishment of the transferred strand. The implications of trans-kingdom transfer are difficult to assess. It is not known if such transfer events can occur in nature. There is, however, phylogenetic evidence to suggest that transfer has occurred, our observations also demonstrate that the potential for transfer exists. As discussed previously transfer in nature would be unproductive, however, the events required for productive transfer, such as integration into the recipient genome, do occur at a finite rate. Therefore, over an evolutionary time scale the promiscuous transfer of DNA could have played an important role in evolution.

Appendix 1

Phenotype of Tetrads

TRP/trp	- Tryptophan non requiring / requiring
URA/ura	- Uracil non requiring / requiring
LEU/leu	- Leucine non requiring / requiring
HIS/his	- Histidine non requiring / requiring
ADE/ade	- Adenine non requiring / requiring
CON^+/con^-	- Wildtype / Mutant (Decreased Transfer efficiency)
EXG^+/exg^-	- Wildtype / Mutant (Increased Transfer Efficiency)
Zym-Sen	- Increased sensitivity to zymolyase
SDS-Res	- Increased resistance to growth in 0.02%SDS

Original *con1* isolate mated with 8HB

A1	MATa	trp	ura	leu	his	ADE	CON^+	
A2	MATa	trp	ura	leu	HIS	ade	CON^+	
A3	MATα	trp	ura	leu	his	ADE	con^-	Zym-Sen
A4	MATα	trp	ura	leu	HIS	ade	con^-	Zym-Sen
B1	MATα	trp	ura	leu	his	ADE	CON^+	
B2	MATα	trp	ura	leu	his	ade	CON^+	
B3	MATa	trp	ura	leu	HIS	ade	con^-	
B4	MATa	trp	ura	leu	HIS	ADE	con^-	
C1	MATa	trp	ura	leu	HIS	ade	con^-	
C2	MATa	trp	ura	leu	his	ADE	CON^+	
C3	MATα	trp	ura	leu	his	ade	CON^+	
C4	MATα	trp	ura	leu	HIS	ADE	con^-	
D1	MATα	trp	ura	leu	HIS	ade	con^-	
D2	MATα	trp	ura	leu	HIS	ADE	con^-	
D3	MATa	trp	ura	leu	his	ADE	CON^+	
D4	MATa	trp	ura	leu	his	ade	CON^+	

E1	MATa	trp	ura	leu	HIS	ADE	CON^+	
E2	MATa	trp	ura	leu	his	ade	con^-	
E3	MATα	trp	ura	leu	his	ade	con^-	
E4	MATα	trp	ura	leu	HIS	ADE	CON^+	
F1	MATα	trp	ura	leu	HIS	ADE	CON^+	
F2	MATa	trp	ura	leu	his	ADE	con^-	Zym-Sen
F3	MATa	trp	ura	leu	HIS	ade	CON^+	
F4	MATα	trp	ura	leu	his	ade	con^-	Zym-Sen
G1	MATα	trp	ura	leu	his	ade	con^-	
G2	MATa	trp	ura	leu	HIS	ade	con^-	
G3	MATa	trp	ura	leu	his	ADE	CON^+	
G4	MATα	trp	ura	leu	HIS	ADE	CON^+	
H1	MATα	trp	ura	leu	HIS	ade	con^-	Zym-Sen
H2	MATa	trp	ura	leu	his	ade	CON^+	
H3	MATα	trp	ura	leu	HIS	ADE	con^-	Zym-Sen
H4	MATa	trp	ura	leu	his	ADE	CON^+	
I1	MATa	trp	ura	leu	HIS	ade	CON^+	
I2	MATa	trp	ura	leu	HIS	ade	CON^+	
I3	MATα	trp	ura	leu	his	ADE	con^-	Zym-Sen
I4	MATα	trp	ura	leu	his	ADE	con^-	Zym-Sen
J1	MATa	trp	ura	leu	HIS	ade	con^-	
J2	MATa	trp	ura	leu	his	ade	CON^+	
J3	MATα	trp	ura	leu	his	ADE	con^-	
J4	MATα	trp	ura	leu	HIS	ADE	CON^+	

Original *con4* isolate mated with MC16

A1	MATa	leu	ADE	trp	HIS	URA	con^-	
A2	MATα	leu	ade	TRP	his	ura	CON^+	SDS-Res
A3	MATa	leu	ade	TRP	HIS	ura	CON^+	SDS-Res
A4	MATα	leu	ADE	trp	his	URA	con^-	
B1	MATα	leu	ADE	trp	his	ura	CON^+	
B2	MATa	leu	ADE	TRP	his	URA	CON^+	SDS-Res
B3	MATα	leu	ade	TRP	HIS	URA	con^-	SDS-Res
B4	MATa	leu	ade	trp	HIS	ura	con^-	
C1	MATa	leu	ade	trp	HIS	ura	CON^+	
C2	MATα	leu	ADE	TRP	his	URA	con^-	SDS-Res
C3	MATa	leu	ade	trp	HIS	ura	CON^+	
C4	MATα	leu	ADE	TRP	his	URA	con^-	SDS-Res
D1	MATα	leu	ADE	TRP	HIS	ura	con^-	
D2	MATa	leu	ade	trp	HIS	URA	CON^+	
D3	MATa	leu	ade	TRP	his	ura	con^-	
D4	MATα	leu	ADE	trp	his	URA	CON^+	
E1	MATα	leu	ADE	TRP	his	URA	con^-	
E2	MATa	leu	ade	trp	his	ura	CON^+	
E3	MATα	leu	ADE	TRP	HIS	URA	CON^+	
E4	MATa	leu	ade	trp	HIS	ura	con^-	
F1	MATα	leu	ADE	TRP	his	URA	con^-	
F2	MATa	leu	ade	trp	HIS	ura	CON^+	
F3	MATα	leu	ADE	TRP	his	URA	con^-	
F4	MATa	leu	ade	trp	HIS	ura	CON^+	
G1	MATα	leu	ADE	TRP	HIS	ura	con^-	
G2	MATa	leu	ade	trp	his	URA	CON^+	
G3	MATa	leu	ade	trp	HIS	URA	CON^+	
G4	MATα	leu	ADE	TRP	his	ura	con^-	

H1	MATα	leu	ade	TRP	HIS	URA	CON^+
H2	MATα	leu	ade	trp	his	URA	con^-
H3	MATa	leu	ADE	TRP	his	ura	CON^+
H4	MATa	leu	ADE	trp	HIS	ura	con^-
I1	MATα	leu	ADE	TRP	HIS	URA	con^-
I2	MATa	leu	ADE	trp	HIS	ura	con^-
I3	MATα	leu	ade	trp	his	URA	CON^+
I4	MATa	leu	ade	TRP	his	ura	CON^+
J1	MATα	leu	ade	trp	his	ura	con^-
J2	MATα	leu	ADE	TRP	HIS	URA	CON^+
J3	MATa	leu	ade	trp	his	ura	con^-
J4	MATα	leu	ADE	TRP	HIS	URA	CON^+

Original *exg1* isolate mated with MC16

A1	MATα	leu	ADE	trp	HIS	URA	EXG^+
A2	MATa	leu	ade	TRP	HIS	ura	exg^-
A3	MATa	leu	ADE	TRP	his	ura	exg^-
A4	MATα	leu	ade	trp	his	URA	EXG^+
B1	MATa	leu	ade	TRP	his	URA	exg^-
B2	MATa	leu	ADE	TRP	his	ura	exg^-
B3	MATα	leu	ade	trp	HIS	ura	EXG^+
B4	MATα	leu	ADE	trp	HIS	URA	EXG^+
C1	MATa	leu	ade	trp	HIS	ura	EXG^+
C2	MATα	leu	ADE	TRP	his	URA	exg^-
C3	MATa	leu	ADE	trp	his	ura	exg^-
C4	MATα	leu	ade	TRP	HIS	ura	EXG^+
D1	MATa	leu	ade	trp	his	ura	exg^-
D2	MATα	leu	ADE	TRP	HIS	URA	EXG^+
D3	MATa	leu	ade	TRP	his	URA	EXG^+
D4	MATα	leu	ADE	trp	HIS	ura	exg^-
E1	MATa	leu	ade	trp	his	ura	EXG^+
E2	MATα	leu	ADE	trp	HIS	ura	EXG^+
E3	MATa	leu	ade	TRP	his	URA	exg^-
E4	MATα	leu	ADE	TRP	HIS	URA	exg^-
F1	MATα	leu	ADE	trp	his	URA	EXG^+
F2	MATa	leu	ade	trp	HIS	URA	exg^-
F3	MATα	leu	ADE	TRP	HIS	ura	EXG^+
F4	MATa	leu	ade	TRP	his	ura	exg^-
G1	MATα	leu	ADE	TRP	HIS	URA	EXG^+
G2	MATα	leu	ade	TRP	his	URA	exg^-
G3	MATa	leu	ADE	trp	his	ura	exg^-
G4	MATa	leu	ade	trp	HIS	ura	EXG^+

H1	MATa	leu	ade	TRP	HIS	ura	exg^-
H2	MATα	leu	ade	trp	his	URA	EXG^+
H3	MATα	leu	ADE	TRP	his	ura	exg^-
H4	MATa	leu	ADE	trp	HIS	URA	EXG^+
I1	MATα	leu	ade	trp	his	URA	exg^-
I2	MATa	leu	ADE	TRP	his	ura	EXG^+
I3	MATa	leu	ade	TRP	HIS	ura	EXG^+
I4	MATα	leu	ADE	trp	HIS	URA	exg^-
J1	MATα	leu	ADE	trp	his	ura	EXG^+
J2	MATa	leu	ade	trp	HIS	ura	exg^-
J3	MATα	leu	ade	TRP	HIS	URA	EXG^+
J4	MATa	leu	ADE	TRP	his	URA	exg^-

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