Analysis of the ribosomes of a micrococcin-resistant strain of *Bacillus megaterium*

by

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Preface

This dissertation contains the results of work carried out in the Department of Biochemistry at Leicester University during the period October 1980 to September 1983.

Some of the results presented in this dissertation have already been accepted for publication:

Gary Spedding and Eric Cundliffe
"Identification of the altered ribosomal component responsible for resistance to micrococcin in mutants of Bacillus megaterium."
European Journal of Biochemistry
(in press).

This dissertation represents the results of my own unaided work. None of this material has been submitted for another degree in this or any other university.

Acknowledgments

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For encouragement, support and for debts I shall never be able to repay I must also acknowledge my parents, Linda, Ms Elizabeth Allen, Mr Murray Fletcher, Ms A. Roberts, Ms C. Dain, and also my excellent typist Mrs Amelia Dunning. Apologies for those I have missed.

"We judge ourselves by what we feel capable of doing, while others judge us by what we have already done."

Henry Wadsworth Longfellow

"A person is a sum of parts of all the people he has ever known."

Gary Spedding

Contents

Abbreviations

Most abbreviations have been defined where they first appear in the text. The most frequent or unusual ones are also listed here.

Ax	:	absorbance at a wavelength of Xnm in a 1 cm light path.
aa-tRNA	:	aminoacyl-transfer RNA.
A.T.C.C.	:	American Type Culture Collection.
c.p.m.	:	counts per minute.
DMSO	:	dimethyl sulphoxide.
DNase	:	deoxyribonuclease.
D.T.T.	:	DL-dithiothreitol.
EDTA	:	ethylenediaminetetraacetic acid.
EF-G	:	elongation factor G.
EF-Tu, Ts	:	elongation factor Tu or Ts.
GDPCP) GMP-PCP)	:	5'-guanylyl-methylene diphosphonate. (β,γ-methylene guanosine 5'-triphosphate).
Hepes	:	N-2-hydroxyethylpiperazine N'-2-ethanesulphonic acid.
MBA	:	N,N'-methylene bisacrylamide.
MSI, MSII, MSIII	:	ppGpp, pppGpp or ppGp (see below).
NAD	:	nicotinamide-adenine dinucleotide.
N.C.I.B.	:	National Collection of Industrial Bacteria.
N.C.T.C.	:	National Collection of Type Cultures.
PBD	:	2-(4'-tert-butyl-phenylyl) -5(4"-biphenylyl) 1,3,4-oxadiazole.
PEI	:	polyethyleneimine.
Poly(U)	:	polyuridylic acid (5').

ppGp	:	guanosine 5'-diphosphate, 3'-monophosphate.
ppGpp	:	guanosine 5'-diphosphate, 3'-diphosphate.
pppGpp	:	guanosine 5'-triphosphate, 3'-diphosphate.
RNA 70, 50	:	total ribosomal RNA derived from 70S ribosomes or 50S ribosomal subunits.
RNase	:	ribonuclease.
r.p.m.	:	revolutions per minute.
S.	:	Svedberg unit (x 10^{-13} sec^{-1})
SDS	:	sodium dodecyl-sulphate.
TCA	:	trichloroacetic acid.
TEMED	:	N,N,N',N-tetramethylethylenediamine.
TP70, 50	:	total proteins derived from 70S ribosomes or 50S ribosomal subunits.
Tris	:	tris(hydroxy methyl) amino methane.

Buffers

Buffer compositions have been abbreviated to letters and superscripts, for example, T^{10} M^{10} A^{50} β^{3} etc. The number refers to the millimolar concentration and the letters used represent:-

T	Tris-HCl (pH 7.6 at 20°C unless otherwise indicated).
M	$MgCl_2$
MgAC	Magnesium acetate.
A	NH_4Cl.
K	KCl
β	2-mercaptoethanol (2 hydroxyethylmercaptan).
E	EDTA.
N	NaCl.

Dialysis

Information concerning dialysis has been given, for example, as (3 x 1l x 2 hr). This means that the dialysis buffer (1 litre) was changed three times at two hour intervals.

CHAPTER 1

INTRODUCTION

S. aureus and S. epidermidis (Pulverer and Jeljaszewicz, 1975) and such substances have been called staphylococcal micrococcins or micrococcin M. The strains from the genus micrococcus were found to produce two antibiotically active substances, M1 and M3 in a ratio of 8:1 (Breiter et al., 1975). Finally, three new antibiotics designated thiocillins I, II and III were isolated from Bacillus species and shown to be related to micrococcin P (Shoji et al., 1976; 1981). Thiocillins I and II have been isolated from B. cereus and compounds II and III from B. badius. It was suggested that as the name micrococcin was too narrowly suggestive of origin that the name 'thiocillin' should be applied to this whole group of substances (Shoji et al., 1976).

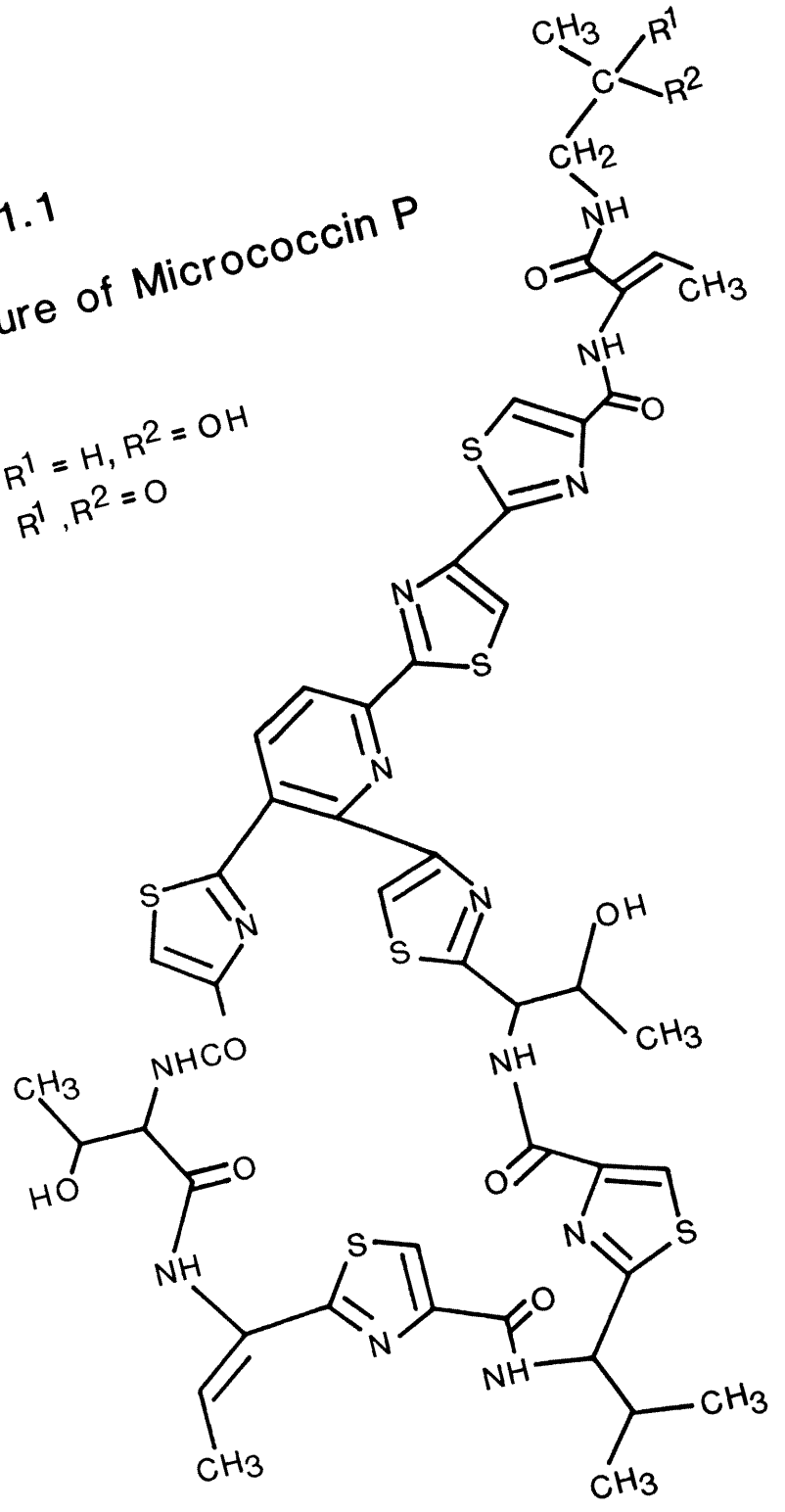
The micrococcins are highly modified peptide (sulphur containing) antibiotics. The structure of micrococcin P was elucidated by [^{13}C] nuclear magnetic resonance spectroscopy (Walker et al., 1977) and slight modifications to the structure (including P_1 and P_2) were reported by Bycroft and Gowland, (1978). It is a polythiazole containing antibiotic with the empirical formula $C_{48}\ H_{49}\ N_{13}\ O_9\ S_6$ and has a molecular weight of 1143 daltons (Walker et al., 1977; Bycroft and Gowland, 1978). The molecule consists mainly of an extended heterocyclic ring system with four thiazoles, three of which are bonded directly to a pyridine nucleus (James and Watson, 1966). These thiazole derivatives are considered to be formed from the incorporation of cysteine and other requisite amino-acids into a peptide chain (probably composed of four molecules of cysteine, one of α-aminobutyric acid and one of α-aminoadipic acid) followed by thiazole ring closure. Subsequent dehydrogenation generates thiazole rings. The α-aminoadipic acid residue by ring closure

Figure 1.1

Structure of Micrococcin P

P_1 R^1 = H, R^2 = OH

P_2 R^1 , R^2 = O



and dehydrogenation produces the pyridine ring (Hall et al., 1966). A product containing the structural features outlined above, and designated micrococcinic acid is liberated from micrococcin upon hydrolysis and is a key feature for the identification of new antibiotics related to micrococcin (Breiter et al., 1975; Shoji et al., 1981). Two other thiazole rings exist in the complete molecule as can be seen in Figure 1.1. Micrococcin is thus related in structure to the better known compound, thiostrepton (Tori et al., 1981) and nosiheptide (Endo and Yonehara, 1978). The importance of cysteine for the synthesis of micrococcin P was confirmed much earlier when it was shown that drug production was markedly stimulated when the amino-acid was supplemented to the growth medium (Brookes et al., 1960).

Micrococcin and thiostrepton have been shown to possess similar modes of action (Cundliffe et al., 1975 and for details of thiostrepton, Cundliffe, 1979a) and to exert similar effects upon protein synthesis both in intact Gram positive bacteria and in prokaryotic cell extracts.

Micrococcin was first claimed to be an inhibitor of translocation (Pestka and Brot, 1971; Otaka and Kaji, 1974). The latter group based their conclusion upon the results of three independent assays which were supposed to measure translocation. One of these assays, once considered to be a direct method for measuring the translocational event, was based upon the release of deacylated tRNA from the ribosome. It was assumed that the action of elongation factor G was to cause the release of deacylated tRNA from the ribosomal P site and that translocation was a consequence of this release. It

has since, however, been realised that the extent of translocation (as measured by the release of peptidyl puromycin for example) generally exceeds the amount of released tRNA. Furthermore these data can be re-interpreted according to a model for three tRNA binding sites on the ribosome (Rheinberger et al., 1981) which states that deacylated tRNA is not expelled from the ribosomal P-site but is translocated to an adjacent site located "upstream" from the P-site, designated the "E" or exit site. Moreover, the release of deacylated tRNA from this latter site is primarily dependent upon the binding of aminoacyl tRNA to the ribosomal A-site (Rheinberger and Nierhaus 1983; Rheinberger et al., 1983) and is not a consequence of nor a prerequisite to translocation. The other two assays employed (Otaka and Kaji, 1974) relied upon factor EF-G dependent translocation to vacate the ribosomal A-site. Following this, the extent of translocation was measured either by the release of N-acetyl[^{14}C]phenylalanine from the P-site by puromycin or by binding of N-acetyl[^{14}C]phenylalanyl-tRNA (N-acetyl[^{14}C]phe-tRNA) to any vacant ribosomal A-sites. In this work it was assumed that two N-acetyl[^{14}C]phe-tRNA molecules were bound per ribosome. This has since been shown not to be possible however, as an exclusion principle determines that not more than one N-acetylaminoacyl-tRNA can be bound per ribosome (Rheinberger et al., 1981). Thus the interpretations of Otaka and Kaji are, arguably, questionable. However, this subject will not be considered any further here.

It was also reported (Otaka and Kaji, 1974) that micrococcin could inhibit factor EF-Tu dependent binding of phenylalanyl-tRNA to the ribosomal A-site, *in vitro*, when the P-site was blocked with deacylated tRNA. This was also checked out in a system directed by MS2 RNA in which formyl--methionyl tRNA was directed to the ribosomal P-site. [^{14}C] alanyl-tRNA binding to the A-site, dependent upon factor EF-Tu, was measured and found to be inhibited by micrococcin. These results were also confirmed by the use of intact bacterial protoplasts (Cundliffe and Dixon, 1975). The drug was shown to preserve, intact, bacterial polysomes (Pestka and Hintikka, 1971) but did not prevent the subsequent release by puromycin of the majority of nascent peptides from the polysomes (Cundliffe and Dixon, 1975). Thus translocation could not be the inhibited reaction *in vivo*.

That the drug is a specific inhibitor of the ribosomal A-site was indicated when it was observed to inhibit the formation of ternary complexes consisting of ribosomes, elongation factor G and guanine nucleotides (Cundliffe and Thompson, 1981a). See the section on the "single GTPase" centre (page 21).

A novel effect of micrococcin was then demonstrated,

besides its inhibitory effects upon the direct reactions of protein synthesis. It was shown that the drug stimulated, quite dramatically, the uncoupled GTP hydrolysis, dependent upon ribosomes and factor EF-G (Cundliffe and Thompson, 1981a). Thiostrepton, on the other hand, markedly inhibits this reaction (Pestka, 1970 and see page 19 for details) and moreover, it is only in their effects upon this reaction that thiostrepton and micrococcin have been shown to act differently. Thiostrepton however, generally acts the more potently. For example, micrococcin will inhibit synthesis of the regulatory nucleotides guanosine tetra- and pentaphosphate dependent upon ribosomes and stringent factor (Cundliffe and Thompson, 1981a; this dissertation page 88). However the inhibition caused by this drug is not as marked as that caused by thiostrepton (Stark and Cundliffe, 1979a). The stringent response is covered in more detail later in this introduction (page 36).

In keeping with the similar modes of action of these two drugs (micrococcin and thiostrepton) their ribosomal binding sites have been shown to be closely related. Both these antibiotics can bind to complexes formed between bacterial 23S rRNA and ribosomal protein L11 (Thompson *et al.*, 1979; Cundliffe and Thompson, 1981a). Moreover, when micrococcin was bound first it was shown to inhibit the subsequent binding of radiolabelled thiostrepton (Cundliffe and Thompson, 1981a). Micrococcin had previously been shown to inhibit, weakly the binding of [^{35}S] thiostrepton to ribosomes of *B. megaterium* (Cundliffe and Dixon, 1975) and to exert a similar effect with 50S ribosomal subunits of *E. coli* (Cundliffe and Thompson, 1981a). From the data obtained however, it appeared that thiostrepton was bound with

the greater affinity. Ribosomes from the thiostrepton producing organism Streptomyces azureus are known to be totally resistant to the thiostrepton group of antibiotics, including micrococcin (Thompson and Cundliffe, 1980). Moreover it was ultimately shown that resistance to this group of drugs could be conferred by the action of a methylase, from S. azureus, upon 23S rRNA (Cundliffe, 1978). Notably, the methylase introduces only a single methyl group into adenosine residue 1067 of 23S rRNA (Cundliffe and Thompson, 1979; Thompson et al., 1982a). Furthermore, resistant strains of B. subtilis and B. megaterium selected on thiostrepton have also been shown to be cross--resistant to micrococcin. Subsequent studies indicated that the 50S ribosomal subunits from these thiostrepton-resistant mutants were devoid of a single protein, homologous with ribosomal protein L11 of E. coli and this deficiency evidently accounted for the phenotype of those strains (Wienen et al., 1979; Cundliffe et al., 1979). It is of importance to note that total resistance to micrococcin and thiostrepton is conferred by the action of a methylase on 23S rRNA whereas only partial resistance is observed in those strains possessing ribosomes which lack protein L11. This suggests that the primary binding site for these drugs is on 23S rRNA but that binding is significantly enhanced by ribosomal protein L11 (or its homologues).

To conclude this introductory section on micrococcin it is of interest to note that the thiostrepton producing organism S. azureus and (at least some of) the micrococcin producing strains employ different modes of self-protection. The method of protection in S. azureus has been discussed above and moreover the same mechanism of resistance was also found in another

thiostrepton producing organism, S. laurentii (Thompson and Cundliffe, 1980). The ribosomes of Su's micrococcus and B. pumilus however are susceptible to micrococcin in vitro although the whole cells of these organisms are resistant to their own toxic products (Dixon et al., 1975). Thus, different modes of self-protection are employed by the respective drug producing organisms even though they produce closely similar toxic compounds.

Translocation

Early in the 1960's it was discovered that cell-free extracts of E. coli could support the transfer and polymerization of amino-acids from aminoacyl-tRNA into protein (Nathans and Lipmann, 1961). (Most of the studies to be discussed here were made with E. coli and this should be assumed throughout; exceptions will be indicated where appropriate.) Subsequently a GTP-hydrolysing activity was discovered upon supplementation of salt-washed ribosomes with a partially purified protein fraction from the supernatant (Nathans et al., 1962). This fraction was further resolved into two complementary components, originally termed "A" and "B" (Allende et al., 1962; 1964). Furthermore, it was subsequently shown that fraction "A" was relatively unstable and responsible for the polymerization activity whereas "B" possessed a GTPase capability and was a heat stable component. (At around this time the "A" component became known as factor T for transfer activity and "B" was re-named factor G for GTPase component.)

The ribosome-linked guanosine triphosphatase was further characterized and it was shown that both 70S ribosomes plus

factor G were required together for activity. The 30S ribosomal subunit was essentially free of such GTP-hydrolysing ability, whereas the 50S subunit could support activity (albeit at a much lower level than that supported by the intact 70S ribosome). Addition of polyuridylic acid, or other polynucleotides and sRNA (tRNA) were found to stimulate the ribosome and factor G-dependent GTP hydrolysis reaction. Thus, GTP hydrolysis and amino-acid polymerization were shown to be coupled under normal circumstances but the two could be separated *in vitro* (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966a). Subsequently, the protein factor T was resolved into two components which were designated Ts and Tu, since the former was stable whereas the latter was unstable, when heated (Lucas-Lenard and Lipmann, 1966). Evidence was then presented (Allende *et al.*, 1967) that factor Tu might interact with and bind GTP, following the detection upon Millipore filters of a complex containing [^{3}H]-GTP and protein. Confirmation that factor Tu could interact with GTP came about when this nucleotide was shown to protect and stabilize the otherwise labile polymerization factor (Seeds and Conway, 1967). Incorporation of phenylalanine was measured after heating the factor Tu in the presence and absence of GTP. Under the latter conditions, activity of the factor was reduced. Moreover, the nucleotide also protected factor Tu against inactivation by urea. Guanosine diphosphate or the non-hydrolysable analogue of GTP, GMP-PCP (5'-guanylyl-methylene diphosphonate otherwise known as β,γ-methylene-guanosine 5'-triphosphate) were also shown to protect factor Tu but other nucleotides e.g. ATP, ADP, UTP etc. did not do so.

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A protein fraction containing unresolved factor T (i.e. Tu and Ts) was found to catalyse the binding of aminoacyl-tRNA to ribosomes in the presence of mRNA and GTP. This supernatant fraction was subsequently shown to have a high affinity for GTP and to possess a latent GTPase activity. Moreover, this activity could be stimulated by aminoacyl-tRNA but not by deacylated-tRNA (Ravel et al., 1967). Protein from this fraction containing the factor T components could form a complex with [^{3}H]-GTP which was retained on Millipore filters. Following on from this, it was shown that when aminoacyl-tRNA was added to GTP and factor T a (Millipore filtrable) ternary complex was formed (Gordon, 1968). This ternary complex was shown, in a series of classic experiments, to bind very readily to ribosomes (Ravel et al., 1967; Lucas-Lenard and Haenni, 1968). Moreover, in the absence of GTP no factor T dependent binding of aminoacyl-tRNA to ribosomes occurred. The currently accepted series of events involves the binding to ribosomes of the ternary complex, containing factor Tu (now known as elongation factor Tu or EF-Tu) GTP and aminoacyl-tRNA and is followed by the release of the binary [EF-Tu·GDP] complex and inorganic phosphate. Elongation factor Ts is required for the recycling of [EF-Tu·GDP] to yield a noncovalent [EF-Tu·EF-Ts] complex which then interacts with GTP and aminoacyl-tRNA with release of factor EF-Ts.

It was proposed (Nishizuka and Lipmann, 1966a) that GTP and factor G are involved concurrently in translocation of the newly synthesized peptidyl-tRNA from the aminoacyl-tRNA (A) site to the peptidyl-tRNA (P) site. This became apparent following a series of elegant experiments performed in several

laboratories (Pestka, 1968; Erbe and Leder, 1968; Erbe et al., 1969; Haenni and Lucas-Lenard, 1968). These studies involved highly purified systems for protein synthesis derived from E. coli and made use of synthetic mRNA's, initiated by the formyl-methionyl codon AUG, followed by 3, 6 or 9 uridylic acid residues (Erbe and Leder, 1968; Erbe et al., 1969). Synthesis of di, tri and tetra peptides, each initiated by formyl--methionine, was followed and the products were analysed by electrophoresis. It soon became apparent that after one peptide bond had been formed, incubation with factor G and GTP was required before the peptide chain could be lengthened by another amino-acid residue. The experiments of Haenni and Lucas-Lenard (1968) utilized the stepwise addition of phe-tRNA to ribosomes initiated with N-acetyl[^{14}C]phe-tRNA and programmed with polyuridylic acid. In the presence of factor T and GTP (in the absence of factor G) polymerization stopped at the dipeptide stage. Puromycin was then employed in order to look for the release of N-acetyldiphenylalanine, and this release of peptidyl-puromycin was shown to be dependent upon factor G and GTP. A reduced level of synthesis of peptidyl--puromycin in the absence of factor G and the guanosine trinucleotide was also noted independently by others (Brot et al., 1968; Tanaka et al., 1968; Erbe et al., 1969). Tripeptide product formation was therefore dependent upon added factor G and nucleotide. Furthermore, these results were consistent with the participation of factor G in the translocation of peptidyl-tRNA which also exposes the next mRNA codon for translation. The cycle of reactions are repeated in a propagative fashion in the presence of transfer factor Tu,

elongation factor G (now known as factor EF-G) and GTP.

Preparation and characterization of a homogeneous enzyme from the protein fraction of E. coli, containing factor G was made (Kaziro and Inoue, 1968) and it was reported that the molecular weight of the enzyme was 99,000 daltons, as determined by equilibrium sedimentation. It was then discovered that the rate of protein synthesis, in vitro was linearly dependent upon the concentration of pure enzyme (here reported as a monomeric protein of molecular weight 72,000 daltons) until approximately one molecule of translocase (elongation factor G) was present per ribosome (Leder et al., 1969). A value of 83-84,000 daltons for the molecular weight of the purified G-factor began to emerge, following work in several laboratories (Parmeggiani and Gottschalk, 1969a; Kaziro et al., 1972). Then, from a new method for preparing EF-G, based upon complex formation involving factor EF-G, ribosomes and GDP, in the presence of fusidic acid, (Rohrbach et al., 1974) a figure of 81,000 daltons was quoted for the molecular weight of the factor. The transfer factor EF-Tu and elongation factor G were subsequently shown by antibody equivalence studies to be present in the cell at roughly one mole of each per mole of ribosomes and the relative amount of the factors to ribosomes remained constant at different growth rates (Gordon, 1970).

The binding of factor G to E. coli ribosomes was shown to be stimulated by GTP and other guanosine nucleotides, using for example [^{3}H]GTP in complex formation with ribosomes and factor G and subsequent elution of the complex from a Sephadex column (Brot et al., 1969). No radioactivity was associated with the high molecular weight complex when [γ^{32}P]-GTP was employed.

However when GTP was equally labelled in both the β and γ-phosphate moieties [^{32}P]-radioactivity was found in the complex, suggesting that GDP and not GTP was present. Moreover, it was discovered that binding of factor G to ribosomes did not actually require the hydrolysis of GTP because non-hydrolysable analogues of this nucleotide also promoted binding (Brot et al., 1969; Parmeggiani and Gottschalk, 1969b; Kuriki et al., 1974). It was confirmed that GDP was present in the ternary complex (formed from ribosomes, GTP and factor G) by chromatographic analysis of the product (Bodley et al., 1969).

At this time the 50S ribosomal subunit was shown to possess the binding site for factor EF-G and to promote, together with the factor, the GTPase reaction. The 30S ribosomal subunit was inert in these respects. However, compared with 70S particles, the 50S subunit exhibited only about 30% activity in uncoupled GTP hydrolysis, which suggested that the 30S ribosomal subunit has a functional role in this process (Bodley and Lin, 1970).

Inhibitors of Elongation Cycle Events

Fusidic acid and related antibiotics were observed to inhibit the ribosome and factor G dependent GTPase activity and also to inhibit polypeptide synthesis (Tanaka et al., 1968). Puromycin dependent release of peptide from the ribosomes was not significantly altered by fusidic acid in the absence of GTP and factor G but the in vitro reaction, enhanced by these two compounds was inhibited. The results obtained suggested that fusidic acid was affecting polypeptide synthesis by inhibition of G factor dependent GTPase and also translocation of peptidyl

tRNA on ribosomes. Two independent reports then indicated that fusidic acid did not exert its effects through a previously proposed inhibition of ternary complex formation. More of this complex [Ribosome·EF-G·GDP] was in fact formed in the presence of the drug (Bodley et al., 1969; Kuriki et al., 1974). It was speculated that this antibiotic inhibited GTP hydrolysis not directly but rather by preventing the dissociation of the GDP containing complex. Later reports stated that fusidic acid promoted the formation of a relatively stable quaternary complex containing ribosomes, EF-G, GDP and fusidic acid in equimolar amounts (Bodley et al., 1970a). A crucial report was then made concerning the hydrolysis of a single round of guanosine triphosphate in the presence of fusidic acid (Bodley et al., 1970b). When GTP was limiting (i.e. ribosomes and factor EF-G were present in excess) the rate and extent of uncoupled GTP hydrolysis was unaffected by fusidic acid. Moreover, when GTP was present in excess over ribosomes and factor G a "burst" of fusidic acid resistant hydrolysis was observed. This was approximately equivalent to the molar amount of factor G present when the molar amount of ribosomes was greater than that of the factor. Fusidic acid then inhibited subsequent hydrolysis. Thus, single rounds of GTP hydrolysis were not inhibited by fusidic acid but multiple rounds were. It indeed appeared that the antibiotic inhibited the overall-uncoupled hydrolysis of GTP by preventing only the dissociation of the ternary complex [ribosome EF-G·GDP]. Furthermore in the presence of fusidic acid, when ribosomes and factor EF-G were present in excess over GTP all the nucleotide could be sequestered upon ribosomes.

Up to now each of the effects of fusidic acid had been

interpreted according to a model whereby the drug inhibited the translocation reaction. However, the results of experiments carried out *in vivo* in bacterial protoplasts revealed that fusidic acid was not an inhibitor of translocation after all (Cundliffe, 1972). Following treatment with fusidic acid (which stopped peptide chain elongation) it was found that puromycin caused a significant release of nascent peptides from ribosomes. An inhibitor of translocation ought to have prevented the puromycin reaction by restricting peptides to the A-sites of the ribosomes. It was thus concluded that fusidic acid was similar in action to chlortetracycline and that it restricted nascent peptides to the ribosomal P sites. Further, it was argued that this effect could only have been brought about by prevention of functional binding of aa-tRNA to the ribosomal A-sites (Cundliffe, 1972). These data began to argue in favour of a previously proposed "single GTPase" site on the ribosome with which both factors Tu and G may interact, near the ribosomal A-site. According to this model fusidic acid stabilizes the [factor G·GDP] complex on the 50S ribosomal subunit and thereby prevents aa-tRNA binding to the A-site, catalysed by factor Tu and GTP. Such results were also supported by *in vitro* experiments (Celma *et al.*, 1972). These authors also observed that phe-tRNA bound to the ribosomal A--site with or without EF-Tu and GTP, blocked the subsequent binding of factor EF-G, in the presence of fusidic acid. This drug was then subsequently shown not to inhibit the translocation of N-acetylphenylalanyl-tRNA bound non--enzymatically to the ribosomal A-site, when the amount of ribosomes in the reaction mix was not sufficient to bind all

the available factor EF-G in the form of complexes (Modolell et al., 1973). In these experiments, translocation from the ribosomal A to P-site was followed by increased release of peptides by puromycin. Moreover the finding that an excess of uncharged ribosomes, more than sufficient to trap all the available factor EF-G, was required before fusidic acid could inhibit translocation helped to resolve the conflicting reports concerning the mode of action of this drug. These were that in some purified systems the antibiotic clearly inhibited translocation whereas in other (more physiological) systems it did not. Rather the drug seemed primarily to inhibit aa-tRNA binding, probably through formation of the complex containing ribosomes, factor EF-G, GDP and fusidic acid. It was also argued that the above complex could only form on ribosomes whose A-sites were devoid of tRNA and that in earlier studies, this might have occurred upon otherwise inactive ribosomes which usually predominated in such systems. Accordingly, the observed inhibition of translocation by fusidic acid arose indirectly and was due to sequestration of the elongation factor G, which was therefore not able to promote translocation upon active ribosomes (Modolell et al., 1973; Burns et al., 1974).

In the same studies designed to determine the true mode of action of fusidic acid, experiments were performed which followed GTP hydrolysis associated with translocation, in the presence of the antibiotic. It was found that one molecule of GTP was hydrolysed per molecule of N-acetylphenylalanyl-tRNA translocated (Modolell et al., 1973).

The related peptide antibiotics siomycin and thiostrepton have contributed greatly to the understanding of factor EF-G

dependent GTP hydrolysis and the GTPase centre of the ribosome. These antibiotics were also initially reported to inhibit the translocation reaction dependent upon EF-G and GTP but not peptide bond formation (Tanaka et al., 1970; Pestka, 1970). On the one hand (Pestka, 1970) it had been reported that thiostrepton inhibited factor G dependent GTPase whilst other workers showed that this antibiotic prevented formation of a complex containing ribosomes, factor G and guanine nucleotides and that it also inhibited GTPase dependent upon factor EF-G (Bodley et al., 1970c; Weisblum and Demohn, 1970). Thiostrepton proved to be different from fusidic acid in that the latter drug only seemed to inhibit enzymic translocation but not the non--enzymic reaction (i.e. a translocation not dependent upon factor EF-G and GTP). Thiostrepton inhibited both reactions and prevented the formation of the ternary complex containing ribosomes, GDP and EF-G whereas fusidic acid stabilised such complexes. Conversely ribosomes were protected from the action of thiostrepton by prior binding of factor G and GTP (Highland et al., 1971). Besides leading to loss, by ribosomes, of the ability to bind GDP in response to EF-G, thiostrepton also inhibited their ability to polymerize amino-acids into peptides. Again, prior binding of factor G and GDP prevented the inactivation by this drug of this polymerizing ability.

Further information concerning the mode-of-action of siomycin and thiostrepton came from experiments performed by Modolell et al., (1971a;b). Siomycin like thiostrepton was shown to inhibit GTPase and not peptidyl transferase (Modolell, 1971a). Moreover it inhibited the binding of [^{14}C]-GTP to 70S ribosomes or to 50S ribosomal subunits in the presence of fusidic acid and factor

EF-G. Furthermore it was concluded that siomycin prevented interaction of EF-G with the 50S subunit. Both siomycin and thiostrepton were then shown to inhibit the binding of aa-tRNA as well as factor G to ribosomes *in vitro* (Modolell, 1971b). That the inhibition of these two events was a manifestation of the same interaction of siomycin with ribosomes was evident through parallel responses to increasing concentrations of the drug. Siomycin and thiostrepton completely abolished the GTPase activity associated with the binding of aa-tRNA, catalysed by factor EF-Tu. (In this respect these two drugs are unlike other inhibitors of aa-tRNA binding, e.g. tetracycline, sparsomycin and streptogramin A.) Furthermore it was presumed that siomycin prevented detectable interaction between the ternary complex (containing [factor EF-Tu·GTP·aa-tRNA]) and ribosomes. The mode of action of siomycin and thiostrepton as inhibitors of translocation was placed in question when it was shown that the latter drug inhibited protein synthesis *in vivo* but that subsequent treatment of ribosomes with puromycin led to release of nascent peptides. These results which confirmed the *in vitro* data indicated that the drug achieved its effect by preventing aminoacyl-tRNA-binding to the ribosomal A-site (Cundliffe, 1971). Similar results with thiostrepton were obtained from *in vitro* experiments (Cannon and Burns, 1971) and with siomycin (Celma *et al.*, 1972). Thus, thiostrepton, like siomycin, appeared to inhibit both EF-G and EF-Tu dependent functions and gave rise to the idea that factors EF-Tu and EF-G might have mutually exclusive binding sites on the 50S ribosomal subunit.

The "Single GTPase" Site

Further insight into the "single GTPase" hypothesis came about when it was discovered that EF-G, when bound to ribosomes in the presence of fusidic acid plus GDP or a non-hydrolysable analogue of GTP, could prevent EF-Tu dependent binding of aminoacyl-tRNA to the ribosome. Furthermore GTPase associated with the aa-tRNA binding was also inhibited when ribosomes were prebound with factor EF-G (Cabrer et al., 1972; Miller, 1972 and Richman and Bodley, 1972). Moreover non-enzymic binding of aa-tRNA at high Mg^{++} ion concentrations was also blocked by factor EF-G. Similar results were obtained by Richter (1972) who also showed the converse, namely that ribosomes with pre--bound complex containing [EF-Tu·GMP-PCP·phe-tRNA] were less active in binding [EF-G·GTP]. (Stable complex formation between EF-Tu and ribosomes is dependent upon a non-hydrolysable GTP analogue such as GMP-PCP and aa-tRNA. If GTP is used the complex of factor and nucleotide dissociates rapidly from the ribosomes as [EF-Tu.GDP].)

Thus EF-G can block the ribosomal A-site and this reinforced the observation that the factor G-site was located near enough to influence or overlap the A-site on the 50S ribosomal subunit. Moreover, together with the information gained from employment of siomycin and thiostrepton, the "single GTPase" site was now seriously implicated. (An alternative to a direct mutual exclusion of binding of the two soluble factors would be that they bind to similar but not identical sites and that the exclusion of one factor when the other is bound is due to conformational perturbations of the ribosomal structure.)

It is of interest and importance to state, briefly that the

site of binding for factors EF-Tu and EF-G is also the binding site for ribosomal initiation and release factors. Initiation factor-2 (IF-2) interaction with ribosomes is followed by GTP hydrolysis and thus implicates a universal GTPase centre on the ribosome. It was evidence from the use of thiostrepton and from competition experiments with factor EF-G which suggested that the site of the IF-2-dependent GTP hydrolysis is close to the elongation factor site(s) (Grunberg-Manago et al., 1972). Thiostrepton inhibited IF-2 and EF-G dependent GTPase activities to a significant and similar extent. Moreover, the formation of complexes containing ribosomes, EF-G, GDP and fusidic acid also inhibited IF-2 dependent GTP hydrolysis.

The Components of the GTPase Centre

I shall now attempt to present the evidence for the "building up" of the GTPase centre upon the ribosome (or more specifically, the 50S ribosomal subunit). An acidic ribosomal protein was first implicated as being important for the GTPase centre of the ribosome. This acidic protein designated "A" (Kischa et al., 1971) was subsequently resolved into two components, 'A1' and 'A2'. Furthermore, it very quickly became established that these were identical to ribosomal proteins L7 and L12, respectively. These two proteins, the product of a single gene differ only in that protein L7 is acetylated at its N-terminus (Terhorst et al., 1973). In solution the protein L7/L12 exists as a stable dimer and two dimers are present on the ribosome (reviewed by Matheson et al., 1979). When ribosomes or 50S ribosomal subunits were treated in such a way that they lost these acidic proteins (L7/L12) there was also a selective loss of GTPase activity

(Kischa et al., 1971; Hamel et al., 1972). One such procedure, which became very popular, involved washing ribosomes or 50S ribosomal subunits in 1M NH_4Cl in the presence of ethanol (Hamel et al., 1972). The resultant core particles were markedly deficient in uncoupled GTPase, dependent upon factor EF-G and also in factor dependent binding of [^{3}H]-GTP. However, these activities could be restored upon addition of the NH_4Cl/ethanol extract (Hamel et al., 1972; Sander et al., 1972; Brot et al., 1972; 1973). Moreover, such cores were also somewhat deficient in factor EF-Tu and IF-2 dependent GTP hydrolysis reactions (Hamel et al., 1972). Different core particles were produced when 50S ribosomal subunits were treated with ammonium chloride and 40-50% ethanol at different temperatures. Subunits treated at 0°C yielded 'P_0' cores, lacking proteins L7/L12, together with a corresponding supernatant containing these proteins. (Different groups of workers assigned various names to these cores and 'split protein fractions' but the modern nomenclature is used here for clarity.) Further treatment of the 'P_0' cores at either 30°C or 37°C produced 'P_0-30' or 'P_0-37' core particles which were also deficient in proteins L10 and L11 (Hamel et al., 1972; Brot et al., 1973; Schrier and Möller, 1975; Bernabeu et al., 1976). Moreover, in one instance, it was reported that direct treatment of 50S subunits at 37°C could produce 'P37' cores, deficient in proteins L7/L12 and L10 but not L11 (Highland and Howard, 1975). Other reports however failed to show any difference between cores prepared by treatment of subunits, firstly at 0°C and then at a subsequent higher temperature or those prepared by direct incubation of subunits at the higher temperature. (Following the more

standard convention, these latter core preparations were designated as 'P30' or 'P37' cores and their corresponding proteins as 'P30' or 'P37' split proteins.)

When the split proteins from a 'P30' core preparation were incubated with 'P_0-30' core particles they were found to stimulate, by 2-3 fold, the binding of purified ribosomal proteins L7/L12 to these cores. Assays of factor EF-Tu or EF-G dependent GTP hydrolysis were used to assess activity in these experiments. Such results indicated that other proteins in addition to L7/L12 were involved in the GTPase centre (Brot et al., 1973). The importance of other ribosomal proteins involved in L7/L12 binding to the ribosome was confirmed independently by three groups (Schrier et al., 1973; Stöffler et al., 1974; Highland and Howard, 1975). Ribosomal core particles, prepared by centrifugation of 50S subunits through CsCl, together with groups of individual 'split-proteins' were tested for their capacity to support EF-G dependent GTP hydrolysis. A combination of ribosomal proteins L10, L7 and L12 when incubated with core particles were found to support higher levels of hydrolysis than either L7 or L12 alone. Moreover there was a lower input requirement for protein L12 when L10 was also present. Furthermore, ribosomal protein L6 was also shown to enhance the level of GTP hydrolysis in the presence of L7 or L12 (Schrier et al., 1973). The primary importance of protein L10 for the binding to ribosomes of L7/L12 was confirmed later (Highland and Howard, 1975). Again various core particles were incubated with 'split proteins' and their ability to bind factor EF-G was then measured. It was found that ribosomal proteins L7/L12 could not rebind to 'P37' core particles

(lacking protein L10) unless this latter protein was also present in the incubation mixture. Without the binding of L7/L12 to cores lacking these proteins no factor EF-G could be bound to the ribosome. The other group (Stöffler et al., 1974) found that they could inhibit the binding of protein L7/L12 to 50S ribosomal cores by monovalent antibody fragments, specific for ribosomal proteins L6, L10 and L18 and this subsequently resulted in a loss of interaction of ribosomes with factor EF-G. Thus, all the data suggested that ribosomal proteins L6 and L10 help to comprise the binding site for 50S ribosomal proteins L7/L12.

An important observation, implicating more clearly that L7/L12 formed the site of action of factor EF-G was also established through the use of antibodies raised against 50 of the 55 individually purified ribosomal proteins of E. coli (Highland et al., 1973). Individual antibodies were tested for their ability to interfere with the formation of complexes containing ribosomes, EF-G and [^{3}H]-GDP. Only those antibodies or monovalent antibody fragments raised against L7/L12 inhibited complex formation and moreover, they did so completely. (At the time no antibodies were available to ribosomal proteins L26, L31, L32 or L34.) It is interesting to note that no effect was seen with antibody raised against protein L8, which later proved to be a complex of proteins L7/L12 and L10, and which can exist in solution with the pentameric structure $[L7/L12]_4 \cdot L10$ (Österberg et al., 1977).

The role of protein L7/L12 in ribosome dependent functions of initiation factor IF-2 was also investigated (Kay et al., 1973; Lockwood et al., 1974). Following the removal of proteins L7/L12 from 50S ribosomal subunits both the coupled and uncoupled

GTPase activities, dependent upon factor IF-2 were greatly diminished as was formyl methionyl tRNA binding to ribosomes. Although the activities of factor IF-2 were less affected by the absence of protein L12 than were those of factor EF-G, these data taken together with others outlined above (Grunberg-Manago et al., 1972) suggested that the elongation factors and IF-2 utilized similar sites of action.

A functional importance for ribosomal protein L11 in the EF-G dependent GTP hydrolysis on E. coli ribosomes was then shown (Schrier and Möller, 1975). Core particles ('P_0-37' cores) lacking several proteins, including L11, when supplemented with proteins L10 and L7 were found to exhibit low-level activity in EF-G dependent GTP hydrolysis, but retained a capacity to bind [^{3}H]-GMPPCP in the presence of factor EF-G. A striking increase in GTPase activity was observed upon readdition of ribosomal protein L11. As a result of these observations it seemed in contrast to the GTPase reaction, that the mutually dependent binding of nucleotides and factor EF-G to ribosomes did not require protein L11 and moreover, this protein might therefore constitute the active site of hydrolysis (Schrier and Möller, 1975).

Thus, in summary, it appears that the stimulation of EF-G dependent GTP hydrolysis by protein L11 (albeit that this protein is not indispensable for this activity) may be expressed through its interaction with other ribosomal proteins, notably L7/L12 and L10, the latter proteins probably being the factor binding site on the ribosome (Schrier and Möller, 1975; Highland et al., 1973). Further evidence for a close proximity of these ribosomal proteins is presented below.

In building up a comprehensive picture of the GTPase centre on the ribosome, crosslinking studies have proved to be of great importance. As stated earlier, ribosomal protein L8 proved to be a pentameric complex of proteins L7/L12 and L10 (Österberg et al., 1977). This complex was shown to be capable of binding to ribosomal 23S rRNA (Dijk et al., 1977) and it has also been reported that the protein complex can bind ribosomal protein L11 in solution (Behlke and Gudkov, 1980). The latter fact was in support of crosslinking data which located ribosomal proteins L10 and L11 in close proximity on the ribosome. The protein-specific crosslinking reagent, dimethylsuberimidate was employed and crosslinked products were analysed by gel electrophoresis. From these studies multiple forms of the L10-L11 complex were identified which probably represented different degrees of intermolecular crosslinking (Expert-Bezançon et al., 1975). Through the use of dimethylsuberimidate other results obtained led to a tentative suggestion that ribosomal proteins L8 (therefore L7/L12 is included by hindsight) L9, (L10) and L11 were near neighbours (Clegg and Hayes, 1974). A close proximity of E. coli 50S ribosomal subunit proteins L7/L12, L10 and L11 was also implied from the use of another crosslinker, dimethyladipimidate (Expert--Bezançon et al., 1976).

Elongation factor G dependent binding of a photoreactive GTP analogue to ribosomes from E. coli was found to label ribosomal protein L11 (Maassen and Möller, 1978). Here a photoreactive derivative of GTP [^{3}H]-azidosalicyl-GTP was prepared and found to be a competitive inhibitor with respect to GTP for EF-G dependent GTP hydrolysis. Specific incorporation of [^{3}H]--radioactivity into ribosomal protein L11 and to a lesser extent

into L5 and L18 was detected. As azidosalicyl GTP inhibited EF-G dependent binding of a non-hydrolysable analogue of GTP to ribosomes it was argued that the photo-affinity analogue binds to the same site as the physiological nucleotide, GTP. Thus, it was shown to fulfil the most important requirement for its use in photo-affinity labelling studies. Azidosalicyl-GTP is in itself non-hydrolysable and consequently the photolabile group remained attached to the γ-phosphate group of GTP during the experiments. That L11 was the most highly labelled protein identified its location near the γ-phosphate of azidosalicyl-GTP.

Results from the same laboratory (Maassen and Möller, 1974) using a photoreactive GDP derivative, azidophenyl GDP had also implicated ribosomal protein L11 as well as L5, L18 and L30 as being involved in the binding of GDP.

The notion that ribosomal proteins L5 and L18 might form part of the guanine nucleotide binding site on the E. coli ribosome could be correlated with earlier studies of Horne and Erdmann (1973,1974) who worked both with E. coli and B. stearothermophilus. In either case, complexes of 5S rRNA together with two or three ribosomal proteins were claimed to possess ATPase and GTPase activities, which were independent of factor EF-G. Those complexes from B. stearothermophilus contained principally, proteins BL5 and BL22 (corresponding to E. coli ribosomal proteins L5 and L18 respectively) whereas those from E. coli contained proteins L5, L18 and L25. It was also claimed that the GTPase activity supported by the complexes from B. stearothermophilus was inhibited by thiostrepton and fusidic acid; however this was not the case with complexes containing components from E. coli (Horne and Erdmann, 1973; 1974; Gaunt-Klöpfer and Erdmann, 1975).

When complexes of *B. stearothermophilus* 5S rRNA plus proteins BL2, BL10 and BL22, supplemented with factor EF-G and fusidic acid, were photoirradiated in the presence of azidophenyl-GDP, proteins BL10 and BL22 were selectively labelled as was the case when the same GDP analogue was incorporated into 70S ribosomes from this organism (Maassen and Möller, 1975). These results suggested that the guanine nucleotide binding sites in the 5S RNA-protein complex and in the ribosome were similar. Furthermore, homologous proteins in *E. coli* and *B. stearothermophilus* appeared to be involved.

Chemical crosslinking studies have also been of great help in attempts to define more closely the actual sites of binding of the elongation factors.

Acharya *et al.* (1973) crosslinked elongation factor G (EF-G) to *E. coli* 50S ribosomal subunits and found proteins L7/L12 as the major target. Other proteins were implicated though not identified. Photochemical crosslinking of factor EF-G to 70S ribosomes of *E. coli* was performed by Maassen and Möller (1981) and chemical crosslinking by Sköld (1982). The former showed that ribosomal proteins L7/L12 were not crosslinked to EF-G but that L1, L3, L11, S3 and S4 were labelled. Results obtained by Sköld (1982) on the other hand indicated that proteins L7/L12, L6, L14 together with S12 and S19 were important for binding EF-G. Moreover, it was also shown that in the presence of thiostrepton, a drug which normally prevents factor EF-G binding to ribosomes, there was a considerable decrease in the yield of the crosslinked EF-G·protein products (Sköld, 1982).

The factors EF-Tu and EF-G possess related binding sites on the ribosome, as discussed above. It is consequently of

interest to note that factor EF-Tu has been crosslinked to ribosomal proteins L1, L5, L7/12, L15, L20, L30 and L33 (San Jóse et al., 1976). In these experiments, labelled proteins were identified by antibody treatment of complexes formed between [^{14}C]-EF-Tu, ribosomes, GDPCP and phenylalanyl-tRNA in the presence of polyuridylic acid following crosslinking and isolation of the linked complexes. Some of the labelled proteins, it will be noted, show overlap with the group identified as neighbours of factor EF-G.

Factor EF-G has also been localized on the E. coli ribosome by the use of photoactivatable crosslinking reagents followed by immune-electron microscopy. The factor was shown to sit at the base of the L7/L12 stalk appendage at the 50S-30S ribosomal subunit interface (Girshovich et al., 1981). Here, factor EF-G was crosslinked to both subunits. In two very recent studies specific interaction of factor EF-G with 23S rRNA from E. coli has been noted. A photoaffinity derivative of [^{3}H]-EF-G crosslinked to 23S rRNA but not 16S rRNA (Girshovich et al., 1982). Binding was inhibited by native EF-G and was stimulated in the presence of ribosomal proteins L11 and the pentameric $[L7/L12]_4 \cdot L10$ complex. Thus 23S rRNA exposed in the EF-G binding site of ribosomes can play a functional role and possess an affinity for factor EF-G. Moreover, the presence of L7/L12 plus L10 and L11 is sufficient to maintain the preferred state of 23S rRNA for interaction of the elongation factor. Studies were made (Dijk et al., 1979) on the binding of the ribosomal protein complex $[L7/L12]_4 \cdot L10$ and protein L11 to the 5'-one third of 23S rRNA and they showed (and the work of Girshovich et al., 1982, agrees very favourably with this) that the

binding of the $[L7/L12]_4 \cdot L10$ protein complex to 23S rRNA from *E. coli* was stimulated by protein L11 and *vice versa*.

Factor EF-G was also crosslinked to 23S rRNA in 70S ribosomes of *E. coli* using the bifunctional, cleavable reagent diepoxybutane (Sköld, 1983). The [EF-G·23S rRNA] complex was isolated, digested with ribonuclease A and the resultant RNA fragment protected by factor EF-G was isolated and sequenced. It proved to be 27 nucleotides long, embracing residues 1055-1081 of 23S rRNA. This is a functionally important and interesting site as will be discussed in greater detail below. Moreover the antibiotic thiostrepton, which prevents the binding of EF-G to ribosomes was shown to inhibit the formation of the [EF-G·23S rRNA] crosslinked complex.

Most of the information discussed so far, pertains to the best known prokaryotic system, i.e. *E. coli*. However, work from this laboratory, and others, makes several important implications regarding the GTPase centre of the ribosome, from the study of *Bacillus megaterium* and *Bacillus subtilis*. Ribosomes from thiostrepton-resistant mutants of *B. megaterium* lack a single 50S ribosomal subunit protein, designated BM-L11 since it was shown to be immunologically homologous with *E. coli* ribosomal protein L11 (Cundliffe *et al.*, 1979). This protein is required for the tight binding of thiostrepton to the ribosomes of *B. megaterium* as is the case for the homologue, BS-L11 from the ribosomes of *B. subtilis* (E. Cundliffe unpublished observations). Mutant strains of *B. megaterium* possessing ribosomes which lack protein BM-L11, exhibit only a low level of EF-G dependent GTP hydrolysis but this could be restored to wildtype levels upon re-addition of protein BM-L11 purified from wildtype. Moreover the ribosomes of

these mutant strains, lacking protein BM-L11, become more sensitive to the action of thiostrepton when supplemented with the missing protein (Stark and Cundliffe, 1979b). These data suggest that protein BM-L11 is involved in the uncoupled hydrolysis of GTP on the B. megaterium ribosome, dependent upon elongation factor G. Ribosomal protein L11 from E. coli was shown to replace protein BM-L11 in the in vitro complementation of B. megaterium ribosomes lacking the latter protein and so argues for a role of E. coli ribosomal protein L11 (and its homologue in other organisms) in the ribosomal GTPase centre. Furthermore these data fit nicely with those of Maassen and Möller (1974; 1978) as discussed above.

Resistance to thiostrepton can be mediated by methylation of a specific residue (nucleotide 1067) in 23S rRNA, by a methylase enzyme isolated from the thiostrepton producing organism Streptomyces azureus (Thompson et al., 1982a). As a result of this methylation thiostrepton cannot bind to the ribosome. The methylated nucleoside proved to be 2'-O methyl-adenosine (Cundliffe and Thompson, 1979). In other studies thiostrepton was shown to bind with a high affinity and 1:1 stoichiometry to a complex formed between E. coli 23S rRNA and ribosomal protein L11 (Thompson et al., 1979). Moreover, in the presence of protein L11, digestion of 23S rRNA with ribonuclease T1 led to the discovery of a protected RNA fragment, 61 nucleotides long. This was characterized by finger-print analysis which showed that the sequence fitted neatly with residues 1052-1112 of E. coli 23S rRNA (Schmidt et al., 1981). It was proposed that the GTPase centre of E. coli ribosomes embraces these residues of 23S rRNA because of the importance of ribosomal protein L11

in this site. These data are readily reconciled with the work of Sköld (1983) who showed that factor EF-G also protects part of this region of 23S rRNA (in that case residues 1052-1081) and with the observation that methylation of nucleotide A-1067 abolishes the binding of thiostrepton which itself inhibits the ribosomal binding of factor EF-G.

Finally, before I sum up what is the probable site of GTP hydrolysis on the E. coli ribosome I should briefly like to outline a study implicating certain 30S ribosomal subunit proteins as of importance to factor EF-G dependent GTP hydrolysis.

Marsh and Parmeggiani (1973) showed an involvement of 30S ribosomal subunit proteins S5 and S9 in the ribosomal dependent GTPase activity associated with elongation factor G. This observation was made when '30S' ribosomal core particles lacking proteins S1, S2, S3, S5, S9, S10 and S14 were prepared and shown to be unable to stimulate the GTPase activity of EF-G in the presence of 50S ribosomal subunits. Activity could be restored by simultaneous addition of ribosomal proteins S5 and S9, with a minor stimulation caused by S2. Sedimentation analysis revealed that the 30S ribosomal cores were deficient in coupling with 50S ribosomal particles, unless the proteins S5 and S9 were added and this suggested that these were therefore interface proteins. Moreover, the ability of the various protein deficient "30S" particles to couple with 50S ribosomal subunits corresponded closely with their activity in stimulating EF-G dependent GTPase.

Taken all together the results presented suggest very strongly that the following components (see Table one) are of importance to the ribosomal GTP hydrolysing centre, involving interaction with the elongation factors EF-G and EF-Tu and the initiation factor IF-2.

Table 1

Summary-table of components implicated as forming the Ribosomal GTPase centre and factor EF-G† binding site (for E. coli)

Major components:-
Proteins L7/L12, L10 and L11
23S rRNA - Residues 1052-1112
Minor components:-
Proteins L6 and L18
Possible components:-
Proteins L2, L5, L8, L9 and L25

(For a recent review see Liljas, 1982)

† Probably also the factor EF-Tu and initiation factor IF-2, binding site.

CHAPTER 1

Reconstitution

The technique of reconstitution of ribosomes, from their component constituents, has proved to be very powerful in studies of structure, function and assembly of ribosomal particles. Moreover, it has also been useful in the identification of altered components in mutationally or physiologically altered ribosomes.

Initial studies involved partial reconstitution with ribosomal core particles and 'split-proteins'. Although this technique of reconstitution was rapid, a major limitation was found in that the protein composition of the core particles varied considerably with slight variations in the conditions used for their preparation. This meant that to give meaningful interpretations to such experiments, the protein composition of each preparation of core particles and of the corresponding 'split-proteins' had to be fully elucidated. However this method was soon superseded by the development of the total reconstitution system with efficient reconstitution of functionally active 30S ribosomal particles being reported by Traub and Nomura (1968). Furthermore, it was also shown that the entire information for correct assembly of 30S ribosomal particles is contained in the structure of their molecular components (Traub *et al.*, 1971; Held *et al.*, 1973).

The reconstitution of 50S ribosomal subunits proved to be more complicated and was first achieved with 50S particles from *Bacillus stearothermophilus* (Nomura and Erdmann, 1970; Fahnestock *et al.*, 1972; 1973). Little success was initially obtained with reconstitution of 50S ribosomal subunits from *E. coli* and it was reasoned that this was due to a requirement

for a high activation energy for the assembly of particles. Incubation temperatures above 50°C were considered necessary for the reconstitution but the ribosomal proteins of the 50S subunit of E. coli could not tolerate such high temperatures and were thus denatured. As a result of this, the thermophilic organism B. stearothermophilus was chosen for study and it was also shown that all the information for the assembly of 50S particles was contained in its molecular components. Total reconstitution of 50S ribosomal subunits derived from E. coli was eventually achieved by a two-step temperature incubation procedure involving a higher magnesium ion concentration in the second stage (Nierhaus and Dohme, 1974). Thus 23S rRNA, 5S rRNA and the total 50S ribosomal subunit proteins were incubated for 20 minutes at 40°C in the presence of 4 mM Mg^{2+} ions and 400 mM NH_4Cl. This was followed by a 90 minute incubation at 50°C with the Mg^{2+} ion concentration raised to 20 mM. The reconstitution of 50S ribosomal subunits has since been shown to proceed via a series of reconstitution intermediates (Nierhaus, 1979). The second stage of the two-step procedure involves purely a conformational change to convert the final reconstitution intermediate, containing all the 50S subunit components, into the fully functional 50S ribosomal subunit (Dohme and Nierhaus, 1976; Nierhaus, 1979). A modified method for the 50S subunit, involving a slightly higher Mg^{2+} ion concentration in the first step, coupled with longer incubations, has been described by Amils et al. (1978; 1979). Various reports concerning reconstitution of 50S ribosomal subunits from other organisms have also been reported including those for Bacillus lichenformis and B. subtilis (Fahnestock, 1979). For a more recent review of

reconstitution and assembly of ribosomes see Nierhaus, (1982).

Stringent Response

Auxotrophic strains of E. coli and other organisms deprived of any essential amino-acid build up significant levels of deacylated tRNA which elicits a regulatory mechanism known as the stringent response. Such strains are termed stringent and the main effect observed is a curtailment of the synthesis of stable RNA species. Other responses are also involved, including major readjustments of metabolic and transcriptional patterns, some of which are described below (and reviewed by Cashel and Gallant, 1974; Gallant, 1979). Mutant strains which continued to accumulate RNA during amino-acid starvation were soon discovered and termed, "relaxed". The locus of the mutant allele in E. coli was eventually mapped at the "RNA Control" (RC) or relA site of the chromosome, by bacterial conjugation experiments (Alfoldi et al., 1962). A protein controlling the stringent response was identified and became known as stringent factor. The thermolability of this protein in several relaxed strains of E. coli proved that it was indeed the product of the relA gene (Block and Haseltine, 1973). This was also confirmed by experiments in which lambda transducing bacteriophages carrying the E. coli relA gene were introduced into wildtype strains. The amount of stringent factor in strains carrying extra relA genes was ten-fold greater than in the normal strain (Friesen et al., 1976).

The variety of processes regulated by a single gene product initiated a search for intermediate effectors of these alterations. Detection of labelled compounds was made after

growing a stringent strain of E. coli in the presence and absence of amino-acids in a medium supplemented with [^{32}P]--phosphate. When no amino-acids were present, i.e. starvation conditions, two new spots corresponding to novel nucleotides were detected on chromatograms and designated "magic spots" I and II (MSI and MSII). These two nucleotides were not detected in relaxed (rel$^-$) strains (Cashel and Gallant, 1969). So, a correlation was seen in that the formation of the "magic spot" nucleotides and cessation of RNA synthesis occurred promptly in starved stringent strains but neither response was detected in relaxed strains, starved for amino-acids. The "magic spot" nucleotides could of course have been produced as a consequence of a shutdown of RNA synthesis. That this was not the case was shown by starvation of a stringent strain for uracil, whereby RNA synthesis decreased and no spots were produced (Cashel and Gallant, 1969). Moreover, the "magic spot" nucleotides were required to be produced before RNA synthesis declined as was shown in experiments with stringent cells starved for amino-acids in the presence of chloramphenicol. Under such circumstances RNA continued to accumulate since MSI and MSII were not produced (Cashel, 1969). This was also an important observation in that it suggested that MSI and MSII might by synthesized on ribosomes (Cashel, 1969). The cellular pool of GTP was observed to shrink during the stringent response and therefore the "magic spots" were postulated to be derivatives of GTP. "Magic spot" I was subsequently shown to be a tetraphosphate of guanosine and MSII a pentaphosphate. These nucleotides were isolated from stringent cells starved for amino-acids and were shown to be guanosine 5'--triphosphate, 3'-diphosphate (pppGpp or MSII) and guanosine

5'-diphosphate, 3'-diphosphate (ppGpp or MSI). The mechanism of synthesis involved the transfer of the terminal pyrophosphoryl group from ATP to the 3'-OH group of GTP or GDP, respectively (Haseltine et al., 1972; Sy and Lipmann, 1973). Recently, a third nucleotide has been isolated which was postulated to be involved in the stringent response. The molecule, guanosine 5'--diphosphate, 3'-monophosphate (ppGp) has been designated as MSIII (Pao and Gallant, 1979).

It was subsequently confirmed (Haseltine et al., 1972) that ppGpp and pppGpp were synthesized on ribosomes in an 'idling' reaction of protein synthesis. Moreover, this reaction was dependent in vitro on the stringent factor protein which was present in the 0.5M NH_4Cl ribosomal wash of stringent cells. This factor from E. coli was purified to near homogeneity and proved to be a monomeric protein of molecular weight 75,000 daltons (Block and Haseltine, 1975). In addition to the ribosome dependent reaction, outlined above, there is also a non-ribosomal reaction in which the "magic spot" nucleotides are produced in the presence of buffer, salts and stringent factor. This information indicated that the factor itself carried the active catalytic centre. (Refer to Block and Haseltine, 1975, and Richter, 1979 for further details of the ribosome independent reaction.) Antibody raised against highly purified stringent factor was employed in studies designed to determine the relative levels of the factor and ribosomes present in the cell. One report indicated that a single copy of stringent factor was available per 200 ribosomes in a $relA^+$ strain (Pederson and Kjeldgaard, 1977 and see Richter, 1979 for further details).

Stringent factor was localized in 50S ribosomal subunits, 70S

run-off ribosomes and polysomes but not in native 30S ribosomal subunits (Ramagopal and Davis, 1974). The binding of this factor to ribosomes was, however, shown to be dependent upon both 30S and 50S ribosomal subunits and mRNA (Richter, 1973; Richter et al., 1975). Furthermore, the factor has been reported to bind to ribosomes at a site different from that of the elongation factors G and Tu. When ribosomes were prebound with either [^{3}H]-EF-Tu or [^{3}H]-EF-G the interaction with stringent factor was not prevented. Conversely, when stringency factor was prebound to ribosomes they still proved active in factor EF-Tu or EF-G dependent functions. Ribosomal proteins L7/L12 were shown to be essential for factor EF-Tu and EF-G factor binding to ribosomes (as outlined above) but ribosomal particles deficient in these two proteins could still bind stringent factor and produce the "magic spot" nucleotides (Richter, 1973; Richter et al., 1975). Another report however has stated that factor EF-G, when stably bound to the ribosome in the presence of fusidic acid and GDP or the non-hydrolysable guanosine nucleotide GDPCP, prevents both stringent factor binding and its enzymic activity. This could be taken to imply that factor EF-G and stringent factor possess similar or overlapping binding sites on the ribosome (Wagner and Kurland, 1980). However these authors also confirmed that ribosomal core particles lacking proteins L7/L12, which could not bind factor EF-G, were active in the stringent factor dependent synthesis of "magic spots" and they therefore suggested that these two factors might not share an identical binding site on the ribosome after all. Clearly this problem, concerning the relationship between the binding sites for the elongation

factors Tu and G and stringent factor, is not yet fully resolved. A report has also been presented which indicates that ribosomal protein L10 from *E. coli* is essential for the binding of the stringent factor to ribosomes. For example, ribosomal core particles lacking proteins L7/L12 were 50-80% active in "magic spot" nucleotide production compared with intact ribosomes, whereas ribosomal cores also lacking protein L10 or proteins L10 and L11 exhibited no stringent response. So ribosomal protein L10 was shown to be needed for binding the factor but it might not necessarily be the binding site *per se* (Howard *et al.*, 1976).

In addition to the *relA*$^-$ mutants lacking stringent factor, other mutational events can also affect the stringent response. A relaxed strain of *E. coli* termed *relC* was reported, which possessed an altered 50S ribosomal subunit (Friesen *et al.*, 1974). Stringent factor was isolated from the mutant and was found to be active with ribosomes from the wildtype, whereas the normally functional factor from the latter strain was not active in the presence of ribosomes from the *relC* mutant. The 50S ribosomal subunit was shown to be altered and subsequently a 50S ribosomal protein was implicated as the cause of the defect, when it was found that 1M LiCl-split proteins derived from 70S ribosomes of the wildtype could, when added to cores from the mutant strain, restore ppGpp and pppGpp synthesis. No observable difference was noted in the two-dimensional gel pattern of the 50S ribosomal subunit proteins from the wildtype or the *relC* mutant (Søren Molin - communicated in Friesen *et al.*, 1974). It was thus concluded that the *relC* strain either coded for an altered structural gene of a 50S ribosomal subunit protein or for an enzyme that modifies one or more of the

ribosomal proteins (Friesen *et al.*, 1974). In contrast to the *relC* mutant, described above, other *relC* mutants of *E. coli* were isolated which possessed an abnormal ribosomal protein L11 having an altered electrophoretic mobility when compared with the protein from the wildtype (Parker *et al.*, 1976). So, ribosomal protein L11 was also implicated as a participant in the synthesis of (p)ppGpp. (This represents a convenient way for describing both tetra- and pentaphosphate and will now be used, on occasions, for clarity.)

Again of importance to the present work, a thiostrepton-resistant mutant strain of *B. megaterium* possessing ribosomes lacking protein BM-L11 was found to be relaxed and these ribosomes could not support the synthesis of "magic spot" nucleotides unless supplemented with either the missing protein (Stark and Cundliffe, 1979a) or with ribosomal protein L11 from *E. coli* (Stark *et al.*, 1980). Thus it was confirmed that protein (BM)-L11 was indispensable for the synthesis of (p)ppGpp. Moreover, thiostrepton-resistant mutants of *B. subtilis* were seen to be phenotypically relaxed (Smith *et al.*, 1978; Weinen *et al.*, 1979) and ribosomal protein BS-L11, immunologically related to protein L11 from *E. coli* was seen to be missing from these mutants. It also reappeared in revertants which regained the stringent phenotype (Smith *et al.*, 1978).

Early observations had indicated that it was the lack of aminoacyl-tRNA rather than of single free amino-acids which was responsible for the shutdown of RNA synthesis (Neidhardt, 1966). Subsequently it was confirmed that synthesis of the two regulatory nucleotides (tetra- and pentaphosphate) *in vitro*, was dependent upon uncharged tRNA and mRNA in a codon specific

manner (Pederson et al., 1973; Haseltine and Block, 1973). Uncharged tRNA was then shown to be directed to the ribosomal A-site by stringent factor whereas preferential binding to the peptidyl-site of the ribosome occurs in the absence of the factor (Richter, 1976). Subsequently, following the hydrolysis of ATP, tRNA is released from the A-site in a reaction also dependent upon bound stringent factor (Richter, 1976). Furthermore, it was also shown that deacylated-tRNA must be present at the ribosomal A-site for spot production (Haseltine and Block, 1973). The experiments performed to elucidate this involved R17 bacteriophage RNA in a ribosome dependent protein synthesizing system. With ribosomal-initiation complexes formed the next codon of a known message could be probed. Only those deacylated tRNA molecules which could recognize the codon and were therefore in the ribosomal A-site could elicit (p)ppGpp formation (Haseltine and Block, 1973). In the report discussed earlier (Richter et al., 1975; see page 39) which stated that stringent factor binding to ribosomes was not blocked by either of the elongation factors Tu or G it was shown that no (p)ppGpp nucleotides were produced when either factor was prebound to ribosomes. This effect was accounted for by the blockade of the ribosomal A-site with either aminoacyl-tRNA carried by EF-Tu or by factor EF-G (Richter et al., 1975). Furthermore, antibiotics which affect the binding of tRNA to the ribosomal A-site were also shown to block (p)ppGpp production, e.g. tetracycline (outlined in Pederson et al., 1973; Haseltine and Block, 1973).

I now briefly return to some of the most important features of the effects caused by the stringent response, particularly with regard to E. coli.

CHAPTER 1

Reports have stated that the rate of RNA synthesis falls to about 30% of control levels during amino-acid starvation (Lazzarini and Dahlberg, 1971). Moreover, it was observed that a preferential curtailment of stable RNA synthesis occurred, i.e. synthesis of ribosomal and transfer RNA. Thus the RNA produced during starvation for amino-acids became considerably enriched in messenger RNA (Lazzarini and Dahlberg, 1971; Ikemura and Dahlberg, 1973). Subsequently, it was shown that cell free systems of *E. coli*, synthesizing mature sized 16S and 23S rRNA could be significantly inhibited by ppGpp. In this instance no reduction of tRNA synthesis by the tetraphosphate was noted although the tryptophan (trp) and β-galactosidase(lac) operons were in fact stimulated at the transcriptional level (Reiness *et al.*, 1975). It was suggested that guanosine tetraphosphate interacts with RNA polymerase in such a manner as to alter the affinity of the enzyme for promoters, in an operon specific fashion. Degradation of rRNA was not involved (i.e. ppGpp was shown to affect the synthesis of rRNA and not its degradation) but it was not clear whether RNA polymerase was affected directly or indirectly. To test this further it was necessary to use a highly purified system and to initiate a search for mutant strains with possible defects in the polymerase receptor site for ppGpp (Reiness *et al.*, 1975). A purified system of components showed that guanosine tetraphosphate inhibited rRNA synthesis *in vitro* by increasing the transition temperature for formation of stable RNA polymerase-DNA complexes at rRNA promoter sites. The experiments involved the use of DNA from a transducing phage carrying an rRNA cistron (φ80 d_3 *rrnB*$^+$ *ilv*$^+$ *Su*$^+$7). In contrast φ80 RNA synthesis was not affected by ppGpp. Thus this

nucleotide could act as an allosteric effector which directly altered the initiation specificity of RNA polymerase holoenzyme, at least *in vitro* (Travers, 1976; van Ooyen *et al.*, 1976). It was further elaborated (Ryals *et al.*, 1982) that at low levels of guanosine tetraphosphate the RNA polymerase would have a high affinity for rRNA and tRNA promoters whereas at elevated levels of ppGpp the polymerase would be 'tuned' to greater affinity for mRNA promoters. Until recently, however, *in vivo* evidence was lacking for such a model. A search for strains of *E. coli* with mutational alterations in the β-subunit of RNA polymerase resulted in the discovery of polymerase molecules insensitive to the presence of guanosine tetraphosphate. Exchange of certain amino-acids in the third quarter of the β-subunit of *E. coli* RNA polymerase resulted in relaxed control over RNA synthesis (Nene and Glass, 1983).

Stringent control of ribosomal protein gene expression has also been observed (Dennis and Nomura, 1974). Measurement of the rate of ribosomal protein formation and the total protein synthesis rate, together with observations of differential synthesis showed that the formation of ribosomal proteins was regulated either directly or indirectly by the availability of charged tRNA. Here, temperature-sensitive mutants in valyl-tRNA synthetase, in isogenic *rel*$^+$ and *rel*$^-$ strains, were employed to effect starvation conditions. Similar experiments have shown that the state of the *relA* gene can also affect the rates of synthesis of many non-ribosomal proteins in *E. coli*, some being stimulated and some repressed, under different conditions (Furano and Wittel, 1976).

To conclude this section we must consider what happens to the

regulatory nucleotides when amino-acid starvation is lifted. In brief, both ppGpp and pppGpp are rapidly degraded when conditions again become favourable for growth. A gene known as spoT was shown to effect the conversion of ppGpp to some other guanosine nucleotide (Fiil et al., 1977 and references therein). The work of these authors also helped to resolve an early conflict which held that either guanosine tetraphosphate was a precursor to pentaphosphate formation or that the latter nucleotide was the primary product which was then degraded to ppGpp. The currently held belief for the cycling of these poly-phosphates is as follows:- GTP - pppGpp - ppGpp - GDP - GTP (Fiil et al., 1977; Kari et al., 1977; Heinemeyer and Richter, 1978; Somerville and Ahmed, 1979). In spoT mutant strains there is no extensive production of pentaphosphate, but the tetraphosphate is overproduced and shows a slower rate of disappearance than in the wildtype $spoT^+$ strains (Laffler and Gallant, 1974). Subsequently, it has been shown that ppGpp is specifically degraded by an Mn^{2+}- ion dependent pyrophosphorylase present in $spoT^+$ but not in $spoT^-$ strains of E. coli (Heinemeyer and Richter, 1978). The enzyme catalyses the release of the pyrophosphate from the 3' position of ppGpp (and may also act on pppGpp Somerville and Ahmed, 1979) to yield ppG (GDP) which can then be converted to pppG (GTP). The guanosine 3'-5'-bis-diphosphate 3'-pyrophosphohydrolase was found to be present within the ribosomal fraction of cell extracts (Heinemeyer and Richter, 1978; Richter et al., 1979). It was isolated and shown to have a molecular weight of 65,000 daltons; moreover it does not require ribosomes for its activity but is inhibited by uncharged tRNA (reviewed by Richter, 1979). Regarding the degradation of

the pentaphosphate, a class of mutants of E. coli has been isolated, which exhibits altered metabolism of both ppGpp and pppGpp, and these have been mapped at a locus designated gpp (Somerville and Ahmed, 1979). These mutants accumulate elevated levels of pppGpp and show a low rate of degradation of the nucleotide during amino-acid starvation. It has been proposed that these gpp (guanosine pentaphosphatase) mutants are defective in a ribosome independent 5'-β-γ-nucleotidase which specifically hydrolyses the pentaphosphate to ppGpp. The situation is however more complex in that five separable pppGppase activities in the wildtype have been found. Of these, three are ribosome independent and two are dependent upon ribosomes. The latter two may involve hydrolysis of the guanosine pentaphosphate by the elongation factors Tu and G (see Somerville and Ahmed, 1979 for details). (I refer the reader here, to Richter, 1979 and Somerville and Ahmed, 1979 for more details regarding the complexities of degradation of the guanosine polyphosphates.)

CHAPTER 2

MATERIALS AND GENERAL METHODS

CHAPTER 2

Materials and General Methods

This chapter describes the Materials and Methods which have been used throughout this course of research. Certain techniques specific to one part of the work will be found in the appropriate chapter as will details of individual experiments.

Materials and Sources

Microorganisms

Bacillus megaterium KM (NCIB 9521)

Streptomyces laurentii (ATCC 31255)

E. coli MRE 600 (NTCC 8164)

E. coli prm-1 (K12 1031 *relA* met ara (λ^-) HFrc) from Dr. Charles Colson, Université de Louvain.

Microbiological Growth Media

Difco Casamino-acids, Malt extract, Nutrient Agar and Tryptic Soy Broth.

Oxoid Bacteriological Peptone, Beef Extract (Lab Lemco Powder), Nutrient Broth No. 2 and Yeast Extract.

Maltose (Hopkin and Williams).

Enzymes

Boehringer Glyceraldehyde-3-phosphate dehydrogenase (from rabbit muscle EC 1.2.1.12), 3-Phosphoglycerate kinase (from yeast EC 2.7.2.3).

Sigma Creatine phosphokinase (from rabbit muscle EC 2.7.3.2), DNase I (DN-EP from bovine pancreas EC 3.1.4.5), Lysozyme (Grade I, from chicken egg white EC 3.2.1.17), Pyruvate kinase (Type III, from rabbit muscle EC 2.7.1.40).

CHAPTER 2

Biochemicals

Boehringer Adenosine-5'-triphosphate (di-sodium salt), Dithiothreitol (DTT, Clelands reagent), Guanosine-5'-triphosphate (di-lithium salt), transfer Ribonucleic acid, phenylalanine-specific ($tRNA^{phe}$, from E. coli MRE 600 RNase negative).

Sigma Bovine serum albumin, DL-Dithiothreitol, Guanosine-5'-triphosphate (di-lithium salt, from equine muscle).

Phosphoenolpyruvate (tri-sodium salt), Polyuridylic acid [5'] (Type II, potassium salt) and transfer ribonucleic acid (Type XXI, from E. coli strain W).

Highly purified elongation factor G was provided by Professor James W. Bodley (University of Minnesota).

Purified ribosomal proteins from E. coli were provided by Dr. Jan Dijk (Max-Planck-Institut für Molekulare Genetik, Berlin-Dahlem, West Germany).

Radiochemicals

All radiochemicals were obtained from Amersham International.

[γ^{32}P] ATP, triethylammonium salt (14 Ci/mmol.)

Carrier free [^{32}P] orthophosphate, carrier-free [^{35}S] sulphate.

[γ^{32}P] GTP, triethylammonium salt (about 25 mCi/mmol.)

L-[U-^{14}C] phenylalanine in 2% (^{v}/v) ethanol (495-513 mCi/mmol.)

5[^{3}H] uridine (27 Ci/mmol.)

Antibiotics

Micrococcin was kindly provided by Dr. N.G. Heatley, University of Oxford and Dr J. Walker, National Institute for Medical Research, Mill Hill, London.

Sodium pseudomonate (pseudomonic acid Dr N. Rogers, Beecham Pharmaceuticals, Research Division, Betchworth, U.K.)

Thiostrepton Miss B. Stearns, Squibb Institute for Medical Research, Princeton, N.J.

Micrococcin and thiostrepton were dissolved in DMSO and sodium pseudomonate was dissolved in water.

Fine Chemicals

All chemicals, listed below, were of the purest quality (usually analytical reagent grade) except where indicated otherwise.

Acrylamide (Serva and Uniscience)

Agarose (electrophoresis grade, BDH)

β-Alanine (Kodak)

Alumina (Norton Abrasives)

Bentonite (Serva)

CM-23 Carboxymethyl-cellulose (Whatman)

Cyanogum 41 Gelling Agent (Sigma)

DEAE Sephadex A25 (Pharmacia)

DE52 (preswollen, Whatman)

Dimethylsulphoxide (DMSO, Fisons)

EDTA (di-sodium salt, Fisons)

Formamide (BDH)

D-Glucose (Fisons)

Glycine (Fisons)

N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid [HEPES] (Sigma)

Lithium Chloride (Merck)

N,N'-Methylene bisacrylamide [MBA] (Uniscience)

2-mercaptoethanol (Sigma)

Phenol (Fisons)

Sodium dodecyl sulphate [SDS] (Serva or BDH, electrophoresis grade)

Sucrose (Ultrapure, Ribonuclease free, Schwarz/Mann)

Triethylamine (Sequanal grade, Pierce)

Trizma Base and Trizma-HCl (Sigma)

N,N,N'N'-Tetramethylethylenediamine [TEMED] (Sigma or Kodak)

Urea (Ultrapure reagent, BRL)

Dyes and Indicators

Bromophenol Blue (Fisons)

Coomassie Brilliant Blue G (Sigma or Raymond A. Lamb, London)

Basic Fuchsin (Sigma)

Methylene Blue (Fisons)

Other General Chemicals (AR grade) were from commonly available sources.

Scintillation Fluids

Radioactivity was estimated by liquid-scintillation spectrometry using a Packard Tri-carb spectrometer and the following scintillation fluors:

Toluene/PBD This scintillant was used to determine radioactivity present in dry samples (e.g. on dried filters). It contained 0.4% ($^{w}/v$) 2-(4'-tert-butylphenyl)-5-(4"-biphenyl)--1,3,4-oxadiazole (butyl-PBD, Fisons) in toluene.

Fisofluor No. 1 (for aqueous samples) and Fisofluor No. 3 (for non-aqueous samples) were supplied by Fisons.

Other Materials

Norit (activated charcoal, from Sigma). This was refluxed in 2N-HCl followed by recovery by filtration according to Thompson (1960). It was stored as the dry powder.

Polygram^{R} cel 300 PEI/UV254 chromatography plates. Cellulose MN300 impregnated with polyethyl-eneimine (Camlab.). These PEI plates were presoaked in water for 30 minutes then dried before use.

X-ray Film Fuji RX - X-ray Film was used for autoradiography.

Dialysis Tubing For dialysis of postribosomal supernatants "Visking" dialysis tubing (Mwt cutoff 14000 daltons) was generally used. However for more exacting work Spectrapor No. 1 (Mwt cutoff 6000-8000 daltons) or Spectrapor No. 3 (Mwt cutoff 3500 daltons) supplied by Spectrum Medical Industries Inc. was used. Dialysis tubing was prepared for use by autoclaving for 20 minutes at 15 lb in^{-2} in a solution containing about 5% ($^{w}/v$) $NaHCO_3$ and 10 mM EDTA (di-sodium salt) and then washed thoroughly with distilled water and stored in the same at 4°C.

Urea solutions (for preparation of RNA) were generally treated with a small amount of autoclaved Bentonite to adsorb ribonuclease and cyanate ions. After 15-30 minutes the Bentonite was removed by centrifugation and the supernatant was filtered through a Millipore filter unit (Swinnex-13, 0.45 µm pore size). Solutions containing T^{30} and 8 M urea (for preparation of total ribosomal proteins) were treated with a small quantity of Norit followed by filtration through a Whatman No. 1 filter paper on a Buchner funnel. All other solutions and glassware for the preparation of rRNA were autoclaved prior to use to destroy ribonuclease.

Deionized water (Millipore, Milli Q) having a conductivity of

less than 1 μmho was used for the preparation of all solutions employed in the manipulation of subcellular components.

Methods

Growth of microorganisms

The following procedures were used routinely for the growth of both wildtype and mutant strains of B. megaterium. Methods for the isolation of these mutant strains are discussed in Chapter 3.

Starter cultures were established by inoculation of a loopful of cells from a nutrient agar plate into 100 ml 'C' medium supplemented with 0.01% ($^{w}/v$) glucose and 0.01% ($^{w}/v$) peptone. This medium contained (per litre) 2g NH_4Cl, 6g Na_2HPO_4, 3g KH_2PO_4, 3g NaCl, 85mg $MgCl_2.6H_2O$ and 75mg Na_2SO_4. The pH of this medium was 6.8 at 20°C. Growth was allowed overnight at 37°C with vigorous shaking of growth flasks. A600 readings after this period were usually between 0.15-0.30. Then 10-20 ml of the starter culture was transferred to 2l flasks containing 1l of Tryptic Soy Broth (Difco) and growth followed by regular monitoring of the A600. Cells were harvested in mid-late exponential phase usually at an A600 reading of 0.7 (see Figure 3.1).

Harvesting Cells

Cells were harvested by centrifugation at 9000 rpm in a Beckman JA-10 rotor for 10 minutes at 5°C or, when larger quantities were grown, in a Sharples continuous flow centrifuge. Bacterial pellets were resuspended and washed twice in T^{10} M^{10} A^{50} $E^{0.5}$ β^{3} buffer followed by recovery of cells by centrifugation as above.

Preparation of S30 extracts

The washed bacterial pellet (when less than 10g) was placed in a precooled (-20°C) sterilized mortar and ground for 5-10 minutes with autoclaved levigated alumina (approximately 2-2½ times the volume of cell paste). Following this, DNase (5 μg ml^{-1} final concentration) was added and grinding continued for about 2 minutes. The paste was then worked to a pourable consistency by addition of T^{10} M^{10} A^{50} β^{3} buffer, usually 1 ml for every 0.5g wet weight of cells. This suspension was then centrifuged at 18000 rpm in a Beckman JA-21 rotor for 30 minutes at 2°C. The supernatant was designated S30. At this stage it was usual to keep a little S30 for assay of protein synthetic activity but the bulk was used to prepare ribosomes and post ribosomal supernatant.

When quantities of bacterial pastes in excess of 10g were to be processed the cells were broken by passing them twice through a chilled French Press operated at approximately 12000 lb in^{-2}. During this procedure the concentration of cell paste was again approximately 0.5g wet weight of cells per ml in T^{10} M^{10} A^{50} β^{3} buffer and DNase was added, as above, after the first passage through the pressure cell. The S30 supernatants were then prepared as described above.

Preparation of Ribosomes and Post Ribosomal Supernatants

The S30 suspension was centrifuged at 40000 rpm and 3-4°C for about 4 hr to yield a 'crude' ribosomal pellet and an S100 post ribosomal supernatant. The 'crude' ribosomes were resuspended in a small volume of T^{10} M^{10} A^{50} β^{3} buffer, frozen rapidly in a dry ice/methanol bath and stored at -70°C. The

S100 supernatant was dialysed against the same buffer (3 x 1l x 1 hr) before storage as small samples at -70°C.

Usually however, cleaner preparations of ribosomes were required (i.e. for most experiments and preparations described in this dissertation). In this case S30 suspensions were layered over about $^{1}/_{3}$ volume of a buffer containing T^{10} M^{30} A^{1000} β^{3} and 20% ($^{w}/v$) sucrose. This was followed by centrifugation in a Beckman Ti75 rotor at 40000 rpm for a minimum of 7 hrs at 4°C. The supernatant, designated S100*, following centrifugation was dialysed at 4°C against T^{10} M^{10} A^{50} β^{3} buffer (3 x 1l x 1 hr) before being frozen rapidly and stored at -70°C as small samples. The pellet of salt washed ribosomes was cleared of any overlaying cell membrane material. This was teased off gently with a glass rod and the clear ribosomal pellet was resuspended in T^{10} M^{10} A^{50} β^{3} buffer. Any remaining cellular debris was cleared away by centrifugation at 12000 x g and 4°C in a microfuge. At this stage the cleared supernatant was layered over the ribosome resuspension buffer containing 40% ($^{w}/v$) sucrose and the ribosomes were pelleted by centrifugation at 40-45000 rpm and 4°C for a minimum of 7 hrs but usually overnight. Ribosomes were finally resuspended in T^{10} M^{10} A^{50} β^{3} and stored as aliquots at -70°C.

Quantitation of Ribosomes

The quantitation of ribosomes was done by measuring the A260 value of suspensions in T^{10} M^{10} A^{50} buffer and using the following conversion values:

1A260 unit = 27.2 pmol 70S ribosomes.

1A260 unit represents 60 µg of ribosomal particles.

CHAPTER 2

Preparation of Ribosomal Subunits

Ribosomal subunits were prepared from salt washed ribosomes. About 50-60 A260 units of 70S ribosomes were layered over 34 ml 10-30% ($^{w}/v$) sucrose gradients made up in T^{10} M^{1} K^{100} β^{3}. After centrifugation for 16 hr at 18000 rpm and 2°C in a Beckman SW.27 rotor the gradients were pumped through an ISCO UA-5 analyser by displacement with 60% ($^{v}/v$) glycerol and the absorbance was monitored continuously at 254 nm. Fractions containing 50S and 30S ribosomal subunits were pooled separately and the Mg^{2+} ion concentration raised to 10 mM. The subunits were then pelleted by centrifugation for 16 hr at 40000 rpm and 4°C then resuspended in T^{10} M^{10} A^{50} β^{3} and stored at -70°C. The concentrations of subunit preparations were determined by assuming that for the 30S subunit 1A260 unit was equivalent to 81.6 pmol and for the 50S subunit the corresponding value was taken to be 40.8 pmol.

Homogeneity of the subunit preparations was tested by loading 1A260 unit of the respective subunits onto 5 ml 10-25% ($^{w}/v$) sucrose density gradients made up in T^{10} M^{5} N^{100} β^{3} buffer. Following centrifugation for 100 minutes in a Beckman SW.50.1 rotor at 45000 rpm and 3°C the gradients were again analysed by pumping through an ISCO UA-5 analyser, as above. The areas of peaks recorded on a chart recorder were measured, the percentage cross-contamination of subunits determined and was never found to be greater than about 3%.

Preparation of Total Ribosomal RNA (RNA 70)

Two methods have been employed to prepare total ribosomal RNA from 70S ribosomes (see below). For reconstitution of ribosomal particles rRNA was used that had been extracted using a slightly

modified acetic acid/urea procedure of Hochkeppel et al. (1976). In assays of [^{35}S] thiostrepton binding (see Chapter 7) to complexes of 23S rRNA and protein BM-L11, RNA 70 was prepared by extraction of ribosomes with phenol or by treatment of acetic acid/urea rRNA preparations with phenol.

Acetic acid and urea method

'Crude' i.e. unsalt washed ribosomes (approximately 20 nmoles ml^{-1}) in T^{10} M^{10} A^{50} β^{3} were mixed with an equal volume of 8 M urea. Solid magnesium acetate was then added to a final concentration of 0.8 M and dissolved. To this suspension, 3 volumes of glacial acetic acid was added and this was followed by a 2 hr incubation on ice with occasional agitation. The precipitated RNA was recovered by centrifugation for 20 minutes at 18000 rpm in a Beckman JA-21 rotor. The RNA was resuspended and washed twice in 25 ml of a buffer containing T^{30} M^{20} with recovery by centrifugation for 5 minutes in an MSE Minor Bench centrifuge. The rRNA was finally resuspended in about 1 ml of T^{30} buffer and on some occasions required heating at 37°C for about 5 minutes before it was all fully dissolved. The concentration of RNA 70 was estimated from A260 measurements in water and taking 1A260 unit to be equal to 27 pmol or 60 µg of RNA.

Phenol extraction

Phenol extracted RNA was obtained from either freshly prepared ribosomes (unsalt washed 10 mg ml^{-1}) which had been redissolved in water or from acetic acid and urea extracted RNA. Phenol extraction was done to ensure the removal of all ribosomal

proteins from all the rRNA. Ribosomes or acetic acid/urea extracted RNA were mixed vigorously for 1 minute with an equal volume of redistilled phenol (saturated with water). The two phases were then clarified by centrifugation for 5 minutes in a bench centrifuge. The upper (aqueous) phase was removed and the lower (phenol) layer was 'back-extracted' with an equal volume of water. Following separation of the two phases the upper layer was carefully removed and pooled with the first aqueous layer. The pooled aqueous phases were re-extracted with phenol in the same manner (usually twice, until no white precipitate was visible at the interface of the aqueous and phenol layers after centrifugation). The aqueous phase was made 300 mM in sodium acetate by addition of a $^{1}/10$th volume of a 3 M stock solution and then $2\frac{1}{2}$-3 volumes of ethanol were added. After at least 1 hr at -20°C the RNA was recovered by centrifugation for 30 minutes at 8000 rpm and 4°C. The ethanol was decanted from the pellet which was then rinsed with 80% ($^{v}/v$) ice-cold ethanol before being redissolved in water. Again a $^{1}/10$th volume of 3 M sodium acetate solution and $2\frac{1}{2}$-3 volumes of ethanol were added followed by incubation in a dry ice and methanol bath for 10 minutes or at -20°C for 2 hr. The RNA was pelleted by centrifugation for 5 minutes in a bench microfuge at 4°C. The ethanol washing procedure was repeated until no phenol odour could be detected. Finally the RNA pellet was rinsed with 80% ice-cold ethanol, vacuum dried and dissolved in water (or T^{30}). It was stored as aliquots at -70°C.

RNA gels

The integrity of rRNA obtained was determined by electrophoresis

in 1.0% ($^{w}/v$) agarose gels containing formaldehyde, according to Lehrach et al. (1977).

The buffers used and their compositions were as follows:-

Phosphate Buffer: 0.5 M Na_2HPO_4, 0.055 M $NaH_2PO_4.2H_2O$., pH 8.2 (20°C).

Running Buffer: 120 ml formaldehyde (13.7 M stock), 27 ml phosphate buffer and water to 750 ml, pH 7.8 (20°C).

Sample Buffer: (made up freshly) 160 µl formaldehyde, 36 µl phosphate buffer, 500 µl formamide (stock), 200 µl glycerol and 0.1% ($^{w}/v$) bromophenol blue as tracker dye.

The 1.0% ($^{w}/v$) agarose gels were prepared by suspending 0.3g agarose in 15 ml water and autoclaving for 5 minutes at 10 lb in^{-2} followed by addition of 15 ml prewarmed running buffer (x2). The agarose was poured into a slab gel-plate assembly, similar to that described by Reid and Bieleski (1968), previously sealed with 5 ml of a 10% ($^{w}/v$) solution of Cyanogum 41 gelling agent. The gelling agent itself was polymerized by the addition of a few crystals of ammonium persulphate and 20 µl TEMED. When the agarose solution was poured a teflon comb was inserted into the slab-gel assembly. Gels were then placed into a vertical box assembly (Reid and Bieleski, 1968) and running buffer added. Ribonucleic acid (rRNA 70) was mixed with sample buffer to give a final concentration of 0.5 µg μl^{-1} and then samples were heated at 60°C for 5 minutes. Approximately 2.5 µg of rRNA was loaded per gel track and subjected to electrophoresis at 120V (constant, about 60 mA) for 30-40 minutes. Gels were removed from gel plates stained for 1 hr in 0.1% ($^{w}/v$) methylene blue in 0.2 M sodium acetate and destained in water. Only RNA preparations which yielded 3 distinct bands, 23S, 16S and 5S rRNA

were used for reconstitution experiments.

Preparation of Ribosomal Proteins

Proteins prepared from salt-washed 70S ribosomes or from 50S ribosomal subunits were designated TP70 or TP50, respectively. One pmol equivalent of TP70 or TP50 was defined as those total proteins derived from 1 pmol 70S or 50S particles, respectively. Proteins were prepared, following extraction of rRNA from 70S ribosomes or 50S subunits with acetic acid and urea, as described above. To supernatants containing proteins was added 5 volumes of acetone and the solutions were kept for 10 minutes at -20°C. Precipitated proteins were recovered by centrifugation at full speed in an MSE bench centrifuge for 5 minutes and then the supernatant was decanted. The pellet was dried under vacuum for about 30 minutes and then dissolved in T^{30} buffer containing 8 M urea before being dialysed against the same for 16 hr at 4°C. Those proteins to be used in experiments involving total reconstitution of 50S ribosomal subunits were then dialysed against buffer containing T^{30} M^{20} K^{1000} β^{3} (3 x 1l x 1 hr). Those to be used to complement 70S ribosomes from B. megaterium strain MJ1 were dialysed against buffer containing NH_4^+ instead of K^+. Proteins were stored at -70°C usually at 200-300 equivalent units ml^{-1}.

Analysis of Proteins by One-Dimensional Polyacrylamide Gel Electrophoresis

Two methods of polyacrylamide gel electrophoresis, in one-dimension, have been used during this study to examine ribosomal proteins.

(a) 0.1% ($^{W}/v$) SDS/polyacrylamide gels

Gels containing 13% ($^{W}/v$) acrylamide and 0.1% ($^{W}/v$) sodium dodecyl-sulphate were prepared according to Laemmli (1970).

Solutions and Buffers:-

30% ($^{W}/v$) acrylamide and 0.8% ($^{W}/v$) methylene bisacrylamide (MBA)

Lower Tris (4x) 1.5 M Tris-HCl pH 8.8 (20°C); 0.4% ($^{W}/v$) sodium dodecyl sulphate (SDS).

Upper Tris (4x) 0.5 M Tris-HCl pH 6.8 (20°C); 0.4% ($^{W}/v$) SDS.

Tris-glycine reservoir buffer (4x) 12g Tris base, 57.6g glycine. 4g SDS, water to 1000 ml. This running buffer was stored at 4°C and diluted four-fold immediately prior to use.

Ammonium persulphate (AP) 2% ($^{W}/v$) in distilled water.

Sample buffer 4.0 ml glycerol, 0.1 ml DTT, 3.0 ml 10% ($^{W}/v$) SDS, 1.25 ml Upper Tris-HCl (4x), water to 10 ml.

Slab gels (Reid and Bieleski, 1968) 1.5 mm thick were prepared. These consisted of a lower separating gel 10 cm long and an upper stacking gel about 2 cm long. Sample wells were formed in the stacking gel by insertion of a comb prior to polymerisation and up to 12-14 samples could be run concurrently with this system.

Separating (Lower) gel

Each slab required about 20 ml of gel solution which was made up as follows:

H_2O	6.06 ml	
Lower Tris (4x)	5 ml	
30% acrylamide/0.8% MBA	8.65 ml	
2% AP	0.3 ml	added after degassing
TEMED	5 µl	

The solution was thoroughly degassed under vacuum-suction,

poured and overlaid with water-saturated butanol to aid the formation of a flat surface. Polymerization usually took 20 minutes after which time the butanol was washed away with running buffer.

Stacking (Upper) gel

For each slab gel the composition was as follows:

H_2O	6.34 ml	
Upper Tris (4x)	2.5 ml	
30% acrylamide/0.8% MBA	1.0 ml	
2% AP	150 µl	added after degassing
TEMED	10 µl	

The solution was degassed and layered over the separating gel. A comb was inserted and the gel allowed to polymerize for about 20 minutes.

Sample Preparation and Electrophoresis

Protein samples (10-50 µl containing about 1-4 µg of each species) were prepared for electrophoresis by heating for 2 minutes at 90°C together with 50 µl of sample buffer followed by the addition of 5 µl 0.1% ($^{W}/v$) bromophenol blue. Samples were then layered under the running buffer into the wells cast into the stacking gels. Electrophoresis was carried out at 60V (constant) for 1½ hr followed by 120V (constant) for 2½ hrs until the bromophenol blue marker had reached the bottom of the gel (alternatively gels were subjected to 20 mA constant for about 3-4 hr). Gels were soaked for 12-16 hrs in 10% ($^{W}/v$) TCA, 15% ($^{V}/v$) methanol (with several changes of solution) to remove SDS and to 'fix' proteins. Protein bands were then stained for 1 hr using 0.04% ($^{W}/v$) Coomassie Brilliant Blue G250 in 3.5% ($^{W}/v$) perchloric acid. Destaining was performed using several changes

of solution containing 10% (^{V}/v) methanol and 7% (^{V}/v) glacial acetic acid.

(b) 8 M-urea/polyacrylamide gels

The method used was that of Traub et al. (1971). Separating gels containing 10% (^{W}/v) acrylamide, 0.15% (^{W}/v) N,N'-methylene bisacrylamide (MBA) and 8 M urea were poured as slabs and electrophoresis was conducted at pH 4.5.

Solutions and Buffers:-

A) 24 ml 1N-KOH, 8.6 ml glacial acetic acid, 2.0 ml TEMED, 24g urea, water to 50 ml final volume.

B) 24 ml 1N-KOH, 1.44 ml glacial acetic acid, 0.23 ml TEMED, 24g urea and water to 50 ml.

C) 6.65g acrylamide, 0.1g MBA, 24g urea and water to 50 ml.

D) 2.5g acrylamide, 0.625g MBA, 24g urea and water to 50 ml.

E) 0.1 mg Riboflavin in 5.0 ml 8 m urea. Made up freshly.

Ammonium persulphate (AP) 0.056g in 5.0 ml 8 M urea. Made up freshly.

Sample buffer 5 ml 8 M urea containing 10 mM DTT and 0.0005% (^{W}/v) Basic Fuchsin.

Running Buffer 31.2g β-alanine, 8.0 ml glacial acetic acid, adjusted to pH 4.5 and brought to 1l final volume with water.

Separating gel This was poured without degassing and contained 1 part of solution A, 6 parts C and 1 part AP. The poured gel solution was overlaid with water to aid a flat surface during polymerisation.

Stacking gel This was layered over the separating gel and was made up with 1 part solution B, 4 parts D, 2 parts 8 M urea and 1 part of solution E. Polymerisation of the stacking gel was

initiated by exposure to an ultraviolet lamp for approximately 2 minutes.

Sample Preparation and Electrophoresis

Samples (10-50 µl) were mixed with 100 µl of sample buffer and loaded under the running buffer (x1) into gel wells cast into the stacking gel. Electrophoresis was carried out at 4°C for 12-16 hr at 120V constant from anode to cathode (i.e. reverse polarity). Gels were stained directly and destained as described above.

Estimation of Protein Concentration

Both crude and accurate methods for determining protein concentrations are detailed in Chapter 7, page 95.

Total Reconstitution of 50S Ribosomal Subunits

This was carried out by a two step procedure based upon that of Nierhaus and Dohme (1979). Total ribosomal RNA (RNA 70; 10 µl - approximately 2A260 units), 70 µl buffer containing $T^{13.75}$ A^{250} $EDTA^{0.025}$ β^{2} and 20 µl TP50 (approximately 5 equivalent units, defined above) were mixed together to give a final ionic composition $T^{18.6}$ M^{4} A^{175} K^{200} $EDTA^{0.018}$ $\beta^{1.8}$. This mix differed only slightly from the published procedure except that in the published recipe the final monovalent cation concentration was 400 mM NH_4Cl. This mixture was incubated for 20 minutes at 44°C and then the Mg^{2+} concentration was raised to 20 mM followed by incubation at 50°C for a further 90 minutes. Products of reconstitution incubations were stored at -70°C and were used without further fractionation. In assessing the activity of reconstituted subunits it was assumed that all the relevant RNA species (23S and 5S rRNA) had been quantitatively incorporated

into 50S particles and the A260 measurement was used to estimate the number of particles.

Preparation of [γ^{32}P] Labelled Nucleoside Triphosphates (ATP and GTP)

Experiments involving the use of [γ^{32}P] ATP or [γ^{32}P] GTP were done with commercial preparations of these nucleotides (as discussed above) or were prepared as described below.

The method employed for the preparation of [γ^{32}P] labelled nucleoside triphosphates of high specific radioactivity was based on the procedure of Glynn and Chappel (1964). This involves an enzyme catalysed exchange reaction between [^{32}P] inorganic phosphate and the terminal phosphate group of ATP or GTP.

To 0.5 ml of 200 mM Tris-HCl, pH 8.0 (20°C), containing 1.5-3.0 mCi of carrier free [^{32}P] orthophosphate was added 0.1 ml of buffer containing T^{100} pH 8.0 at 20°C, M^4 and 12 mM dithiothreitol; 3 mM, 3-phosphoglyceric acid; 1 mM NAD; 14 µg rabbit muscle glyceraldehyde-3-phosphate dehydrogenase and 1.4 µg yeast-3-phosphoglycerate kinase. This was followed by addition of either 40 µl 100 mM ATP or 70 µl 100 mM GTP. After 20 minutes at 20°C approximately 9 volumes of ice cold water was added and the diluted mixture was then loaded to a DEAE Sephadex A25 column (2 ml bed volume) pre-equilibrated with water at room temperature. The column was developed using a 0-1.0 M concentration gradient of NH_4HCO_3 (10 ml). Fractions (0.5 ml) were collected and the radioactivity in each fraction determined by taking 2 µl into Fisofluor No. 1 scintillant and counting. Two peaks of radioactive material were eluted from the column, the first being free [^{32}P] phosphate and the second the radiolabelled nucleotide.

The most active fractions corresponding to the second peak were analysed for purity on PEI plates (as above). The 'cleanest' [γ^{32}P] nucleoside triphosphate fractions were then pooled, diluted to 5 ml, made 10% (^{v}/v) with triethylamine and lyophilised overnight. Two further cycles of dilution with water, addition of triethylamine and lyophilization were performed before the dried [γ^{32}P] NTP was dissolved in approximately 0.5 ml H_2O. The purity was again checked on PEI plates and by adsorption of the labelled nucleotides to activated charcoal followed by measurement of any free [^{32}P] phosphate in the cleared supernatant. Such analysis showed that greater than 99% of the radioactivity was present in the nucleoside triphosphates.

The ultraviolet absorbance of ATP preparations was read at 259 nm and that of GTP preparations at 253 nm. Molar extinction coefficients of ATP and GTP at these wavelengths are 15400 M^{-1} cm^{-1} and 13700 M^{-1} cm^{-1} respectively. From these data the specific radioactivity of the compounds was determined and values ranged between 90-185 Ci/mol. These radionucleotides were stored at -70°C.

Preparation of [^{35}S] labelled Thiostrepton

During the course of this work several preparations of [^{35}S] thiostrepton were used. They were prepared in this laboratory under similar conditions by Dr J. Thompson or by this author as described below.

Streptomyces laurentii and not S. azureus was used in these preparations because this producing organism has routinely (in this laboratory) been found to give better yields of the antibiotic.

S. laurentii was grown at 30°C on plates containing solidified NE Medium (this contained per litre: 10g glucose; 2g yeast extract; 1g beef extract; 2g Casaminoacids; 20g Agar and was adjusted to pH 7.0 with 1M-KOH) until copious sporulation occurred. Loopfuls of spores were then transferred to 1 ml Fermentor medium to establish starter cultures (Fermentor medium contained per litre: 10g Peptone; 15g malt extract; 20g glucose; 3g yeast extract and 20g of maltose). The starter cultures were grown overnight at 30°C without agitation and were used to inoculate flasks containing 50 ml of Fermentor medium and growth was continued at 30°C in a gyrotary water bath. At approximately 18 hrs, 26 hrs and 40 hrs 2.5 mCi of carrier free [^{35}S] sulphate was added and growth continued for 7 days with continuous shaking at 30°C.

Extraction of [^{35}S] Thiostrepton

The culture was extracted with 100 ml chloroform and the phases clarified in a separating funnel. The lower chloroform layer was collected and the upper aqueous layer was re-extracted with 100 ml of chloroform. Again the solvent layer was collected and pooled with the previous chloroform extract. This was then placed in a round bottomed flask attached to a rotary evaporator and the chloroform distilled-off at 35°C under reduced pressure. The residue remaining was washed twice with 10 ml petroleum ether in which thiostrepton is not soluble. After again drying the residue it was dissolved in 10 ml chloroform. The suspension was then passed through a small column of preswollen DE-52 sephadex resin (about $\frac{1}{2}$ x 3 cm) and washed through with an equal volume of chloroform. The eluate was taken and this procedure

repeated through two fresh DE-52 columns. The chloroform was then distilled-off and the [^{35}S] thiostrepton was taken up in 20 mls DMSO and characterized.

Characterization of [^{35}S] Thiostrepton

The preparation of labelled antibiotic made by this author was analysed for its ability to bind to ribosomes from B. megaterium wildtype and to phenol extracted rRNA 70 from E. coli. These assays were similar to the [^{35}S] thiostrepton binding assays described in Chapter 7. The specific radioactivity of the preparation was determined by plotting a curve for the binding of [^{35}S] thiostrepton to 70S ribosomes [to which the drug binds very tightly with a 1:1 stoichiometry (Sopori and Lengyel, 1972; Highland et al., 1975)] or to rRNA 70 as a control. From the slope of such a curve (minus background readings in the presence of RNA 70) it was calculated that the specific activity for this preparation was 715 cpm pmol^{-1} at a concentration of about 40 pmol per 5 µl.

Partial Reactions of Protein Synthesis

Cell-Free Protein Synthesis

Synthesis of polyphenylalanine directed by polyuridylic acid was performed in reaction volumes of 50 or 100 µl.(see Figure legends for precise details of each experiment).

Ribosomes (5-10 pmol) in T^{10} M^{10} A^{50} β^{3} buffer were mixed with S100 or S100* (30% ^{v}/v final concentration) in a total volume of 25 or 50 µl made up with the same buffer. To this was added an equal volume of a Poly(U) cocktail. This contained 40 mM Hepes-KOH (pH 7.5 at 20°C); 100 mM KCl; 20 mM $MgCl_2$; 0.075 mM each of 19 'cold' amino acids (minus phenylalanine);

0.75 mM GTP; 5 mM ATP; 10 mM phosphoenolpyruvate; 200 units ml^{-1} (final concentration) pyruvate kinase; L-[U-^{14}C] phenylalanine (500 mCi/mmol, 10 µCi ml^{-1}); 1.5 nM phenylalanine-specific tRNA from E. coli and polyuridylic acid (1 mg ml^{-1}). The final ionic composition of the assay mixes was therefore 100 mM KCl, and 15 mM $MgCl_2$.

When drug activity was to be measured, drugs or, in controls, DMSO (1% $^{v}/v$) were added and preincubated with ribosomes for 5 minutes at 0°C and then for 5 minutes at 37°C before addition of other components to start the reaction. (When ribosomal protein BM-L11 was added to ribosomes from strain MJ1 the same preincubations were performed before addition of S100(*) etc.) Reactions were normally started by the addition of the Poly(U) mix and incubation continued at 37°C. Samples (10 µl each) were withdrawn into 10% ($^{w}/v$) TCA and then heated at 90°C for 20 minutes. Acid precipitable material was collected by filtration onto Whatman GF/C glass fibre discs (which had been presoaked in 5% ($^{w}/v$) TCA) and then extensively washed with 5% ($^{w}/v$) TCA. Filters were dried under an infrared lamp and radioactivity, measuring phenylalanine incorporation into polyphenylalanine, was estimated by liquid scintillation spectrometry using 0.4% ($^{w}/v$) PBD/toluene scintillation fluid or Fisofluor No. 3. At the specific activity of L-[U-^{14}C] phenylalanine quoted, 1000 cpm represents approximately 1 pmol of phenylalanine residues incorporated.

EF-G dependent uncoupled GTP hydrolysis

Reaction mixtures (75 µl) contained salt-washed 70S ribosomes (1-5 pmol) or ribosomal subunits (see Figure legends) together

with 10 nmol [γ^{32}P] GTP (70-20 Ci/mol 162-44 cpm^{-1} $pmol^{-1}$) and 50 pmoles factor EF-G from E. coli in a buffer containing T^{15} M^{10} A^{80} β^{2}. In experiments involving the addition of protein BM-L11, TP70 or drugs, the protein(s) or drugs (DMSO in controls) were preincubated with ribosomes for 5 minutes at 30 or 37°C before addition of other components (see Figure legends for precise details). Reactions were started by the addition of 10 µl of mixture containing the factor EF-G and labelled GTP and during incubation at 30 or 37°C samples (20 µl each) were removed at successive time intervals into 20 µl ice cold 1 M perchloric acid to terminate hydrolysis. To each sample, 200 µl of a 5% (^{w}/v) suspension of activated charcoal (Norit) in the assay buffer was added to remove free [γ^{32}P] GTP. After 5 minutes the Norit was removed by centrifugation at 12,000 rpm for about 5 minutes. To determine the amounts of GTP hydrolysed, free [^{32}P] phosphate was measured in the supernatant by liquid-scintillation spectrometry using scintillant Fisofluor No. 1.

The Stringent Response

Both in vivo and in vitro methods were used to determine whether strains were relaxed or stringent.

Measurement of RNA synthesis in vivo

Synthesis of RNA was measured as the incorporation of [5-^{3}H] uridine into trichloroacetic acid precipitable material. Bacterial cells were grown in 10 ml of C-minimal salts medium supplemented with 0.01% (^{w}/v) glucose and 0.01% (^{w}/v) peptone as described above (page 52). When cultures had reached exponential growth experiments were started by the addition of [5-^{3}H]uridine (9.61 Ci/mol) at a concentration of 2 µCi ml^{-1}. Samples (0.5 ml)

were removed periodically to 1.0 ml ice cold 10% ($^{w}/v$) TCA and mixed well. Precipitates were collected onto glass-fibre discs (Whatman GF/C) and washed twice with 3 ml cold 5% ($^{w}/v$) TCA. Radioactive material bound on the dried filters was determined by liquid-scintillation spectrometry in scintillant Fisofluor No. 3. Sodium pseudomonate (200 µg ml^{-1} final concentration) was added to certain cultures, as indicated in the Legend to Figure 6.1.

Synthesis of Guanosine tetra- and pentaphosphate in vitro

Synthesis of guanosine polyphosphates *in vitro* was measured in a heterologous system using partially purified stringent factor from *E. coli*. This had been prepared by M. Stark in this laboratory based upon the procedure of Block and Haseltine (1975) and as described previously (Stark *et al.*, 1980).

Reaction mixtures for the measurement of guanosine polyphosphate production contained (in 50 µl total volume) 25 pmol ribosomes (in 10 µl T^{10} M^{10} A^{50} β^{3} buffer); 25 µg polyuridylic acid; 50 µg *E. coli* unfractionated tRNA; 4 mM GTP; 1.25 mM [γ^{32}P] ATP (224-26 cpm^{-1} $pmol^{-1}$ about 100-12 Ci/mol); 16.5 µl crude stringent factor in T^{5} M^{5} A^{500} β^{3}. The final ionic conditions, allowing for the addition of protein BM-L11 or drugs (see Figure legends for details) were T^{25} $MgCl_2^{5}$ $MgAC^{15}$ A^{182} $\beta^{1.8}$. After incubation for 1 hr at 37°C, reaction mixtures were transferred to ice and mixed with 50 µl of a solution containing 2 M ice-cold formic acid and 10% ($^{w}/v$) TCA. Macromolecular precipitates were removed by centrifugation at 12000 rpm for 2 minutes. Then 2-10 µl samples of the supernatant were spotted onto poly(ethyleneimine)-cellulose (PEI) plates, and dried in a cool

air stream to prevent hydrolysis of guanosine polyphosphates. Chromatograms were developed in 1.5 M KH_2PO_4 pH 3.4 and the location of the various nucleotides determined by autoradiography overnight. Radioactively labelled nucleotides were cut out from plates and the [^{32}P] content determined by liquid-scintillation spectrometry in Fisofluor No. 1.

Usually ribosomes and drugs (or DMSO in controls) were incubated together for 5 minutes at 20°C before inclusion in these assays. In some experiments (see Figure legends, Chapter 6) ribosomes and protein BM-L11 were preincubated together for 5 minutes at 0°C followed by 5 minutes at 20°C to allow any integration of the protein into ribosomes before addition of drugs.

CHAPTER 3

SELECTION OF MUTANTS AND GROWTH OF ORGANISMS

CHAPTER 3

Selection of Mutants and Growth of Organisms.

Selection of mutant strains

Mutant strains of B. megaterium KM (NCIB 9521), resistant to micrococcin were isolated in this laboratory by Dr M.J.R. Stark. They were selected by plating the wildtype onto nutrient agar plates containing micrococcin (3 μg ml^{-1} final concentration) and arose spontaneously. A second, unlinked, genetic marker was then introduced by plating the micrococcin resistant mutants on nutrient agar plates containing nalidixic acid (10 μg ml^{-1}). The concentration of nalidixic acid used was chosen following standard liquid culture assays for determination of the minimum inhibitory concentration. It was found that growth of one of the micrococcin-resistant mutants was inhibited at a concentration of nalidixic acid as low as 5 μg ml^{-1} final concentration. One 'double' mutant, which again arose spontaneously on the nalidixic acid plates was chosen for further study. The phenotype of this strain (designated GS2) was checked periodically by growth on nutrient agar plates containing both drugs (each at 10 μg ml^{-1} final concentration) although, routinely the organism was grown in broth at 37°C in the absence of antibiotic, see below for further details. Organisms were stored both in "stab" cultures at room temperature and in 20% ($^{v}/v$) glycerol at -20°C.

Growth of organisms and in vivo response to antibiotics.

The organisms employed here were routinely grown up, in bulk, in broth at 37°C (as described fully in Materials and Methods). Tryptic Soy Broth (Difco) was found to give the best results and typical growth curves for the wildtype and strain GS2 are presented in Figure 3.1. Under such conditions (at 37°C with vigorous shaking) the mean generation time for the wildtype was

found to be 36 minutes whilst that of strain GS2 was only slightly longer at 45 minutes. Another strain of B. megaterium (designated MJ1) which had been selected for resistance to thiostrepton, as described elsewhere (Cundliffe et al., 1979) and employed extensively throughout this present study was also routinely grown up in Tryptic Soy Broth. It was found to have a doubling time of 81 minutes under the above conditions (data not shown). In comparison, growth of the strains in Nutrient Broth ♯2 (Oxoid) was somewhat slower, with a mean generation time of 48 minutes for the wildtype and about 51 minutes for strain GS2, as shown in Figure 3.1.

Preliminary studies with growth of strain GS2 in Tryptic Soy Broth, with successive inoculations to fresh broth (5 x 1l) i.e. continuous culture in the absence of micrococcin, were made in order to check for revertants. Samples from the final flask were serially diluted, plated onto nutrient agar plates and then replica plated onto drug plates (containing nalidixic acid and micrococcin, both at 10 μg ml^{-1} final concentration). Results of such experiments gave no indication of reversion of strain GS2 to sensitivity to either drug.

Minimum inhibitory concentrations of micrococcin and thiostrepton for wildtype and strains GS2 and MJ1.

It was decided to check the minimum inhibitory concentrations (MIC values) of micrococcin and also of the closely related antibiotic thiostrepton (see Introduction for details) for the B. megaterium strains, wildtype, GS2 and MJ1. The data are presented in Table 3.1. Both the mutant strains GS2 and MJ1 were highly resistant to micrococcin (drug concentrations of 100 μg ml^{-1} correspond to saturated aqueous solutions) and

moreover, strain MJ1 was more resistant to micrococcin than to thiostrepton, the drug on which it had originally been selected. Neither of the mutant strains, however, was totally insensitive to micrococcin and this is shown for strain GS2, Figure 3.2. At an input of 30 $\mu g\ ml^{-1}$, micrococcin extended the mean generation time of strain GS2, growing in broth at 30°C from about 33 minutes to about 42 minutes.

Thus, B. megaterium strain GS2 was shown to be highly resistant to micrococcin. Moreover a strain (MJ1) selected for resistance to the closely related drug, thiostrepton, was cross--resistant to the former drug. It was already known that strain MJ1 possessed ribosomes which were lacking a single protein, BM-L11 and that this protein was involved in determining the thiostrepton-resistance phenotype (Cundliffe et al., 1979). These data prompted the further characterization of strain GS2 and the next objective was to prepare cell-free extracts in order to localize the source of resistance to micrococcin.

Legend to Figure 3.1

Growth of microorganisms in broth

Overnight starter cultures were established in 'C' minimal salts medium supplemented with 0.01% ($^{w}/v$) glucose and 0.01% ($^{w}/v$) peptone as described in Materials and Methods. Then 10 ml of culture was transferred to flasks containing broth (1 l) and growth was continued at 37°C with continuous shaking. Growth was monitored by measuring the absorbance at 600 nm. (●,○) growth of organisms in Tryptic Soy Broth; (■□) growth in Nutrient Broth No. 2. Closed symbols represent the wildtype and open symbols strain GS2.

Figure 3.1

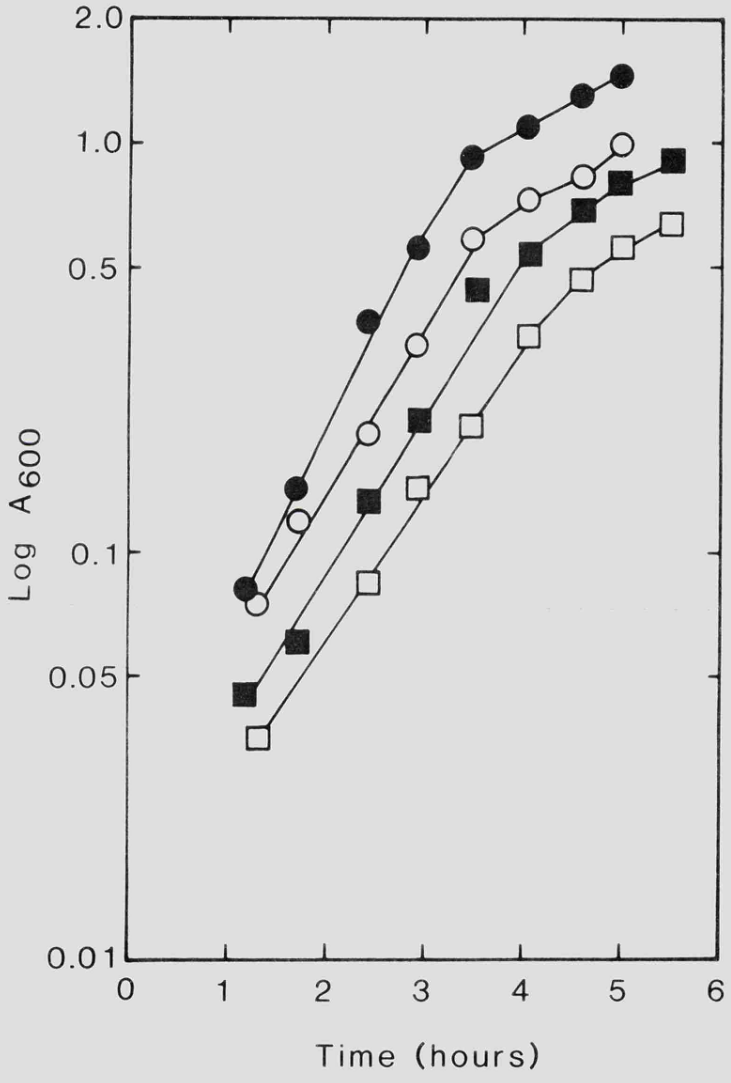


Table 3.1

Minimum inhibitory concentrations of micrococcin and thiostrepton for three strains of B. megaterium.

Strain	Minimum inhibitory concentration ($\mu g\ ml^{-1}$)	
	Micrococcin	Thiostrepton
Wildtype	0.03	0.05
GS2	>100	0.03
MJ1	>100	30

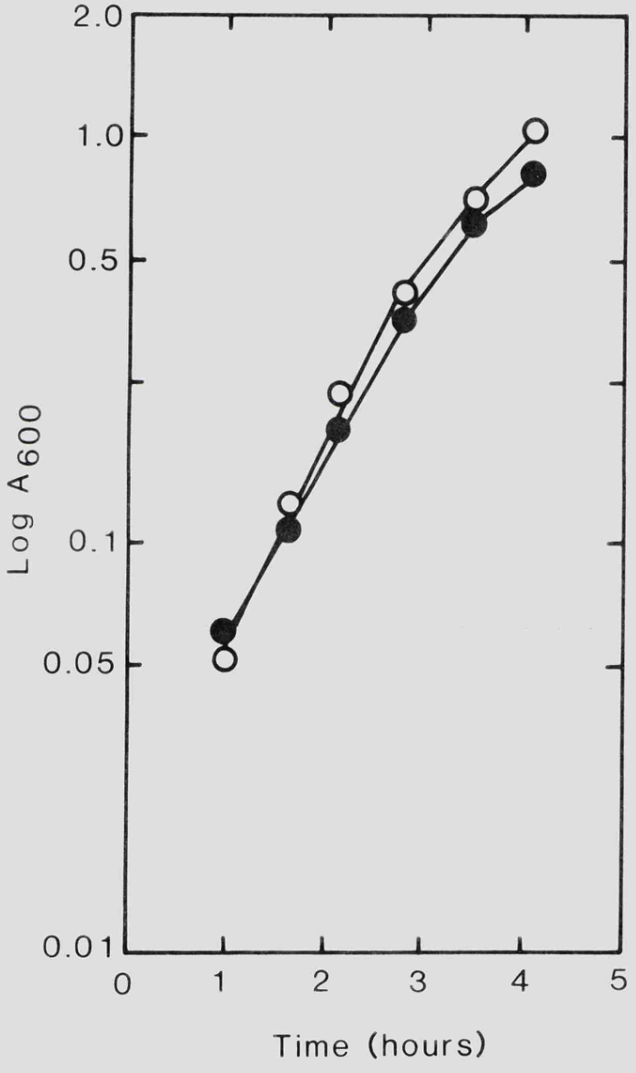
Organisms grown in broth were streaked out on nutrient agar plates containing drug or DMSO (controls) and incubated at 37°C. Plates were examined for growth after 16h (wildtype and strain GS2) or 36h (strain MJ1) and were merely scored as positive or negative.

Legend to Figure 3.2

Effect of micrococcin on the growth of strain GS2

Overnight starter cultures of strain GS2 were established as described in the Legend to Figure 3.1. Then 10 ml of culture was transferred to 45 ml of Tryptic Soy Broth (prewarmed, 37°C) and growth was continued for 3 hr at 37°C. After this time 5 ml of this culture was transferred to flasks containing 45 ml of Tryptic Soy Broth and (○) DMSO (0.3% $^{v}/v$) as a control or (●) micrococcin at 30 μg ml^{-1} final concentration. Growth was then followed by monitoring the absorbance at 600 nm.

Figure 3.2



CHAPTER 4

CELL-FREE PROTEIN SYNTHESIS

CHAPTER 4

CELL-FREE PROTEIN SYNTHESIS

Introduction

The classical method of measuring the protein synthesizing capability of ribosomes *in vitro*, (as developed by Nirenberg and Matthaei) and involving the synthetic polynucleotide, polyuridylic acid (Poly-U) was employed in the preliminary characterization of the various strains of *B. megaterium*, outlined here.

Nirenberg and Matthaei (1961) succeeded in preparing stable cell-free extracts from *E. coli*, capable of synthesizing protein. Following on from crucial observations of others, namely that DNase inhibited amino-acid incorporation into protein in such systems, they noted that RNase also markedly inhibited incorporation of [^{14}C]-L-valine into protein. These results fitted in neatly with the contemporary realization that the DNA sequence codes for the RNA sequence and that RNA specifies the order of amino-acids in protein. These workers then successfully separated mRNA away from the ribosomal fraction and showed that naturally occurring RNA as well as Poly(U) could act as a template and stimulate the incorporation of amino-acids into protein (Nirenberg and Matthaei, 1961; 1963).

Polyuridylic acid was found to direct the incorporation of phenylalanine into oligophenylalanine. Although this assay (the Poly(U) assay) relies on non-physiological conditions (for example it requires an abnormally high Mg^{2+} concentration) it is a readily utilizable assay for studying the modes of action of those inhibitors of protein synthesis which affect elongation cycle events. It can also be employed to determine which ribosomal subunit contains the site of action of a particular

drug, as in the classic crossover experiments utilizing ribosomal subunits from sensitive and resistant strains. Precisely such experiments were done to show the site of action of streptomycin. When 30S and 50S ribosomal subunits were prepared from streptomycin sensitive and resistant strains and then reaggregated in all combinations to yield 70S ribosomes it was found that only those particles which contained the 30S subunit from the sensitive strain were inhibited by the drug (Davies, 1964; Cox et al., 1964).

Results

Micrococcin is known to be an inhibitor of protein synthesis and acts directly upon the ribosome, probably within the ribosomal A-site. (See the Introduction, page 7 for details). The synthesis of polyphenylalanine by ribosomes from a micrococcin resistant mutant of Bacillus subtilis was shown to be resistant to this drug (Goldthwaite and Smith, 1972). With those results in mind, cell-free extracts from B. megaterium strain GS2, resistant to micrococcin, were examined for their response to this antibiotic. Comparisons were made with extracts from the wildtype (which is sensitive to micrococcin) and with those from mutant strain MJ1 (which is resistant to this drug, although it was originally selected as being resistant to thiostrepton).

It had quickly been established (data not shown) that crude cell-free extracts from the wildtype, subjected to assays of protein synthesis, were sensitive to micrococcin. Moreover extracts from strain GS2 were resistant to this antibiotic. Cell-free extracts were then fractionated into S100*,

postribosomal supernatant and salt washed ribosomes (see Materials and Methods for details). Ribosomes and S100* from wildtype and from strain GS2 were combined in all four possible ways to yield protein synthesizing systems which were incubated with and without micrococcin (Figure 4.1). In this way it was shown unequivocally that resistance to micrococcin is a feature of the ribosomes from strain GS2 and not some property of any supernatant factor.

In these crossover experiments the activity of ribosomes from strain GS2 was not the same as that with ribosomes from the wildtype (Figure 4.1) but in other experiments the levels of activity were comparable. The only exception to this was the combination of ribosomes from strain GS2 supplemented with S100* from the wildtype. This combination, for unknown reasons, never proved to be very active in these experiments despite several attempts to produce more active preparations.

It had now been established that the ribosomes were the source of resistance to micrococcin in strain GS2. Moreover, it was interesting that strain MJ1 also possessed micrococcin-resistant ribosomes. It was therefore decided to check out the dose response (with the various strains) more carefully over a range of antibiotic concentrations. Furthermore, since strain MJ1 had originally been selected as being resistant to thiostrepton, ribosomes from both mutants were assayed with both drugs and compared with each other and the wildtype.

The effects of micrococcin upon the ribosomes from the various strains are shown in Figure 4.2. Ribosomes from the wildtype were very sensitive to this antibiotic, with greater than 50% inhibition at an equimolar input of drug. In contrast, the

ribosomes from strains GS2 and MJ1 were both highly resistant to micrococcin and gave, for example, around 30% inhibition when drug was present at 100-fold molar excess over ribosomes. It should be noted that in this experiment (in contrast to the last one) S100 and not S100* was used together with ribosomes to support protein synthesis. This was because difficulties were often encountered in producing active S100*'s from all the B. megaterium strains, whereas similar problems did not arise in the preparation of S100. (For details see Methods, Page 53). Under these conditions, the activities of the ribosomes from the three strains were not affected by the source of S100 employed, nor were their responses to antibiotic. With ribosomes from wildtype or from strain GS2, S100 from the wildtype was routinely used. However, ribosomes from strain MJ1 were supplemented with homologous S100. This was done because it was suspected that some preparations of S100 from wildtype were contaminated with traces of protein BM-L11. Since ribosomes from strain MJ1 lack this particular protein, it was necessary to ensure that they were not supplemented with any protein BM-L11 from the S100. Otherwise a true picture of their sensitivity or resistance to added drug might not have been apparent.

The resistance pattern of the ribosomes from strain GS2 was seen to resemble that of strain MJ1 (Figure 4.2). This led to an initial speculation that the mutational events in these two strains might have been similar. Other evidence, however, suggested that this was unlikely. Notably, strain MJ1 grows very slowly compared with the wildtype, doubtless reflecting the absence of a protein from the ribosomes. In keeping with this supposition ribosomes from strain MJ1 function in vitro much

less well than do those from wildtype but can be restored to wildtype levels of activity by supplementation with the missing protein (Stark, 1979; Cundliffe *et al.*, 1979: see also Figure 8.1). In contrast, strain GS2 had a similar mean-generation time as the wildtype and moreover, its ribosomes were almost as active as those from wildtype, *in vitro*.

Nevertheless it was decided to carry further the comparison between ribosomes from strains GS2 and MJ1 by studying their response to thiostrepton. Micrococcin and thiostrepton are closely related antibiotics (they possess similar structures and have similar modes of action; see the Introduction for details) and resistance to both drugs can be conferred by the action of a 23S rRNA methylase (Cundliffe and Thompson, 1981a). Furthermore, strain MJ1 had originally been selected on thiostrepton and had only subsequently proved to be resistant to micrococcin. As seen in Figure 4.3 the responses of the two mutant strains to thiostrepton were completely different. This confirms that they had not arisen as a result of similar mutational events. Thus, ribosomes from wildtype and from strain GS2 were both dramatically inhibited by this antibiotic and the latter exhibited only a marginal level of resistance when compared with the former. This partial resistance of the ribosomes was not, however, reflected *in vivo* in raised MIC values. (See Chapter 3, Table 3.1). In contrast (see also Stark, 1979) ribosomes from strain MJ1 were significantly resistant to thiostrepton. This resistance (as outlined above) is known to be due to the absence of protein BM-L11 from the ribosomes of strain MJ1. Normally this protein (BM)-L11 significantly enhances the binding of thiostrepton to ribosomes (Stark, 1979;

Thompson et al., 1979), although the primary binding site for the drug is on 23S rRNA. As might be expected under such circumstances, loss of the protein is accompanied by a decreased affinity of the drug for ribosomes but binding still occurs and the result is only partial resistance.

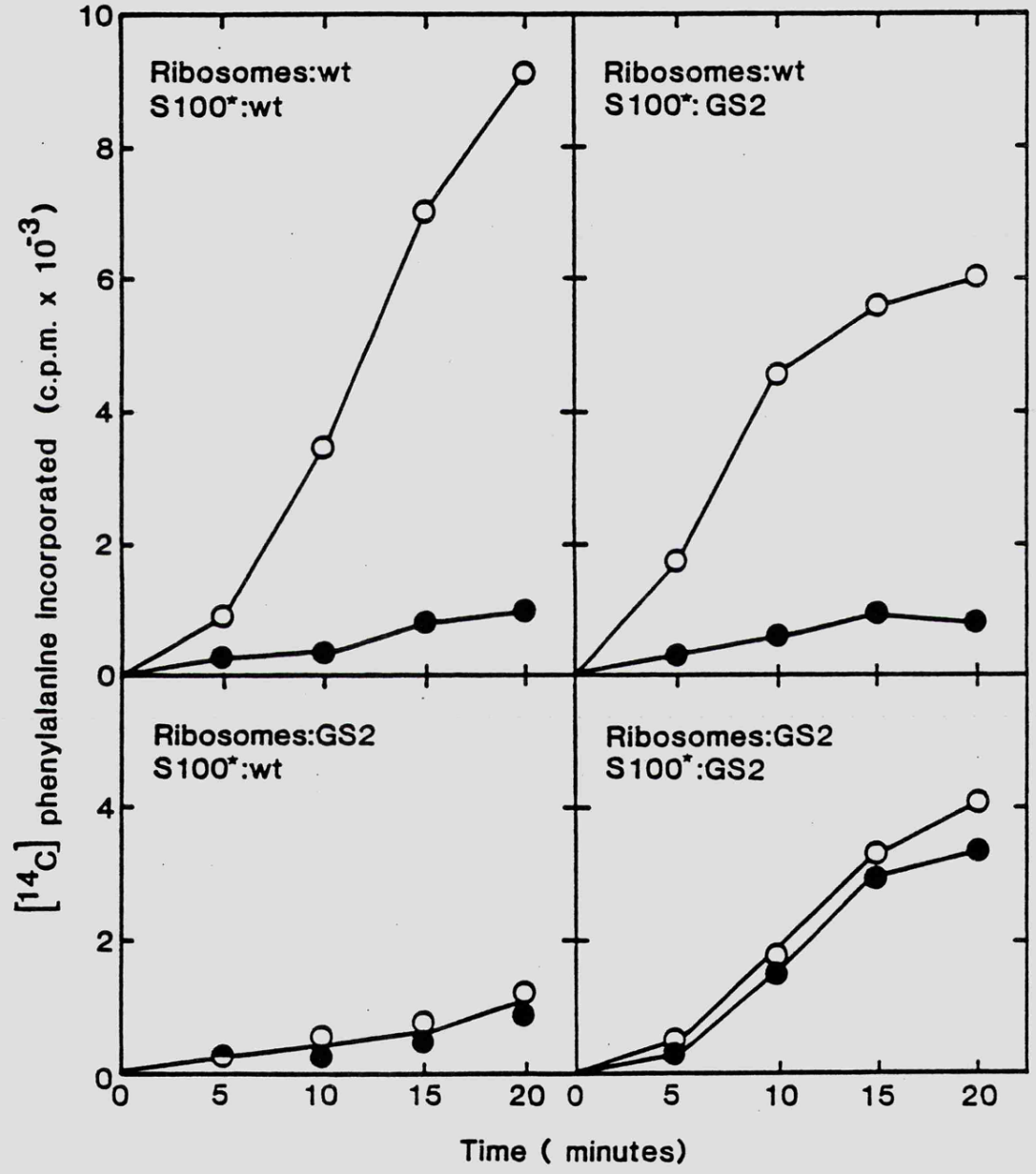
An interesting point to note is that the resistance of the ribosomes from strain MJ1 to micrococcin was much more pronounced than their resistance to the drug on which they had been selected, i.e. thiostrepton. Moreover these results were seen to be in agreement with others (Goldthwaite and Smith, 1972; Pestka et al., 1976). Strains of Bacillus subtilis selected for resistance to thiostrepton were found to be cross-resistant to micrococcin (as is the case with strain MJ1). Conversely, with strains selected for resistance to the latter drug, cross--resistance to thiostrepton was not observed and strain GS2 proves no exception here. Thus, knowing that the genetic lesion within strain GS2 was different to that within strain MJ1 the next task was to find out precisely how it differed.

Legend to Figure 4.1

Effects of micrococcin on cell-free protein synthesis: Combinations of ribosomes and S100*'s from wildtype and strain GS2

Ribosomes (10 pmol in T^{10} M^{10} A^{50} β^{3}) were preincubated with either micrococcin 100 pmol (●) or with DMSO (2% $^{v}/v$ final concentration) in controls (○) for 10 minutes at 37°C. This was followed by the addition of 10 µl S100* to give a final volume of 25 µl. An equal volume of prewarmed poly(U) cocktail was then added and assays were performed at 37°C as described in Materials and Methods. For source of ribosomes and S100* see the figure. In these assays 1000 c.p.m. represents approximately 1 pmol of phenylalanine incorporated.

Figure 4.1

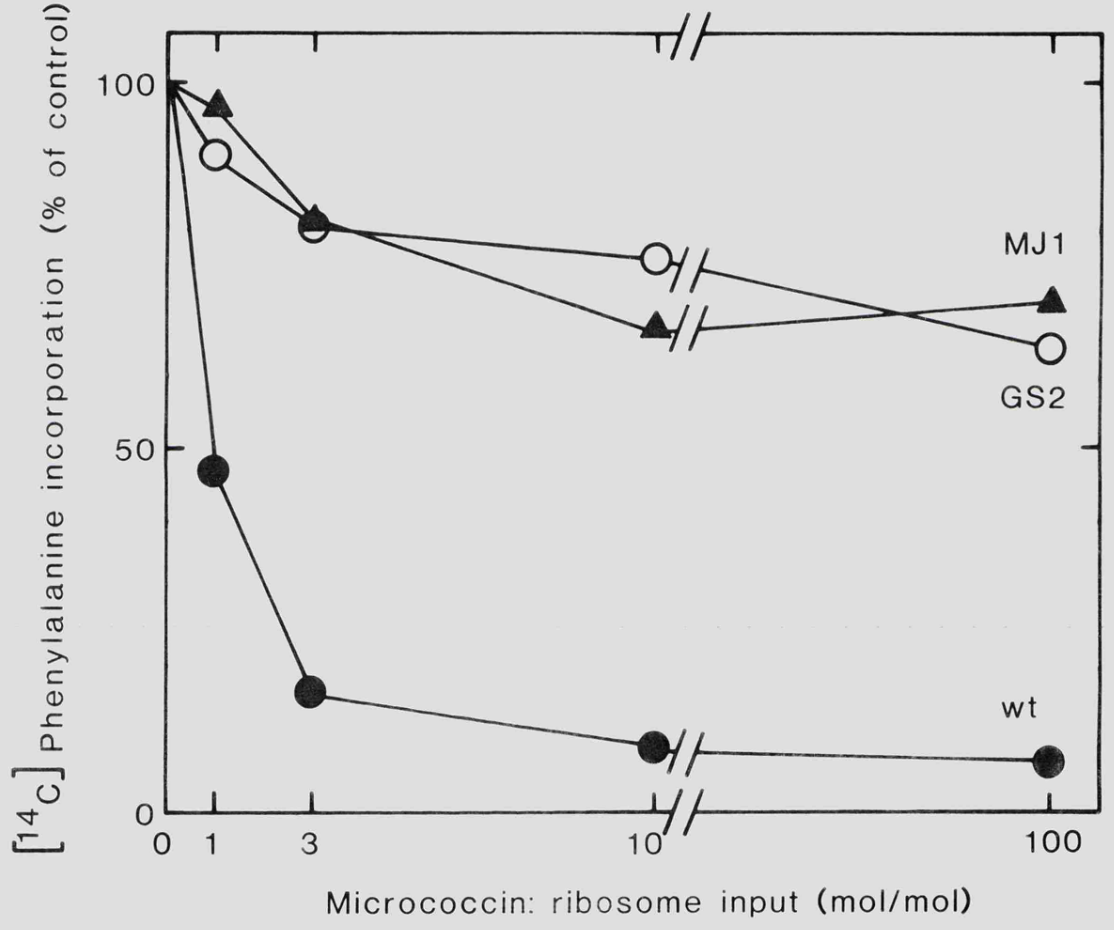

Ribosomes:wt
S100*:wt
Ribosomes:wt
S100*:GS2
Ribosomes:GS2
S100*:wt
Ribosomes:GS2
S100*:GS2
[14C] phenylalanine incorporated (c.p.m. x 10-3)
Time (minutes)
0
2
4
6
8
10
0
5
10
15
20

Legend to Figure 4.2

Effects of micrococcin on protein synthesis in extracts of various strains of B. megaterium

Salt-washed ribosomes were preincubated with micrococcin for 5 minutes at 0°C and then for 5 minutes at 37°C. Controls received DMSO at 1% ($^{v}/v$) final concentration. Then prewarmed S100 was added followed by the poly(U) cocktail to start the reaction. The data given represent the means of several experiments. Typical levels of synthesis (expressed as pmol phenylalanine incorporated/pmol ribosomes) in controls after 30 minutes were about 6 for wildtype or strain GS2 and about 1.5 for strain MJ1. A ten-fold molar excess of micrococcin over ribosomes corresponded to a drug concentration of 1 µM i.e. about 1.1 µg ml^{-1}. Source of ribosomes (●) wild-type; (○) strain GS2; (▲) strain MJ1. For sources of S100 see text.

Figure 4.2

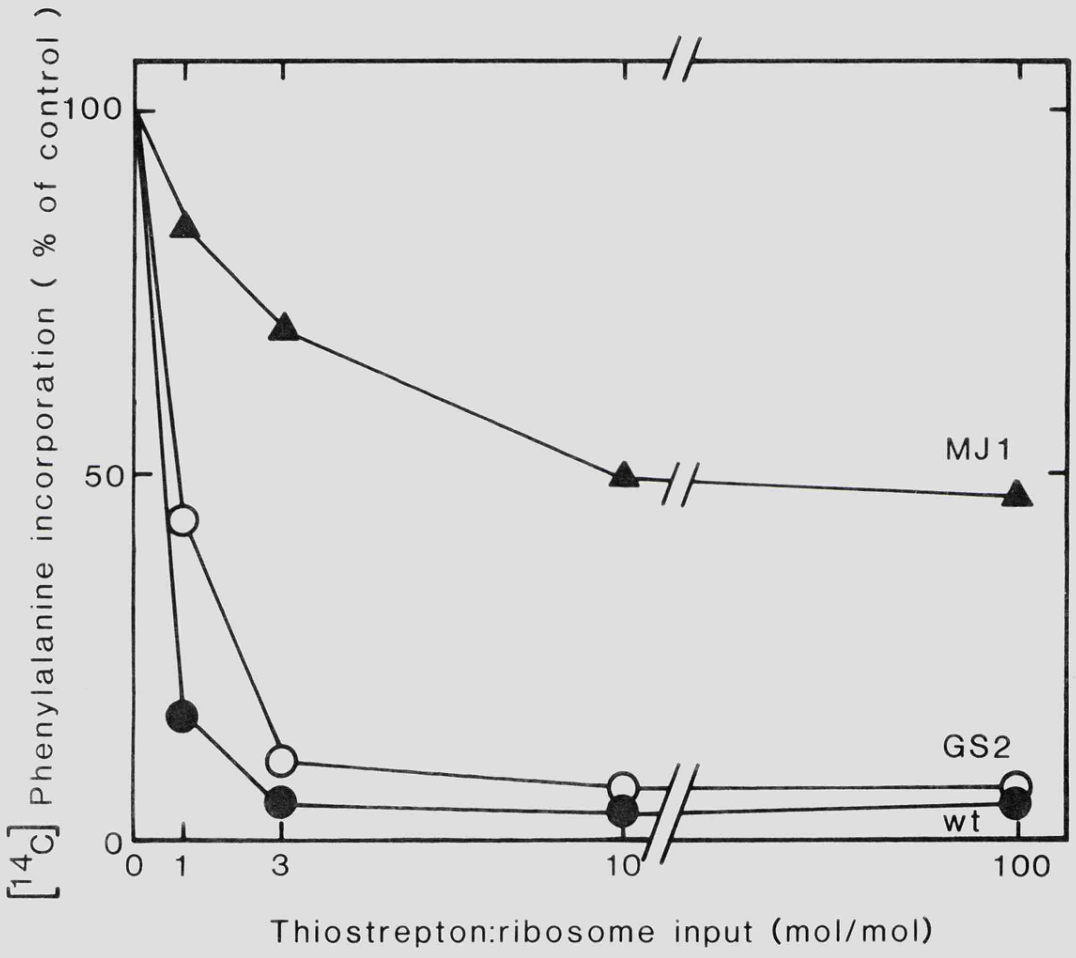

[14C] Phenylalanine incorporation (% of control)
100
50
0
0
1
3
10
100
MJ1
GS2
wt
Micrococcin: ribosome input (mol/mol)

Legend to Figure 4.3

Effects of thiostrepton on protein synthesis in extracts of various strains of B. megaterium

Salt-washed ribosomes were preincubated with thiostrepton or DMSO and assays performed as described in the Legend to Figure 4.2. A ten-fold molar excess of thiostrepton over ribosomes corresponded to a drug concentration of 1 μM or about 1.7 μg ml^{-1}. Source of ribosomes, (●) wildtype; (○) strain GS2; (▲) strain MJ1. For sources of S100 see text.

Figure 4.3


[14C] Phenylalanine incorporation (% of control)
100
50
0
0
1
3
10
100
MJ1
GS2
wt
Thiostrepton:ribosome input (mol/mol)

CHAPTER 5

ELONGATION FACTOR G DEPENDENT GTP HYDROLYSIS AND RECONSTITUTION: RESISTANCE TO MICROCOCCIN, WITHIN STRAIN GS2, IS A FEATURE OF AN ALTERED 50S RIBOSOMAL PROTEIN

CHAPTER 5

Elongation Factor G Dependent GTP hydrolysis and Reconstitution: Resistance to Micrococcin, within strain GS2, is a feature of an altered 50S ribosomal protein.

In the absence of tRNA and mRNA, ribosomes were shown to support an EF-G dependent GTP hydrolysis, uncoupled from translocation (Conway and Lipmann, 1964; Nishizuka and Lipmann, 1966a). This "uncoupled" reaction proved to be the most useful and convenient assay for the studies described here.

Thiostrepton was shown to be a very potent inhibitor of this EF-G dependent reaction (Pestka, 1970) whereas, the related antibiotic micrococcin stimulated the reaction quite markedly (Cundliffe and Thompson, 1981a). The dramatic levels of stimulation of GTP hydrolysis caused by micrococcin together with the use of a catalytic ("uncoupled") reaction rather than the normal stoichiometric reaction were considered to be useful features, which would enable ready detection of any differences between the ribosomes from micrococcin-sensitive and resistant strains. In this context the merits of using "uncoupled" reactions in drug-studies with ribosomes were marshalled by Stark and Cundliffe (1979b). For example, it was considered that partial impairment of functions which occurred only once per active ribosome might not be readily detectable. However a catalytic reaction might amplify any minor differences in activity between ribosomes from wildtype and those of mutant strains, in the presence and absence of drugs.

Accordingly the activities of 70S ribosomes from B. megaterium wildtype and those from strains GS2 and MJ1 were examined in the uncoupled hydrolysis of GTP. From Figure 5.1 it can clearly be seen that ribosomes from the wildtype and from strain GS2

supported similar levels of GTP hydrolysis whereas those from strain MJ1 were only about 20% as active. These findings with the ribosomes from strain MJ1 are in agreement with earlier observations (Stark and Cundliffe, 1979b) at which time it was also shown that when such ribosomes were supplemented with protein BM-L11, purified from wildtype, their activity in GTP hydrolysis was stimulated to about wildtype levels (see also this dissertation, Chapter 8).

Before leaving Figure 5.1, two points should be made. Despite extensive washing in buffers containing 1M NH_4Cl, ribosomal preparations usually exhibited "background" levels of GTP hydrolysis in the absence of added G-factor. Such activity which typically corresponded to about 5-8% of that observed in the presence of factor EF-G, was routinely estimated in control experiments and was subtracted from the values shown in Figure 5.1 and (as appropriate) in subsequent figures. Furthermore, it should also be noted that different ribosomal preparations differed in their activities *in vitro*. Thus although the data given in Figure 5.1 are typical of several preparations, some batches of ribosomes from strain GS2 were slightly less active than those shown.

Next, using the same assay, the effects of micrococcin upon ribosomes from the various strains of *B. megaterium* were examined (Figure 5.2). The drug caused a marked stimulation of GTP hydrolysis supported by ribosomes from wildtype whereas a much smaller effect was seen with those from strain GS2. The magnitude of the latter effect was subject to some variation. Thus, the level of stimulation of GTP hydrolysis on ribosomes from strain GS2 was sometimes less than that shown in Figure 5.2

(which indicates about 25% enhancement) and was, on occasion insignificant. In contrast, the drug was virtually without effect upon GTP hydrolysis supported by ribosomes from strain MJ1 (see also Cundliffe and Thompson, 1981a). These results should be compared with those in Figure 4.2 where the effects of micrococcin upon cell-free protein synthesis were examined. A qualitative correlation between the level of stimulation of GTP hydrolysis and the level of inhibition of protein synthesis is clearly seen. As a result of this (see later sections of this dissertation) the ribosomal response to micrococcin in GTPase assays has been used as an indicator of the levels of sensitivity.

Ribosomes from wildtype and from strain GS2 were also compared in their response to thiostrepton. Again, the uncoupled GTPase assay was employed (see Figure 5.3). In both cases, thiostrepton inhibited dramatically GTP hydrolysis supported by ribosomes and factor EF-G. Even so, these levels of inhibition were not as pronounced as those seen in Figure 4.3 where cell-free protein synthesis was measured. Furthermore, ribosomes from strain GS2 were again marginally more resistant to thiostrepton than were those from the wildtype. In comparison, in the GTPase assay, ribosomes from strain MJ1 were shown previously to be inhibited by approximately 50% when thiostrepton was employed at 12-fold molar excess over ribosomes (Stark, 1979; Stark and Cundliffe, 1979b). Clearly ribosomes from strain MJ1 were substantially more resistant to thiostrepton than were those from strain GS2, both in the GTPase assay and in cell-free protein synthesis. They were not, however, totally resistant to thiostrepton and this has been commented upon earlier (Chapter 4, page 79).

In contrast, ribosomes from strains GS2 and MJ1 were about equally resistant to micrococcin regardless of the assay system employed.

A more precise localization of the alteration within the ribosomes of mutant strain GS2 was now sought. Here, again, the "micrococcin effect" upon the EF-G dependent GTPase assay was employed, following the re-combination of 30S and 50S ribosomal subunits from wildtype and from strain GS2 (Figure 5.4). The subunits were re-associated in all the four possible combinations to produce 70S particles which, when supplemented with factor EF-G displayed similar levels of GTPase activity. Micrococcin caused massive stimulations of GTP hydrolysis in those subunit recombinations incorporating 50S ribosomal particles derived from the wildtype. In contrast only slight stimulations were noted when 50S subunits from strain GS2 were employed. Thus, it was concluded that the micrococcin-resistant strain GS2, possessed abnormal 50S ribosomal subunits. These data were in agreement with others (Smith et al., 1976) where it was shown that the 50S ribosomal subunits from a micrococcin-resistant strain of B. subtilis were responsible for resistance to the drug.

The GTPase assay, coupled with the powerful technique of total reconstitution of ribosomal particles, was used to establish a causal connection between resistance to micrococcin and some property of the ribosomal protein fraction from strain GS2. In these experiments 50S ribosomal particles were reconstituted with rRNA from 70S ribosomes (RNA70) and with proteins derived from 50S ribosomal subunits (see Materials and Methods for details). Again, this was done using all four possible combinations of these components prepared from the wildtype and from strain GS2.

To assess the relative efficiency of reconstitution the various particles were assayed for EF-G dependent GTPase, alongside control 50S ribosomal subunits (Figure 5.5). In all cases, the 50S particles were supplemented with native 30S subunits derived from the wildtype. (Supplementation with 30S particles from strain GS2 gave similar results, data not shown.) All the reconstituted particles assayed in this way supported similar levels of GTP hydrolysis and this corresponded to a respectable 33% of the activity of native 50S ribosomal subunits. Next, the reconstituted particles were assayed for their response to micrococcin. Two variants of this assay were employed, one in the absence of 30S ribosomal subunits, in order to eliminate effects due to the presence of any contaminating 50S subunits in the 30S subunit preparations, (Figure 5.6) the other in the presence of 30S particles (Figure 5.7). The 30S particles (themselves devoid of GTPase activity) were included in the latter assay because they normally stimulate the activity of 50S subunits and here they showed that the reconstituted particles responded appropriately to their presence to yield highly active 70S ribosomes. (Compare the activities in Figures 5.6 and 5.7 and with Figure 5.1.) In both these assay systems, micrococcin promoted stimulation of GTPase activity in those particles containing TP50 proteins derived from the wildtype. From these results it was clear that the resistance to micrococcin was associated with the TP50 fraction from the ribosomes of strain GS2 and not with rRNA.

Several courses of action were now open to define whether the ribosomes from strain GS2 contained an altered protein or whether, like those of MJ1, they were devoid of one. Two dimensional gel

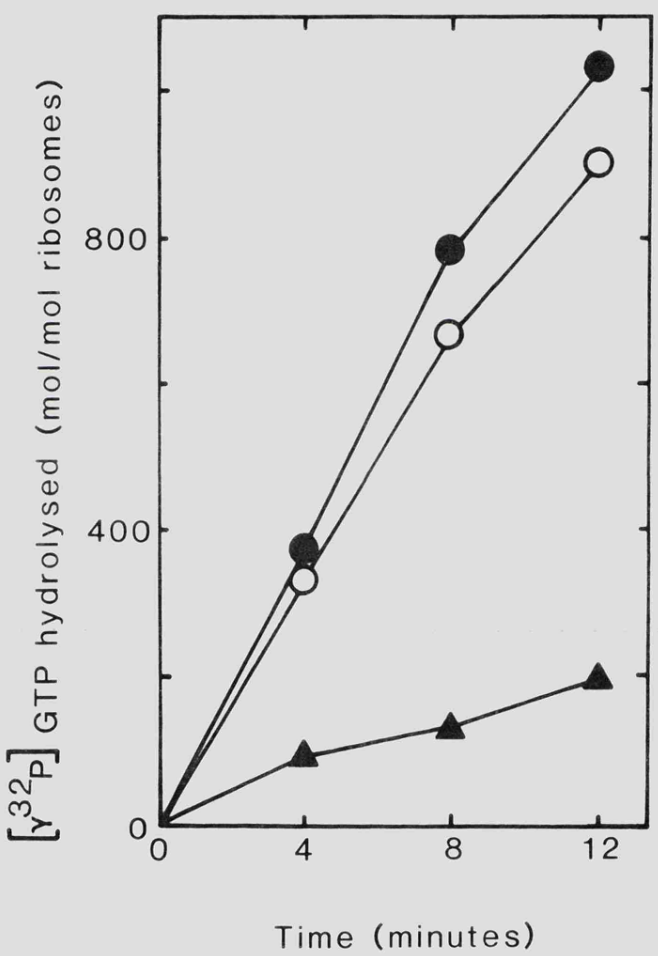
electrophoresis or immunological analysis of the TP50 fraction could have been employed at this stage. However such techniques can only detect quite drastic alterations in proteins and other workers had not detected any abnormalities among the ribosomal proteins from micrococcin-resistant mutants of B. subtilis despite a most rigorous analysis (Wienen et al., 1979). From data already to hand, ribosomal protein BM-L11 was beginning to appear as a likely candidate for the altered protein within the ribosomes of strain GS2. It had previously been shown that ribosomal protein L11 from E. coli formed a complex with 23S rRNA to which micrococcin could bind (Cundliffe and Thompson, 1981a). Furthermore, strains of B. megaterium (such as MJ1) which lack a ribosomal protein BM-L11 are resistant to micrococcin (see Figure 4.2 and Cundliffe et al., 1979). Such strains however, as noted previously, (Chapter 4, page 78) were extremely sick and so it was suspected that the ribosomes from strain GS2 might not actually lack this or any other protein. It was therefore decided to establish whether protein BM-L11 was present in the ribosomes of strain GS2 and, if so, whether it differed from that in the wildtype.

Legend to Figure 5.1

Uncoupled GTP hydrolysis dependent upon factor EF-G (from E. coli) and ribosomes from various strains of B. megaterium

Ribosomes (1 pmol) were incubated for 5 minutes at 30°C in 65 µl T^{15} M^{10} A^{80} β^{2} and reactions then started by the addition of 10 µl of a mix containing factor EF-G (50 pmol) and [γ^{32}P] GTP (10 nmol). Assays were performed as described in Materials and Methods. Source of ribosomes, (●) wildtype; (0) strain GS2; (▲) strain MJ1.

Figure 5.1

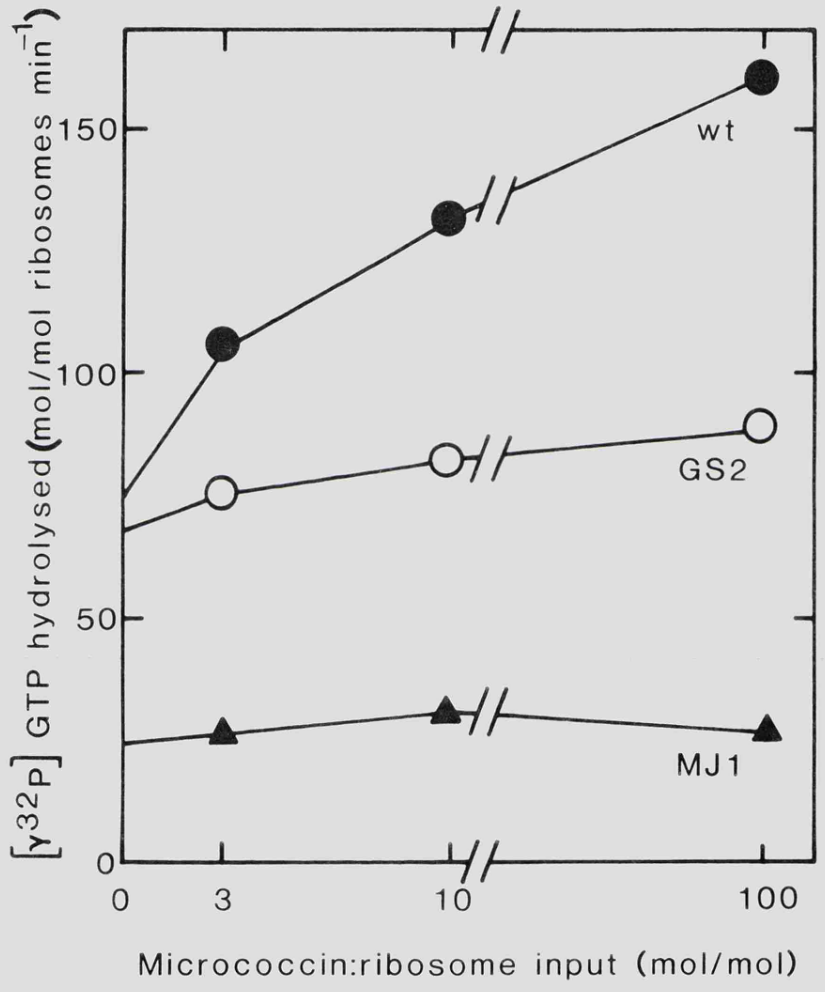

[γ32P] GTP hydrolysed (mol/mol ribosomes)
800
400
0
0
4
8
12
Time (minutes)

Legend to Figure 5.2

Effect of micrococcin on uncoupled hydrolysis of GTP with ribosomes from various strains of B. megaterium

Salt-washed ribosomes (1 pmol) were preincubated with micrococcin or in controls with DMSO (0.66% $^{v}/v$ final concentration) for 5 minutes at 0°C and then for 5 minutes at 37°C. Then [γ^{32}P] GTP and factor EF-G were added in excess over ribosomes. Assays were then performed as described in Materials and Methods. At ten-fold molar excess over ribosomes, micrococcin was present at about 0.15 µg ml^{-1}. Source of ribosomes (●) wildtype; (○) strain GS2; (▲) strain MJ1.

Figure 5.2

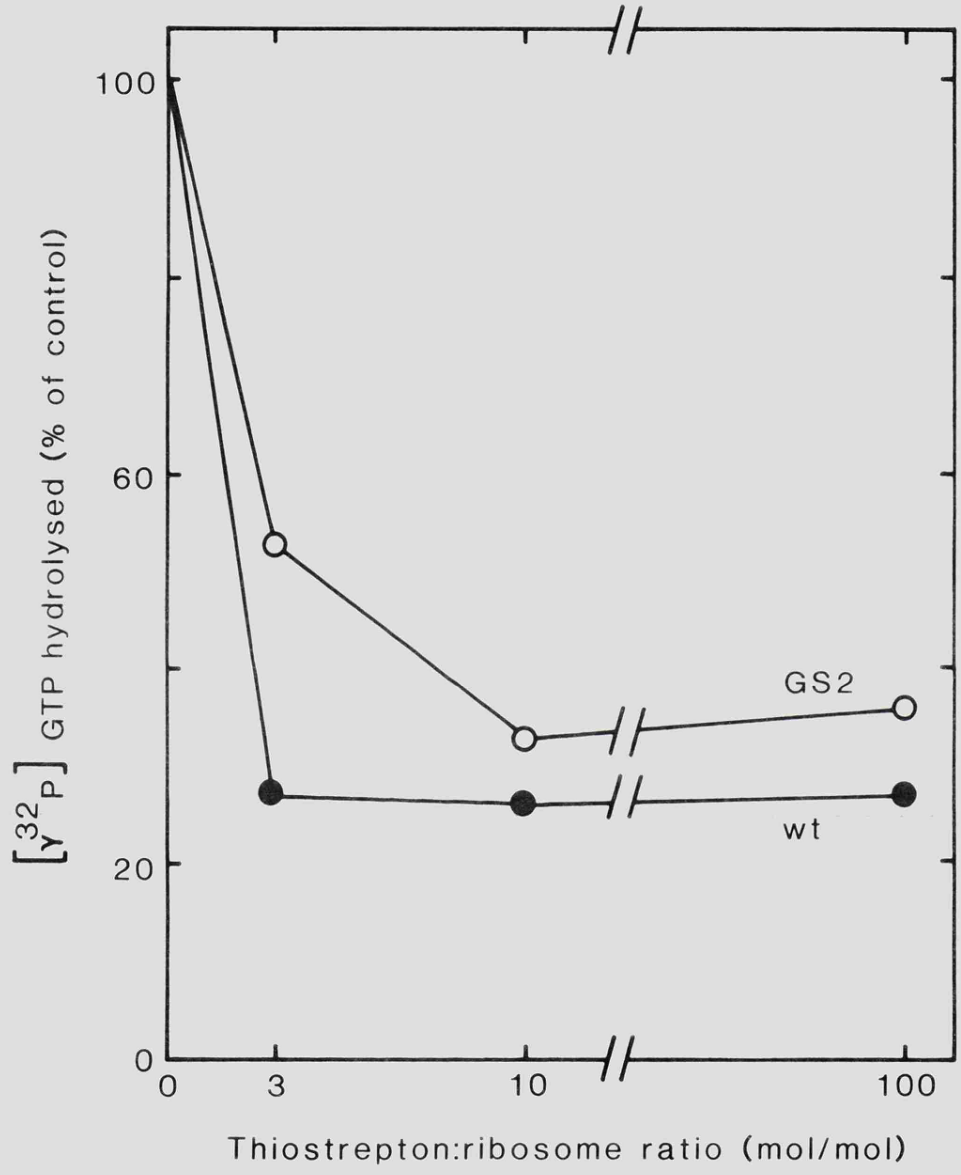

[γ32P] GTP hydrolysed (mol/mol ribosomes min-1)
150
100
50
0
wt
GS2
MJ1
0
3
10
100
Micrococcin:ribosome input (mol/mol)

Legend to Figure 5.3

Effects of thiostrepton on uncoupled hydrolysis of GTP with ribosomes from wildtype and strain GS2

Salt-washed ribosomes (1 pmol) were preincubated with thiostrepton or in controls with DMSO (0.66% $^{v}/v$ final concentration) for 10 minutes at 30°C. Then assays were performed as described in the Legend to Figure 5.1. A ten-fold molar excess of the drug over ribosomes corresponded to a thiostrepton concentration of 0.13 µM i.e. about 0.22 µg ml^{-1}. Source of ribosomes (●) wildtype; (0) strain GS2. Typical levels of hydrolysis (expressed as pmol GTP hydrolysed pmol ribosomes min^{-1}) in controls were about 105 for wildtype and 64 for strain GS2. The data given are the means of duplicate experiments.

Figure 5.3

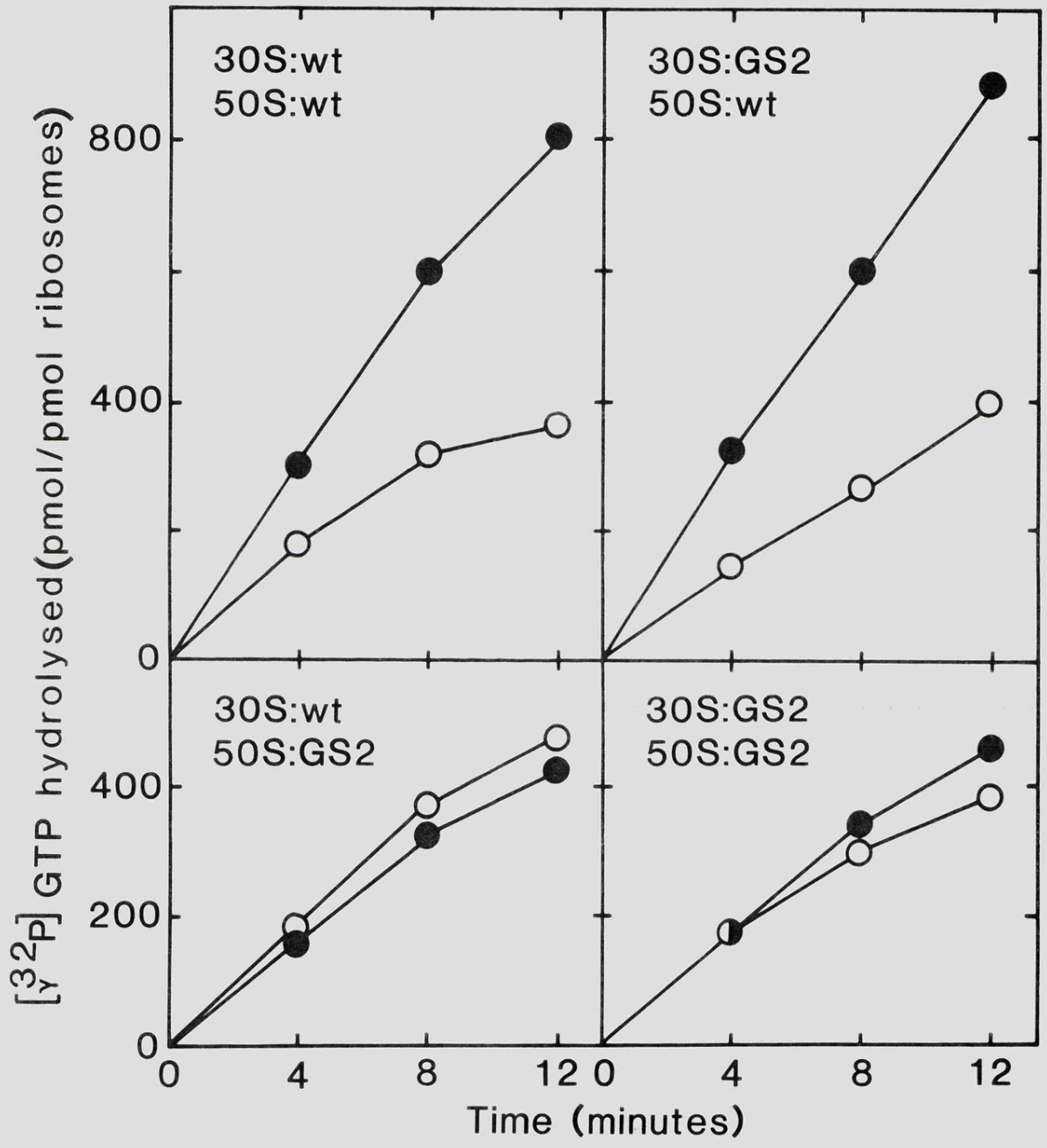

100
60
20
0
0
3
10
100
GS2
wt
[γ32P] GTP hydrolysed (% of control)
Thiostrepton:ribosome ratio (mol/mol)

Legend to Figure 5.4

EF-G dependent GTPase : Subunit localization of resistance to micrococcin within the ribosomes of strain GS2

Ribosomal subunits (30S and 50S, 2 pmol of each) from wildtype and strain GS2 were recombined in all four possible ways followed by incubation at 30°C for 10 minutes in 64 µl T^{15} M^{10} A^{80} β^{2}. Then micrococcin 20 pmol (●) or DMSO 0.66% (^{v}/v) final concentration (O) was added followed by incubation at 30°C for 10 minutes. Assays were then performed as described in the Legend to Figure 5.1. For the sources of the ribosomal subunits and their combinations see the Figure.

Figure 5.4

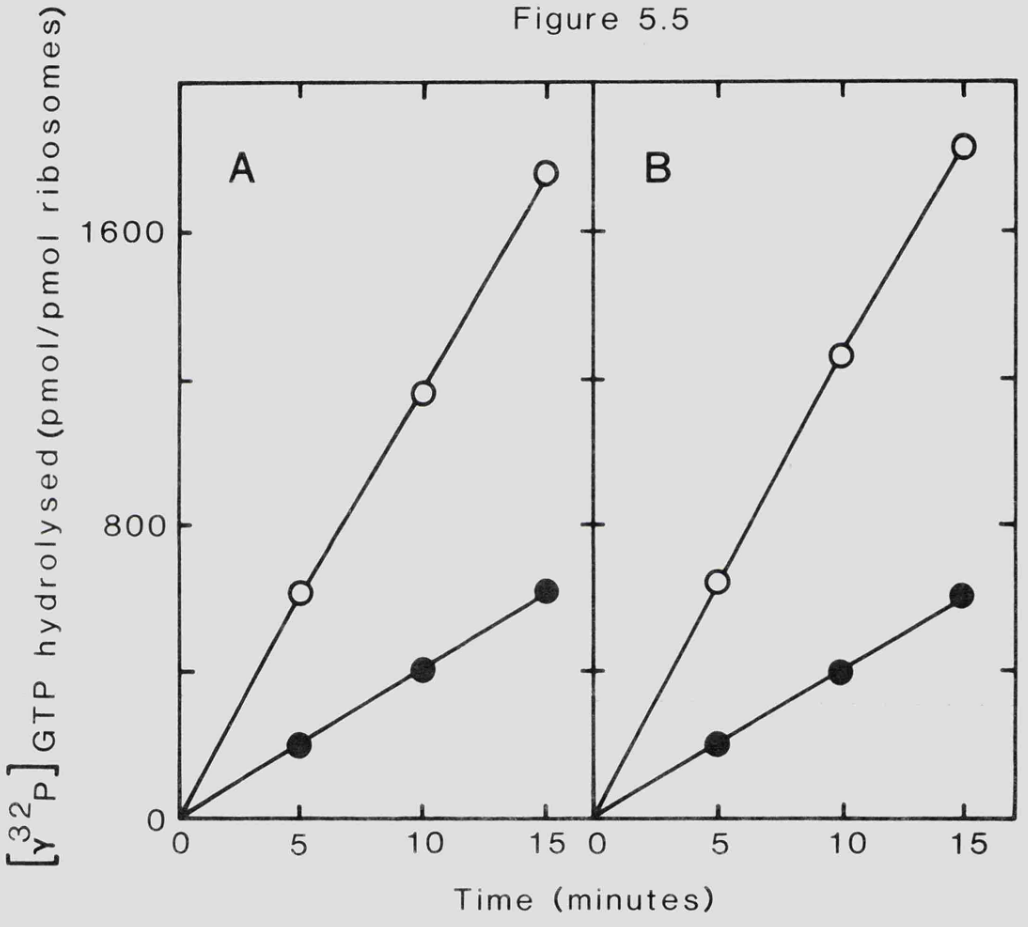

30S:wt
50S:wt
30S:GS2
50S:wt
30S:wt
50S:GS2
30S:GS2
50S:GS2
[γ32P] GTP hydrolysed (pmol/pmol ribosomes)
800
400
0
400
200
0
0
4
8
12
0
4
8
12
Time (minutes)

Legend to Figure 5.5

EF-G dependent GTP hydrolysis : Activity of reconstituted 50S ribosomal particles

Reconstituted 50S subunits were obtained by recombining TP50 proteins and RNA 70, derived from the ribosomes of wildtype and strain GS2, in all four possible ways (see Materials and Methods). The reconstituted particles or native 50S subunits (2 pmol) were supplemented with native 30S ribosomal subunits (4 pmol) derived from wildtype followed by incubation for 10 minutes at 30°C. Assays of GTP hydrolysis were then performed as described in the Legend to Figure 5.1. In (A) the levels of GTP hydrolysed by native 50S subunits from wildtype and reconstituted 50S particles, formed from wildtype components are shown. In (B) the levels of GTP hydrolysed by native 50S subunits from strain GS2 and 50S particles reconstituted from strain GS2 components, are shown. Heterologous reconstituted particles gave similar results. (O) native 50S particles; (●) reconstituted 50S sununits.

Figure 5.5

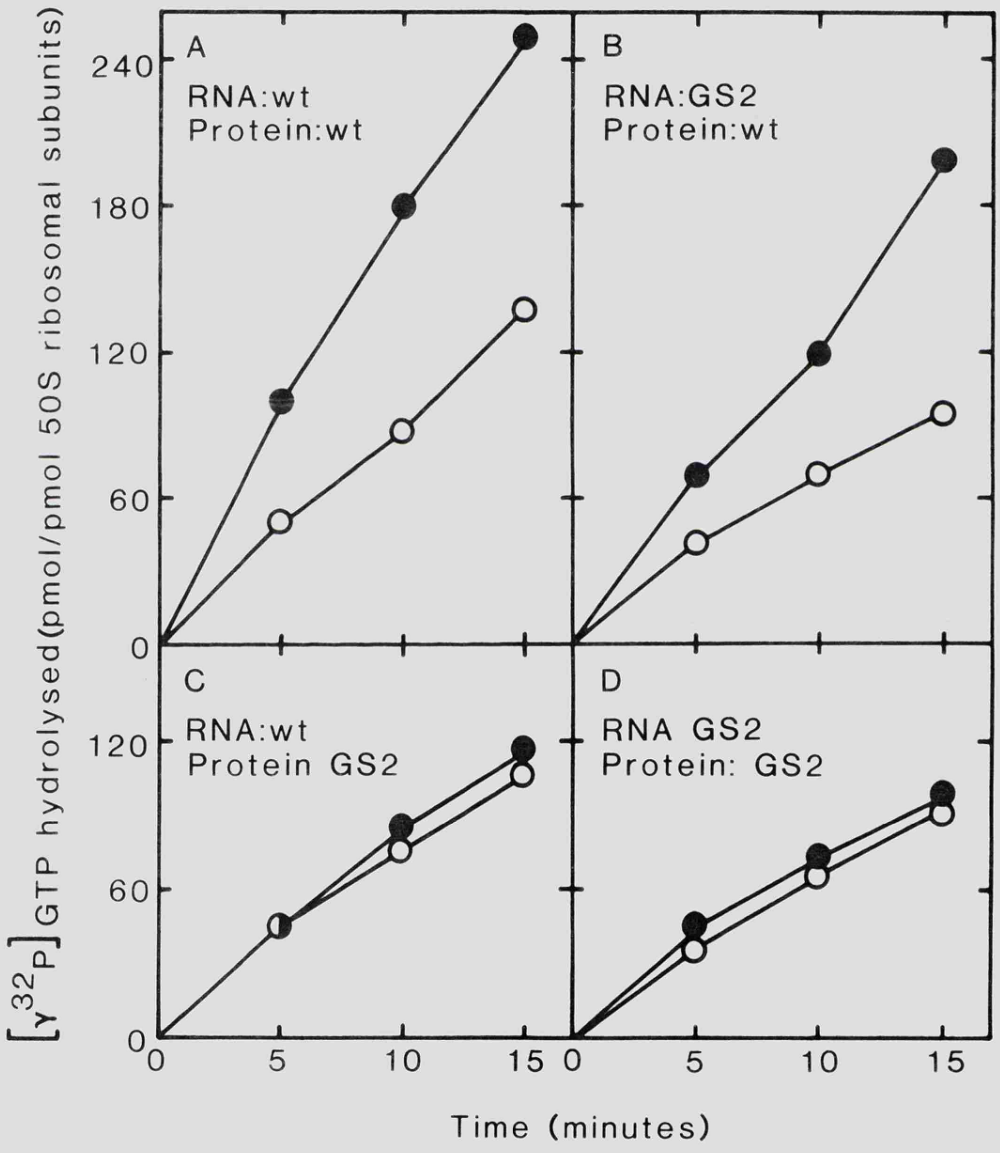

A
B
1600
800
0
0
5
10
15
0
5
10
15
Time (minutes)
[γ32P]GTP hydrolysed (pmol/pmol ribosomes)

Legend to Figure 5.6

Effects of micrococcin on reconstituted 50S ribosomal subunits in the absence of 30S ribosomal particles

Reconstituted 50S subunits were obtained by recombining, in all four possible ways, TP50 proteins and RNA 70 derived from the ribosomes of wildtype and strain GS2 (see Materials and Methods). Products of reconstitution incubations, assumed to include 2 pmol 50S particles, were preincubated for 10 minutes at 30°C with micrococcin, 200 pmol (●) or DMSO 0.66% ($^{v}/v$) final concentration (○). Then [γ^{32}P] GTP and factor EF-G were added in excess over ribosomes and assays performed as previously described. Reconstituted particles contained (A) RNA and proteins from wildtype; (B) RNA from strain GS2 and proteins from wildtype; (C) RNA from wildtype and proteins from strain GS2; (D) RNA and proteins from strain GS2.

Figure 5.6

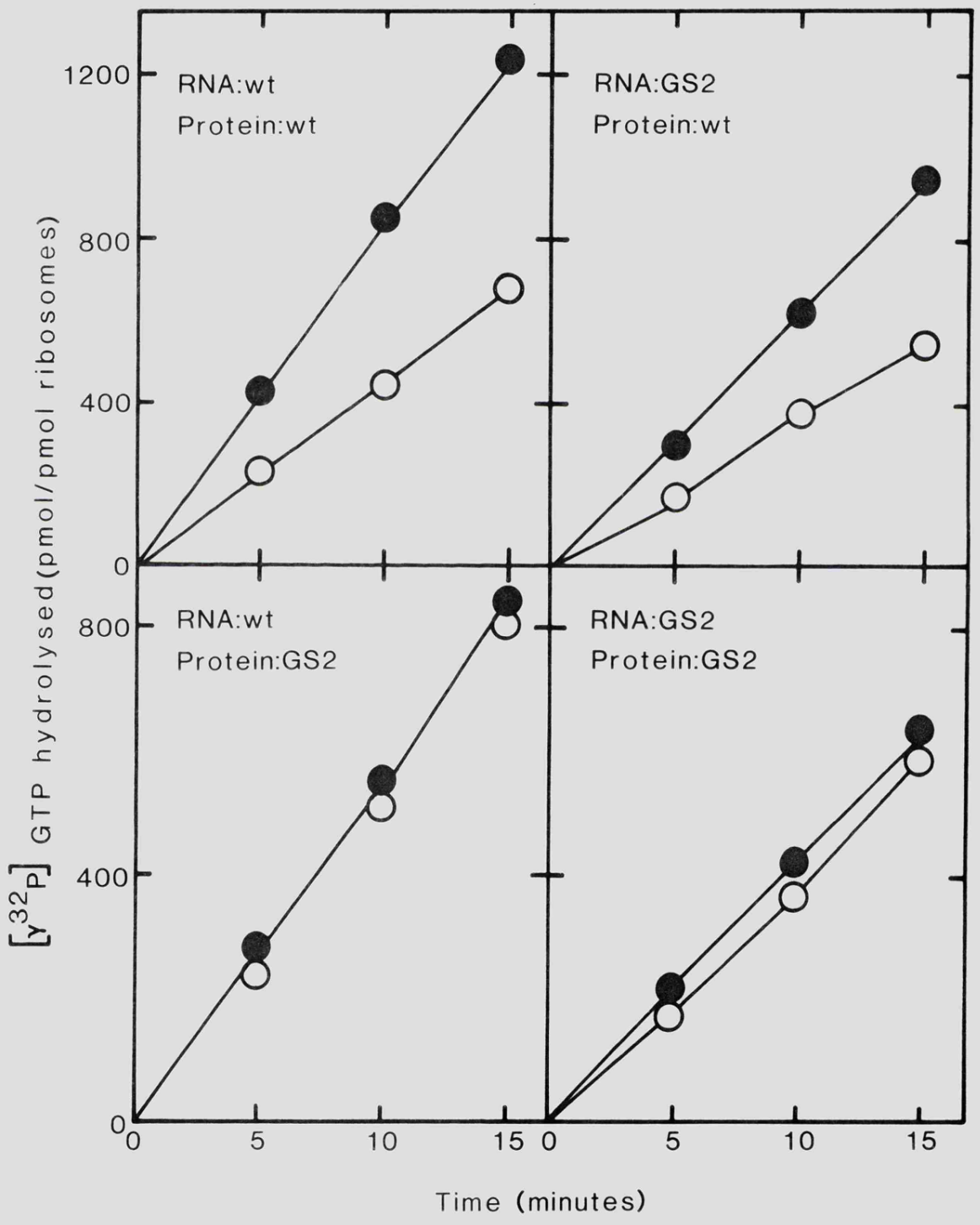

A
RNA:wt
Protein:wt
B
RNA:GS2
Protein:wt
C
RNA:wt
Protein GS2
D
RNA GS2
Protein: GS2
240
180
120
60
0
120
60
0
0
5
10
15
0
5
10
15
[γ32P]GTP hydrolysed(pmol/pmol 50S ribosomal subunits)
Time (minutes)

Legend to Figure 5.7

Effects of micrococcin on reconstituted 50S ribosomal subunits supplemented with wildtype 30S ribosomal subunits

Reconstituted 50S subunits were obtained by recombining, in all four possible ways, TP50 proteins and RNA 70 derived from the ribosomes of wildtype and strain GS2. Products of reconstitution incubations, assumed to include 2 pmol 50S particles were supplemented with 4 pmol 30S ribosomal subunits from wildtype. Uncoupled GTPase activity was then assayed as in the Legend to Figure 5.6, in the absence of (0) or presence (●) of micrococcin (200 pmol). See the Figure for the composition of the reconstituted particles.

Figure 5.7


RNA:wt
Protein:wt
RNA:GS2
Protein:wt
RNA:wt
Protein:GS2
RNA:GS2
Protein:GS2
1200
800
400
0
800
400
0
0
5
10
15
0
5
10
15
[γ32P] GTP hydrolysed (pmol/pmol ribosomes)
Time (minutes)

CHAPTER 6

THE STRINGENT RESPONSE: RIBOSOMES FROM STRAIN GS2 POSSESS PROTEIN BM-L11

CHAPTER 6

The Stringent Response: Ribosomes from strain GS2 possess protein BM-L11.

Two approaches, both of which involved tests for the stringent response, were made to determine whether or not ribosomes of strain GS2 possessed their own functional protein BM-L11.

Data already to hand had suggested strongly that the ribosomes from strain GS2 did not lack protein BM-L11 and those results have been discussed in the previous sections (for example, see Chapter 4, page 78). Since an indispensable role for this protein or its equivalent in the stringent response had already been established (Stark and Cundliffe, 1979a; Smith *et al.*, 1978 and see Introduction for details) any strain with a missing protein (BM)-L11 should be relaxed. Such strains do not show diminished synthesis of RNA during starvation for amino-acids. Moreover, it is ultimately a lack of amino-acylated tRNA which triggers the stringent response (see Introduction). The antibiotic pseudomonic acid is a powerful inhibitor of isoleucyl-tRNA synthetase and consequently exerts its effect in a readily demonstrable fashion upon stringent but not relaxed strains (Hughes and Mellows, 1978). Experiments were designed with these facts in mind. Figure 6.1 shows the results of experiments in which pseudomonic acid was added to growing cultures of the three strains of *B. megaterium*. Both the wildtype and strain GS2 exhibited the typical response of a stringent strain, i.e. curtailment of RNA synthesis in the presence of the drug. For comparison, the effects of the antibiotic upon a relaxed strain (i.e. MJ1) are also shown. Here, RNA continues to accumulate under the induced starvation conditions, in agreement with earlier observations (Stark and

Cundliffe, 1979a).

Experiments were then performed *in vitro* to examine the capability of the ribosomes, from the various strains, to synthesize guanosine tetra- and pentaphosphate and to test their response to micrococcin. Figure 6.2 clearly shows that ribosomes from the wildtype and from strain GS2 were active in the synthesis of guanosine polyphosphates (tracks 1 and 5 respectively). Normally, however, ribosomes from strain MJ1 are completely inactive in the synthesis of guanosine polyphosphates unless supplemented with protein BM-L11 (Stark and Cundliffe 1979a; Stark *et al*., 1980). Unfortunately in this case (track 9) the ribosomes from strain MJ1 did not appear to be totally inactive in the synthesis of guanosine tetraphosphate although no spot corresponding to the penta--phosphate was visible. When these ribosomes were supplemented with protein BM-L11 from the wildtype, an efficient synthesis of both the tetra and pentaphosphate was evident (track 10). In control experiments, similar treatment of ribosomes from the wildtype or mutant GS2 with protein BM-L11 (tracks 2 and 6) had no effect upon their activity. The synthesis of "Magic spot" nucleotides by ribosomes from wildtype or strain GS2, and by ribosomes from strain MJ1 when supplemented with protein BM-L11 derived from wildtype, was almost totally inhibited by thiostrepton (tracks 3, 7 and 11 respectively). The inhibition of this reaction by thiostrepton indicated that this process was predominantly ribosome dependent. Again in other experiments, performed in this laboratory, the synthesis of guanosine polyphosphates by ribosomes from wildtype, or strain MJ1 supplemented with BM-L11, was totally abolished by thiostrepton

(Stark and Cundliffe, 1979a). The 'unusual' material present in track 9 (also present in tracks 14 and 15) and possibly the residual spots in tracks 3, 7 and 11 may represent some tetraphosphate produced in a ribosome-independent reaction. This ribosome-independent synthesis of guanosine polyphosphates was briefly mentioned in the introduction (page 38) and is known to be insensitive to thiostrepton. However, the reaction normally occurs only below 30°C (Block and Haseltine, 1975). Control experiments indicated that neither ribosomes (for example see track 4), nor stringent factor (track 15) nor protein BM-L11 alone (track 14) could support the synthesis of ppGpp (see above) or pppGpp. However, in qualification of that statement, it is to be noted that in the presence of ribosomes alone (tracks 4, 8 and 12), or in the absence of both ribosomes and stringent factor (track 13), some material was detected in the ppGpp region of the autoradiogram. This was a source of contamination in the commercial preparations of (γ32P) ATP used in these experiments (see also for example track 9 in Figure 6.3). Finally, the identity of the material which "caps" the ppGpp region and which is of variable intensity from track to track is unknown. It was now clear, however, from both the *in vivo* and *in vitro* evidence that ribosomes from strain GS2 did possess their own functionally active version of protein BM-L11.

The effect of micrococcin upon the ribosomes of the wildtype and those of mutant GS2 was next examined. Figure 6.3 shows the results of such *in vitro* experiments and Table 6.1 shows the

relative levels (expressed as % inhibition) of ppGpp and pppGpp formed as a function of the concentration of micrococcin present. From these data (see figure legend) it was observed that the ribosomes from strain GS2 were only about half as active as those from the wildtype in the synthesis of guanosine polyphosphates. This was an interesting observation because of the fact that protein BM-L11 was known to be indispensable for this reaction (discussed above). The reduction in the formation of guanosine tetra- and pentaphosphate could be due to the presence of an altered form of protein BM-L11 on the ribosomes of strain GS2 and would therefore bear further investigation. Moreover, these data showed that the synthesis of guanosine polyphosphates supported by ribosomes from the mutant strain was less sensitive to micrococcin than was that supported by ribosomes from wildtype (Table 6.1).

Two points are worthy of discussion here. The ribosomes from wildtype are not affected by micrococcin in this assay as dramatically as they are in poly U-directed protein synthesis or in the GTPase reaction. (Compare the data of Table 6.1 with Figures 4.2 and 5.2.) Moreover there is not such a pronounced discrimination by micrococcin between the ribosomes from wildtype and strain GS2 as is seen in the other assays. Thus, on the one hand the drug would not appear to be such a potent inhibitor of the synthesis of guanosine polyphosphates. On the other, it would appear that the synthesis of nucleotides on ribosomes of strain GS2 can still be inhibited to some extent.

The other point of note, is the presence of the unusual nucleotide which appears on such chromatograms only when ribosomes from strain GS2 are employed in this assay (see

Figure 6.3). This 'nucleotide' migrates to a position between that for guanosine tetraphosphate and GTP. It is not known what this nucleotide is but it would be interesting to isolate and identify it. One obvious speculation is that it might be the so called MSIII. This nucleotide, guanosine 5'-diphosphate--3'-monophosphate (ppGp) was originally not discovered because of its close migration with GTP (Pao and Gallant, 1979). In these experiments also the unknown product is seen to run very close behind GTP. Another possibility is that it could be an adenosine polyphosphate. Such nucleotides have been, arguably, associated with the onset of sporulation in B. subtilis and their synthesis is reported to be dependent upon ribosomes (reviewed by Chambliss, 1979). The important and obvious question arising from this, is why the ribosomes from strain GS2 should produce this odd nucleotide anyway and whether it has any function when synthesized.

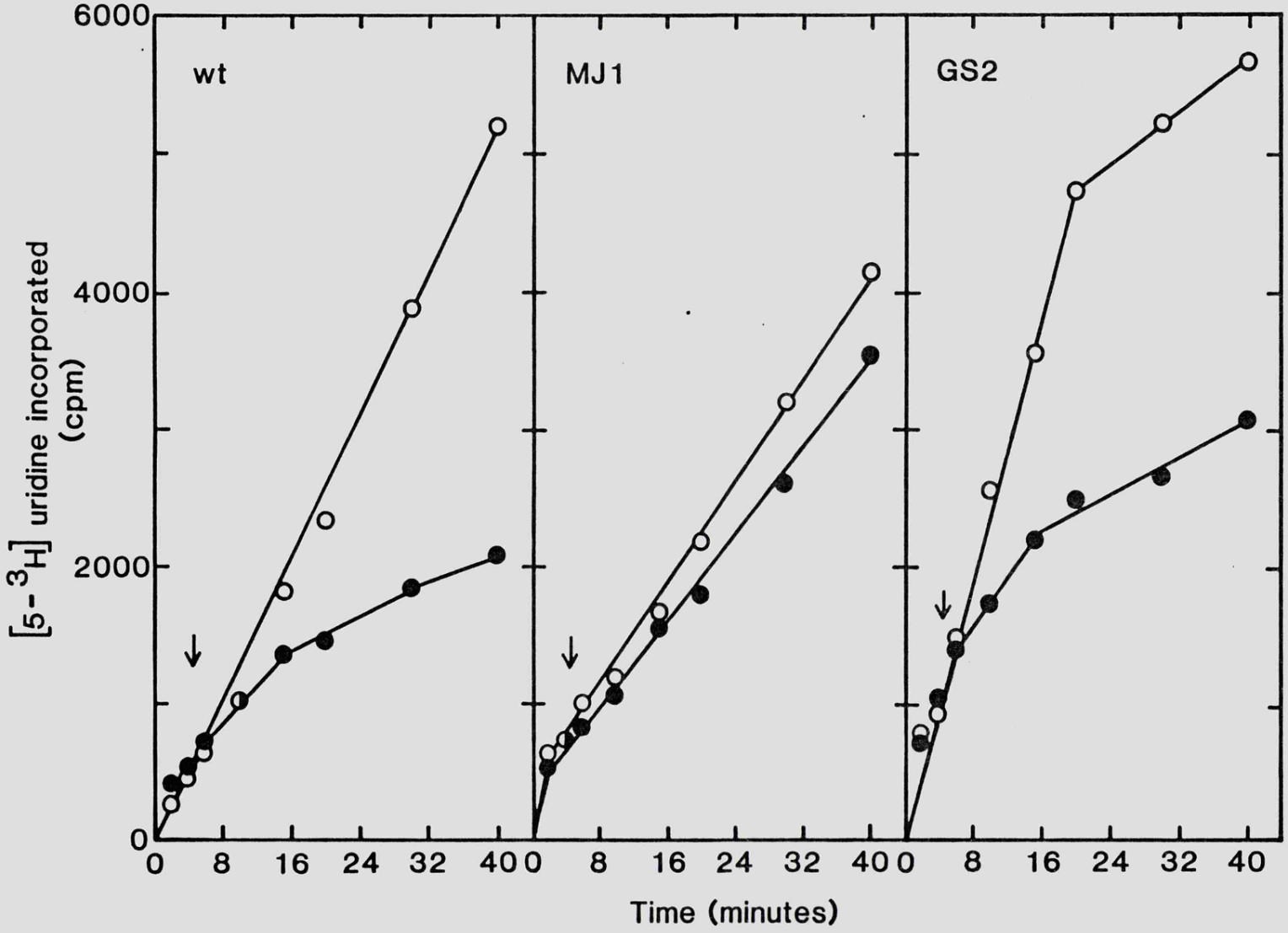
So the next task was to purify ribosomal protein BM-L11 from wildtype and from strain GS2 and then to see if the protein from the mutant differed from its wildtype counterpart.

Legend to Figure 6.1

The effect of pseudomonic acid on the synthesis of RNA by wildtype and strains GS2 and MJ1

Incorporation of [^{3}H]uridine into trichloroacetic acid precipitates was measured in parallel cultures for each strain over a 40 minute time course. Sodium pseudomonate, 200 μg ml^{-1}, was added to some cultures (●) at the times indicated by the arrows. In control cultures (○) growth remained exponential during the course of the experiments (data not shown).

Figure 6.1


6000
4000
2000
0
wt
MJ1
GS2
[5-3H] uridine incorporated (cpm)
0 8 16 24 32 40
Time (minutes)

Legend to Figure 6.2

Synthesis of guanosine polyphosphates by ribosomes from B. megaterium wildtype, mutant strain GS2 and strain MJ1

Assays contained ribosomes (25 pmol), poly(U), deacylated-tRNA, stringent factor, GTP and [γ^{32}P] ATP. Additionally some ribosomes were incubated with protein BM-L11 (250 pmol) for 5 minutes at 0°C followed by 5 minutes at 20°C before addition to reaction mixtures. Thiostrepton (0.42 µg, 250 pmol) was added to some ribosomes with incubation for 5 minutes at 20°C prior to their addition to reaction mixtures. The drug was added as 1 µl of solution in 50% (^{v}/v) DMSO, which was also added to all other assay mixtures. Identification of tracks: (1 - 4) ribosomes from wildtype; (5 - 8) ribosomes from strain GS2; (9 - 12) ribosomes from strain MJ1:-

1,5,9. Control, no additions of protein or drug.

2,6,10. Ribosomes plus protein BM-L11.

3,7,11. Ribosomes plus protein BM-L11 and then thiostrepton.

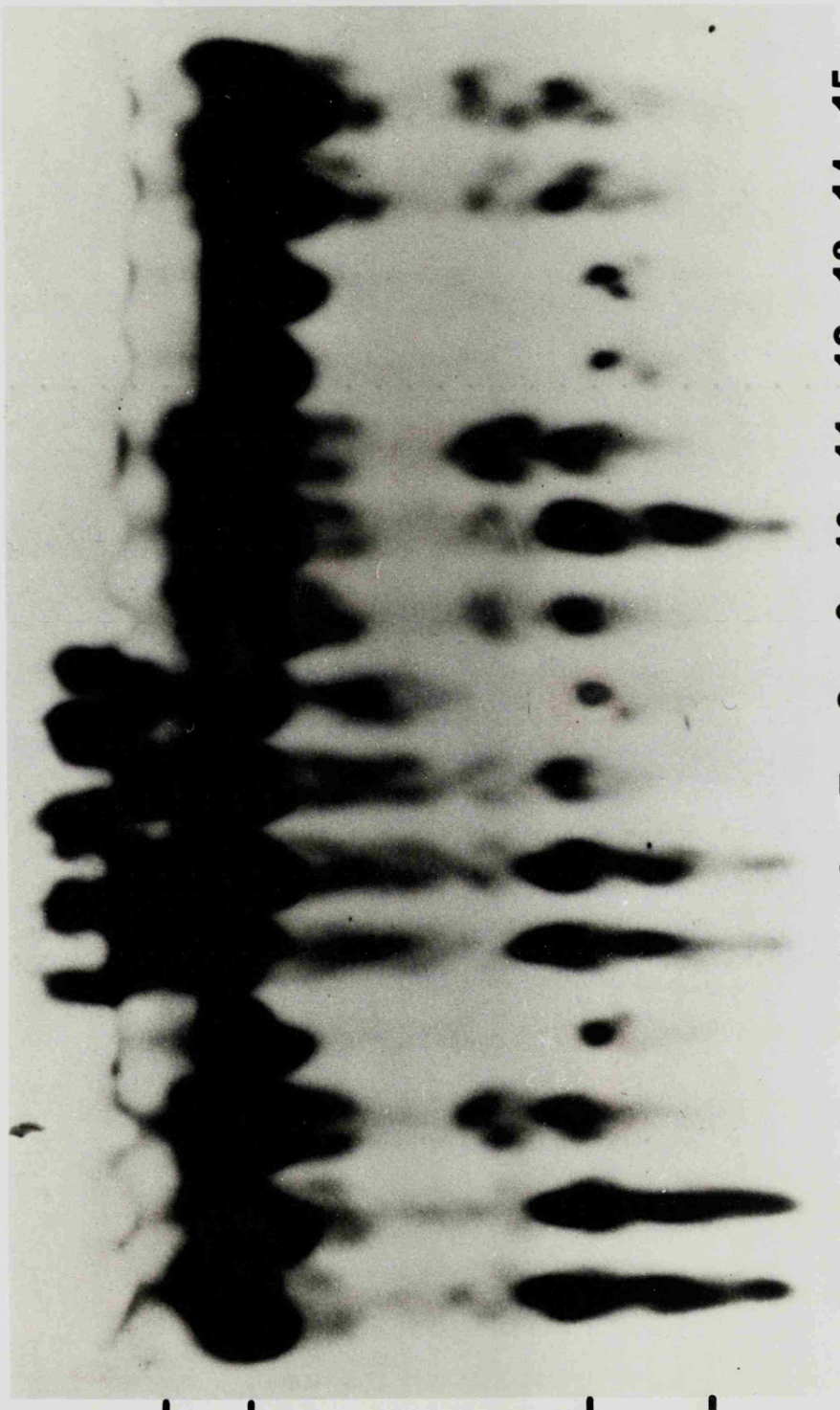
4,8,12. Ribosomes, stringent factor omitted.

13. Both ribosomes and stringent factor omitted.

14. BM-L11 present, no ribosomes.

15. Stringent factor present, ribosomes omitted.

Figure 6.2



Legend to Figure 6.3

Inhibition by micrococcin of guanosine polyphosphate production in vitro (see also Table 6.1)

Assay conditions were as described in the Legend to Figure 6.2 and Materials and Methods. Micrococcin or DMSO (1% $^{v}/_{v}$ final concentration) was preincubated with ribosomes for 5 minutes at 20°C prior to their addition to other reaction mixtures. Identification of tracks: (1 - 4) ribosomes from wildtype; (5 - 8) ribosomes from strain GS2; (9) both ribosomes and stringent factor omitted.

1,5 Controls, no drug.

2,6 Plus 25 pmol micrococcin.

3,7 Plus 75 pmol micrococcin.

4,8 Plus 250 pmol micrococcin.

In a similar experiment 2 µl of each reaction mixture was loaded on to PEI plates and following chromatography the nucleotide spots were cut out and radioactive content determined. These data are presented in Table 6.1.

Figure 6.3

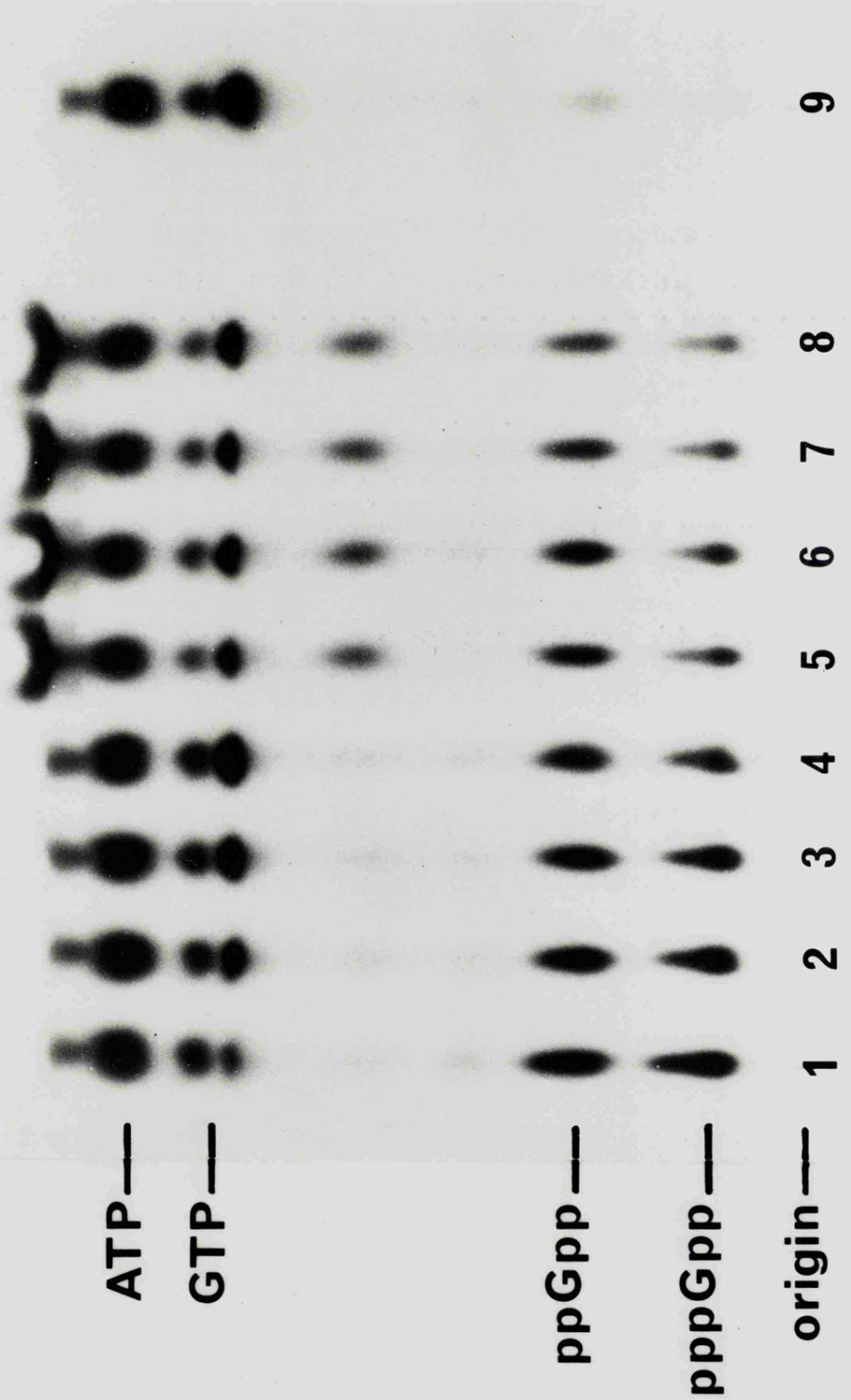


Table 6.1

Inhibition by micrococcin of guanosine polyphosphate production in vitro

In drug-free controls with ribosomes from wildtype, about 38% of the [γ^{32}P]ATP input was converted to guanosine polyphosphates in one hour. This represented about 945 pmol/pmol ribosomes. With ribosomes from strain GS2 the corresponding value was about 400 pmol/pmol ribosomes.

Micrococcin added (molar excess over ribosomes)	Inhibition of guanosine polyphosphate formation	
	wildtype	strain GS2
	%	
1	13	3
3	23	12
10	42	32
100	41	35

CHAPTER 7

PURIFICATION OF RIBOSOMAL PROTEIN BM-L11 FROM WILDTYPE AND STRAIN GS2

CHAPTER 7

Purification of Ribosomal Protein BM-L11 from wildtype and strain GS2.

The purification of ribosomal protein BM-L11 is facilitated by its ability to bind to 23S rRNA in solution and the subsequent ability of this complex to bind [^{35}S] thiostrepton. Consequently, column fractions during protein purification can be tested for the presence of protein BM-L11. The preparation and characterization of this protein will be considered in detail in this section due to its central role in subsequent experiments.

Methods employed here were similar to those used previously in this laboratory (Stark, 1979) and involved the use of "non--denaturing" conditions. Such conditions were considered desirable because the purified protein was to be employed in assays to test its function and its ability to reintegrate into ribosomes. The details were based on earlier studies for the purification of ribosomal proteins which avoided the use of urea, extremes of pH and lyophilization (Dijk and Littlechild, 1979). The procedures adopted for the purification have previously been outlined by Stark (1979) and may be conveniently divided as follows:-

Preparation of CM-cellulose

Whatman CM23 cellulose (30g) was resuspended in distilled water (1l) and allowed to settle for about 20 minutes, after which the supernatant was discarded. This method of removing 'fines' was repeated three times (with water) and twice using 1M-NaOH. The slurry was then poured into a Buchner funnel containing a Whatman No. 1 filter paper. The cellulose resin was washed with water until the pH of the effluent was less than 8. The slurry was then resuspended in 1l of 0.5M-HCl and allowed

to settle as before; the supernatant was again discarded. This procedure was repeated twice and the slurry returned to a Buchner funnel and washed with water until the pH of the eluate was 4.5. The resin was stored until needed at 4°C as an aqueous slurry containing 0.06% ($^{v}/v$) toluene as an antimicrobial agent.

Preparation of a CM-cellulose chromatography column

About 50 ml of the CM-23 cellulose, prepared as above, was brought to 200 ml final volume with 200 mM sodium acetate (pH 5.6, adjusted with acetic acid) and allowed to settle. This procedure was repeated twice and finally three more times with 10 mM sodium acetate. Material thus prepared was used to pour a column (approximately 12 x 1 cm). The resin was supported in a glass tube on a glass fibre disc (Whatman GF/C), itself placed upon a lightly-packed plug of glass wool. The column was poured with the tap open to give a bed volume of approximately 11 ml which was then washed with 200 ml of 10 mM sodium acetate, pH 5.6.

After a chromatographic "run" the column resin was "recycled" in situ by passing about 500 ml of 200 mM sodium acetate, pH 5.6 through the column and then 1 l of 10 mM sodium acetate at the same pH.

Preparation of 1M-LiCl split-proteins

Ribosomes (500-700 mg) were freshly prepared as described under Materials and Methods, from frozen cell pastes of B. megaterium wildtype or strain GS2 (about 100 g in each case). Split proteins were extracted from 70S ribosomes using 1M-LiCl in the following manner. Ribosomes in T^{10} M^{10} A^{50} β^{3} buffer were diluted with the same buffer to give a ribosome concentration of approximately 20 mg ml^{-1}. Each 1 ml suspension of ribosomes was mixed with a buffer of suitable volume and composition to

yield 10 ml of suspension with a final composition of T^{10} M^{1} (A^{5}) β^{3} $LiCl^{1000}$ and 2-2.5 mg ml^{-1} ribosomes. This suspension was then left on ice overnight with occasional agitation. Ribosomal core particles (not used here) were separated from the split proteins by centrifugation overnight at 45K rpm and 4°C in a Beckman Ti50.2 rotor. Split proteins contained in the supernatant were placed in Spectrapor No. 1 or No. 3 tubing depending on the volume, and dialysed against 10 mM sodium acetate pH 5.6 at 4°C (usually 3 x 6 l x 2½ hr). During dialysis a fine white protein precipitate appeared [which according to Dijk and Littlechild (1977) is normal] and was removed by centrifugation in a Beckman Ti50.2 rotor at 10K rpm for 20 minutes and 4°C. The pH of the split proteins (5.6) was then checked before chromatography.

Ion-exchange chromatography of 1M-LiCl split-proteins on CM23 cellulose

The split-proteins (in approximately 250 ml of 10 mM sodium acetate) were loaded onto the column at 60-100 ml hr^{-1} and washed on with 20 ml 10 mM sodium acetate. In one preparation it was estimated, following removal of the proteins which formed a precipitate during the dialysis step (see above), that about 50% of the original protein in the 1M-LiCl supernatant remained for loading onto the column and this amounted to about 35 mg of protein. This was estimated from the absorbance of solutions at 230 nm assuming that $1A_{230}$ unit was equal to 200 µg of protein. From such measurements it was found that about 16% of the protein loaded onto the chromatography column was not bound to the resin. The remaining protein, which was bound, was found to include protein BM-L11.

Material was eluted (flow rate 20 ml hr^{-1}) from the column with 200 ml of a 10-200 mM linear concentration gradient of sodium acetate (pH 5.6) at 4°C and about 80 ($2\frac{1}{2}$ ml) fractions were collected.

Detection of protein BM-L11 in column fractions. (Refer here to Figures 7.1 and 7.2)

The conductivity of the column fractions was measured and values converted to concentration of sodium acetate by reference to a standard curve. Protein BM-L11 was detected usually in fractions 40-60 by its ability to promote tight binding of [^{35}S] thiostrepton to RNA70 from the ribosomes of B. megaterium wildtype (or RNA70 from strain GS2 which proved just as capable under such circumstances) see below for further details. Appropriate [^{35}S] thiostrepton binding fractions were then analysed on 0.1% (^{w}/v) sodium-dodecyl sulphate/13% (^{w}/v) polyacrylamide gels as described in Materials and Methods.

The [^{35}S] thiostrepton binding assay

Incubation mixes containing 20 pmol of rRNA70 from B. megaterium wildtype or strain GS2 and 20 µl of each column fraction were brought to a final volume of 85 µl with $M^{1.5}$ buffer (T^{10} $M^{1.5}$ A^{50} β^{3}). This was followed by the addition of 5 µl (approximately 40 pmol) [^{35}S] thiostrepton in DMSO (see Methods section for production of radiolabelled drug). The reaction mix was kept at 20°C for 20 minutes followed by the addition and mixing of 10 µl 5% (^{w}/v) Norit in $M^{1.5}$ buffer to adsorb free [^{35}S] thiostrepton. After a further 10 minutes at 20°C the Norit was removed by centrifugation at 12000 x g for 2 minutes in a bench micro-centrifuge. Binding of [^{35}S] thiostrepton to any 23S rRNA·protein BM-L11 complexes present in

the supernatant was then determined by liquid scintillation spectrometry using the scintillant Fisofluor No. 1.

Estimation of Protein concentration

Accurate determinations of protein concentration were made according to Lowry et al. (1951) or Spector (1978). The latter assay involved the quantitative formation of a complex between the dye Coomassie brilliant blue G and protein. After 5 minutes in the presence of the dye the protein concentration was estimated by measuring the absorbance of the solution at 595 nm and comparing the readings with a standard curve constructed using Bovine serum albumin.

Discussion of results and handling of the purified proteins from wildtype and strain GS2

The [^{35}S] thiostrepton binding profile together with the SDS-acrylamide gel analysis of the major drug binding fractions is presented in Figure 7.1 for the preparation of protein BM-L11 from wildtype. Figure 7.2 shows similar data for the purification of protein BM-L11 from strain GS2. In both cases elution of BM-L11 occurred within fractions 40-60 at salt concentrations within the range 85-130 mM. In the SDS gel profiles it can be seen that only one protein species was detected within this fraction range and accordingly these respective fractions were pooled and dialysed against T^{10} M^{10} A^{50} β^{3} buffer (3 x 2 l x 2 hr). Following this the protein from each preparation was concentrated in the same buffer containing 20% (^{w}/v) polyethylene $glycol_{20000}$ (about 8 hrs) and finally dialysed against the T^{10} M^{10} A^{50} β^{3} buffer (3 x 2 l x 2 hr) before storage in this buffer, as aliquots at -70°C. Yields of protein BM-L11 estimated as described above were 0.5 mg for the

preparation from wildtype and 0.2 mg from strain GS2 and the average percentage recovery was about 10%.

The concentrated protein samples were then finally checked for purity in two gel systems, as shown in Figure 7.3. On acrylamide gels containing SDS only one protein species was detected in each case. However a minor contaminant was sometimes visible on polyacrylamide gels containing 8M urea run at pH 4.5, see Figure 7.3 (and Materials and Methods for details of urea-acrylamide gels). For unknown reasons the preparation of protein BM-L11 obtained from strain GS2 was more heavily contaminated than that from the wildtype, the latter being particularly clean. In view of the experiments which follow, this contaminant in the protein BM-L11 preparation from strain GS2 was considered to be very important as will be discussed in Chapter 8.

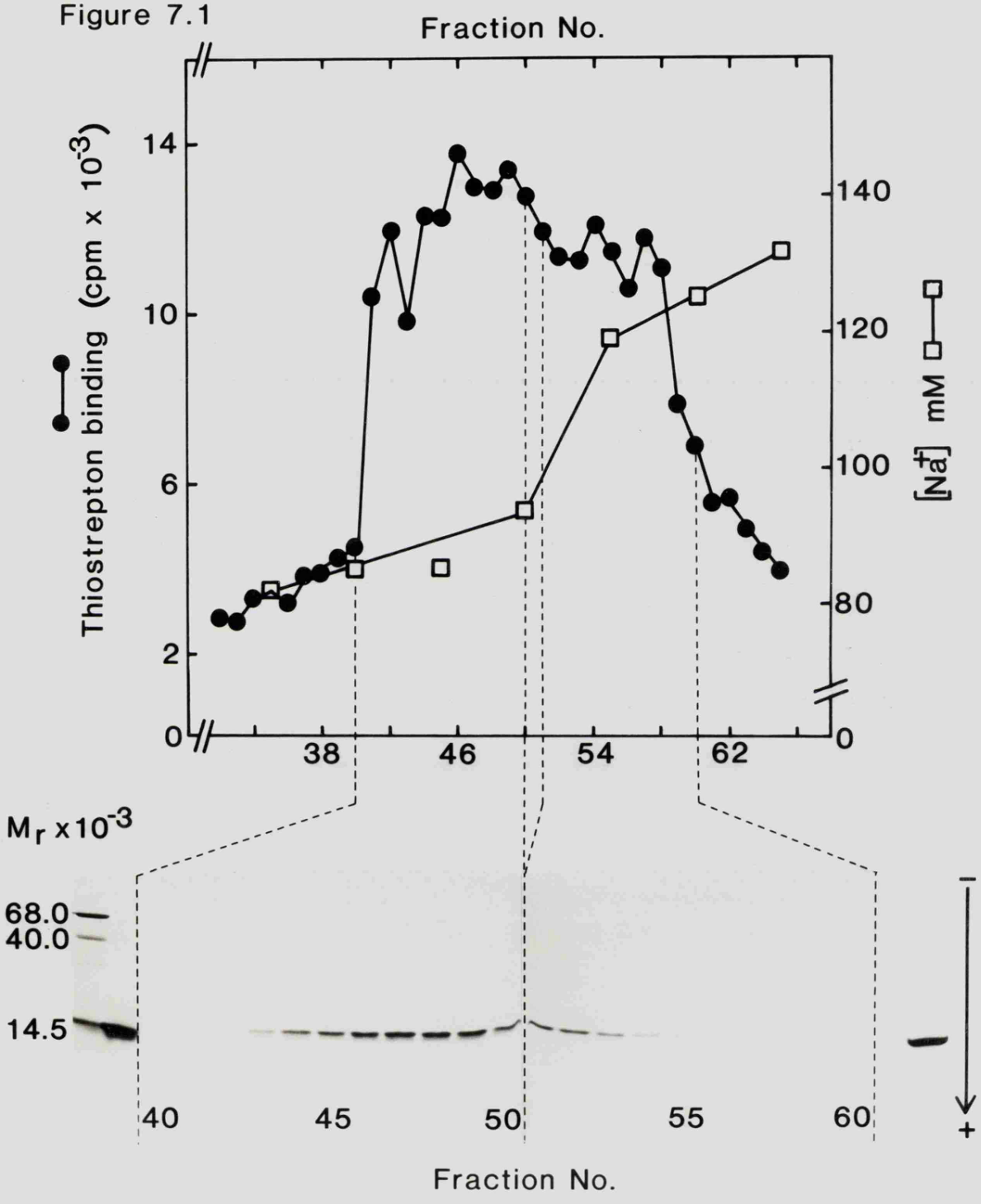
This preliminary characterization of protein BM-L11 from the two strains had indicated that if the protein from mutant GS2 was altered then it could not be drastically so. It eluted from an ion-exchange column under the same conditions as the protein from the wildtype and it migrated to a similar position in two gel systems, one separating on the basis of size and the other on the basis of charge (see Figure 7.3). Protein BM-L11 from the two strains was now available in a reasonably purified state and functional assays could be performed to establish whether or not the protein from strain GS2 was altered with respect to determining the micrococcin-resistance phenotype.

Legend to Figure 7.1

Purification of ribosomal protein BM-L11 from wildtype ribosomes

1M-LiCl split proteins derived from ribosomes of *B. megaterium* wildtype were chromatographed on CM23 cellulose as described in the text. Fractions (2.5 ml) were collected and analysed as follows: The conductivity of every 5th fraction was measured and the Na^+ concentration in each estimated by reference to standard solutions of sodium acetate (□). The presence of protein BM-L11 in fractions was determined by its ability to restore binding of [^{35}S] thiostrepton to 23S rRNA as described on page 94 (●). Active fractions were analysed by electrophoresis on SDS/polyacrylamide gels using 25 µl of appropriate column fractions. Bovine serum albumin (Mwt 68,000), creatine kinase (Mwt 40,000) and lysozyme (Mwt 14,500) were used as molecular weight markers. In the first track, on the left-hand side, 4 µg of each marker protein was loaded. Adjacent to this track, 8 µg of lysozyme was loaded.

Figure 7.1

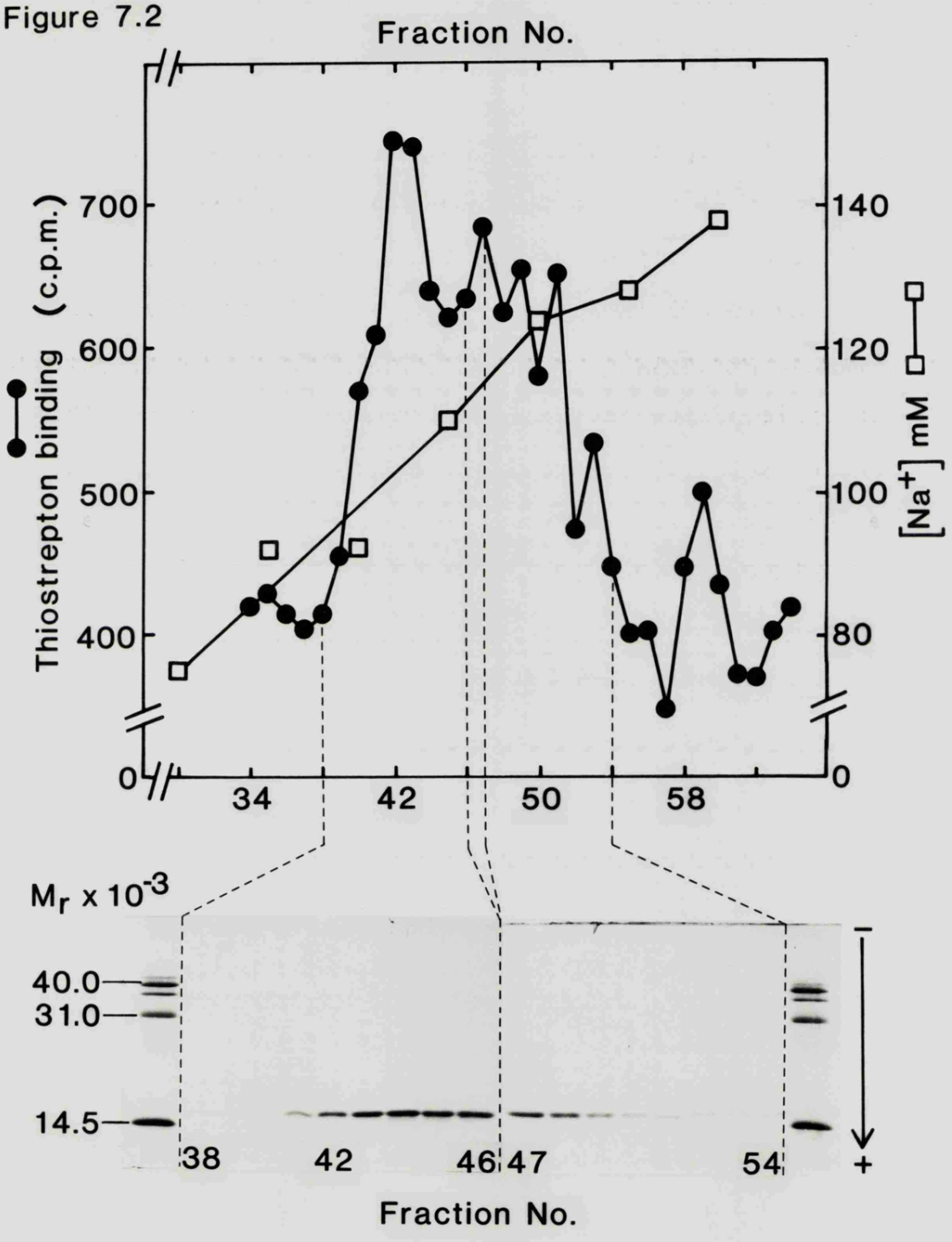

Fraction No.
Thiostrepton binding (cpm x 10^{-3})
14
10
6
2
0
38
46
54
62
140
120
100
80
0
[Na^+] mM
M_r x10^{-3}
68.0
40.0
14.5
40
45
50
55
60
−
+
Fraction No.

Legend to Figure 7.2

Purification of protein BM-L11 from ribosomes of strain GS2

1M-LiCl split proteins derived from ribosomes of strain GS2 were chromatographed on CM23 cellulose as described in the Legend to Figure 7.1. Fractions (2.5 ml) were collected and analysed as also described in the Legend to Figure 7.1. (□) conductivity of column fractions; (●) [^{35}S] thiostrepton binding to 23S rRNA promoted by protein in column fractions. For further details see the text. In the electrophoretic analysis of the column fractions, creatine kinase (Mwt 40,000), DNase (Mwt 31,000) and lysozyme (Mwt 14,500) were used as molecular weight markers. Approximately 4 µg of each marker protein was loaded.

Figure 7.2


Fraction No.
Thiostrepton binding (c.p.m.)
700
600
500
400
0
140
120
100
80
0
[Na+] mM
34
42
50
58
Mr x 10-3
40.0
31.0
14.5
38
42
46
47
54
−
+
Fraction No.

Legend to Figure 7.3

Electrophoretic analysis of preparations of ribosomal protein BM-L11

The protein purified from the ribosomes of *B. megaterium* wildtype and from those of strain GS2 was analysed by one-dimensional electrophoresis on polyacrylamide gels containing (A) 0.1% (^{w}/v) SDS/13% (^{w}/v) acrylamide and (B) 8 M urea/10% (^{w}/v) acrylamide, pH 4.5 as described in Materials and Methods. (A) 1 µg of each protein was applied to the gel; (B) 4 µg of each was loaded. Marker proteins used in (A) were (in order of decreasing molecular weight) *E. coli* ribosomal proteins L2, L1, L3, L6, L11 and L23.

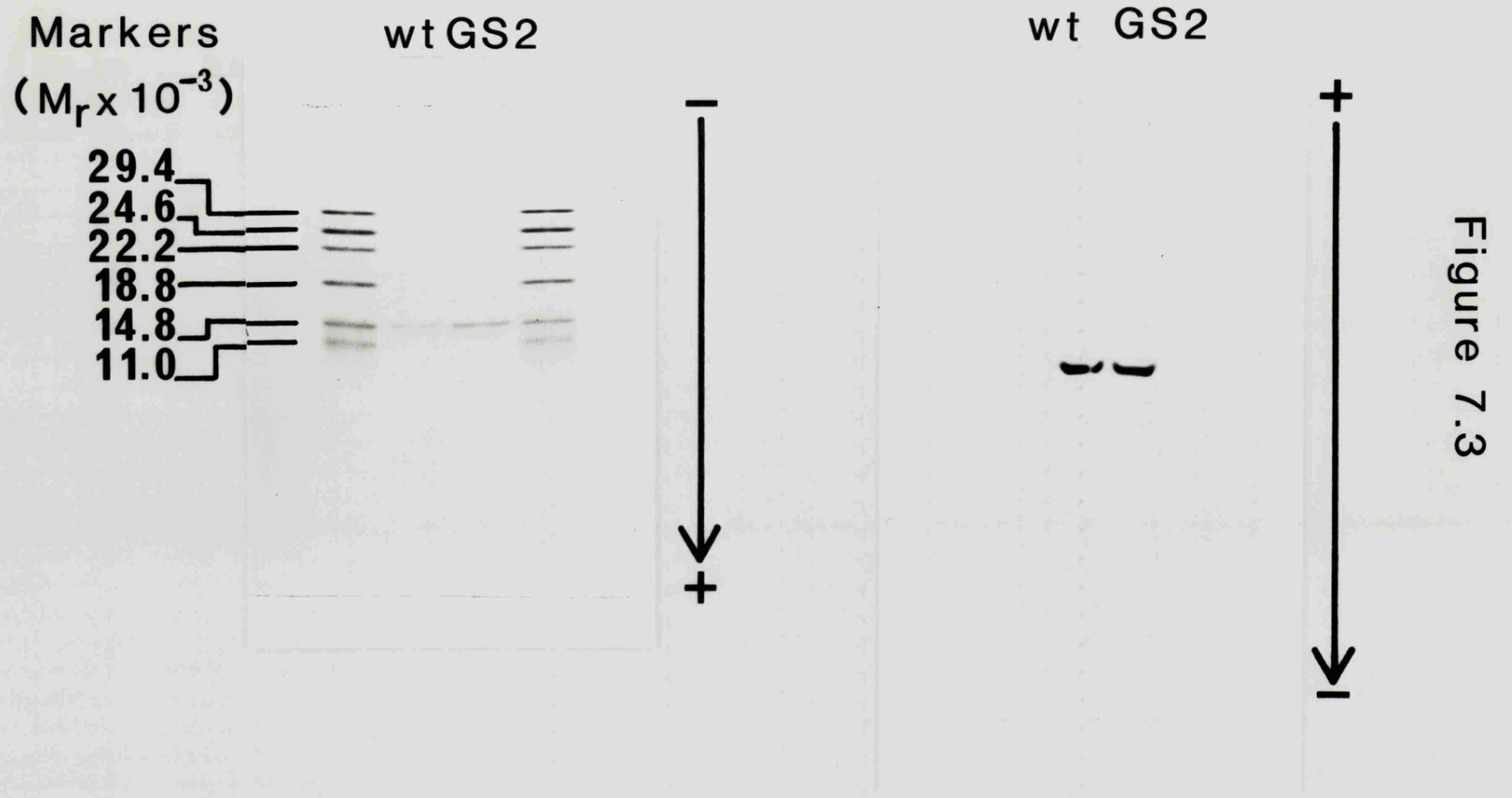

Markers
(M_r x 10^{-3})
29.4
24.6
22.2
18.8
14.8
11.0
wt GS2
−
+
wt GS2
+
−
0.1% SDS/13%
acrylamide gel
(A)
8M urea/10%
acrylamide gel (pH 4.5)
(B)

Figure 7.3

CHAPTER 8

RIBOSOMAL PROTEIN BM-L11 FROM STRAIN GS2 IS ALTERED AND DETERMINES RESISTANCE TO MICROCOCCIN

CHAPTER 8

Ribosomal Protein BM-L11 from strain GS2 is altered and determines resistance to micrococcin.

The rationale behind the following series of experiments was to take ribosomes from strain MJ1 (lacking protein BM-L11) and to supplement them with the purified protein BM-L11 from wildtype or from strain GS2. Following the integration of the respective proteins into 70S ribosomes from strain MJ1, the 'reconstituted' particles were incubated with micrococcin and their responses were determined in the EF-G dependent GTPase assay.

The results of supplementing ribosomes from strain MJ1 with protein BM-L11 purified from the wildtype are shown in Figure 8.1. The protein was without any effect when supplied to ribosomes from the wildtype but could supplement ribosomes from strain MJ1 to give wildtype levels of GTP hydrolysis. Moreover this reaction was then sensitive to the presence of micrococcin. Data presented in Figure 8.2 show the response of ribosomes from strain MJ1 supplemented with protein BM-L11 from strain GS2. In this case the purified protein could integrate into ribosomes from strain MJ1 to give similar levels of GTPase activity as did the purified protein BM-L11 from the wildtype (compare Figures 8.1 and 8.2) but the supplemented ribosomes were resistant to the action of micrococcin. As previously observed (see Chapter 5 page 83) micrococcin was without effect on 70S ribosomes from strain MJ1 in the absence of any added proteins. Furthermore it should be noted that neither preparation of protein BM-L11 alone (i.e. from wildtype or strain GS2) possessed any GTPase activity (data not given).

The simplest explanation for these results is that protein

BM-L11 from strain GS2 is altered and is thereby responsible for resistance to micrococcin. However, as noted in Chapter 7, page 96 since the protein prepared from the mutant was not 100% pure it might be argued that exchange might have taken place between free and bound ribosomal proteins other than protein BM-L11. It has been reported that certain ribosomal proteins undergo exchange among ribosomes in E. coli (Robertson et al., 1977; Subramanian and van Duin, 1977) and this possibility could not therefore be discounted. If ribosomal protein exchange could occur under the conditions of the experiments just described, then the effects demonstrated in Figure 8.2 might not be attributable exclusively to reincorporation of protein BM-L11 into the ribosomes. Consequently a series of competition experiments were designed to test this possibility.

In the first set of experiments (see Figure 8.3A) it was seen that the addition of TP70, from strain GS2, to ribosomes from strain MJ1 conferred the GS2 response. That is, GTP hydrolysis was stimulated but the supplemented ribosomes were not susceptible to the action of micrococcin. Moreover this result was similar to that obtained when ribosomes from strain MJ1 were supplemented with the purified preparation of protein BM-L11, also from strain GS2. Compare the data presented in Figures 8.3(A) and 8.2. Notably, however, when such TP70 proteins were supplied together with an excess of purified protein BM-L11 from the wildtype, the MJ1 ribosomes then gave the wildtype response, Figure 8.3. Thus, the excess of highly purified protein BM-L11 from the wildtype was competing out the effects of protein within the TP70 preparation from strain GS2. In control experiments (data not given) when ribosomes from

strain MJ1 were supplemented with total ribosomal proteins from the wildtype, the resultant particles exhibited the wildtype response. The level of stimulation of GTP hydrolysis and the effect of micrococcin were about the same as when the highly purified protein BM-L11 from wildtype was employed (see Figure 8.1).

Direct competition experiments were also performed by supplementing ribosomes from strain MJ1 with protein BM-L11 from strain GS2 either alone or together with a 10-fold molar excess of the highly purified protein BM-L11 from the wildtype (Figure 8.4). Again it was shown that the protein from the wildtype could compete out the effect of the protein from strain GS2. These experiments, taken together, suggest perhaps that irrespective of any exchange which might have taken place between any proteins present on the ribosomes and any in the TP70 preparations or in the preparation of protein BM-L11 from strain GS2 (i.e. the contaminating species) a single protein was able to determine the ultimate response of ribosomes to micrococcin. It was therefore concluded not only that protein BM-L11 was altered in ribosomes of strain GS2 but also that there was no reason to suppose that any other protein component was altered. The state of protein BM-L11 in the ribosomes of strain GS2 therefore appears to be responsible for resistance to micrococcin in this strain.

Even though the results of the experiments described above appeared conclusive and although there was no reason to doubt the presence of an altered form of protein BM-L11 within the ribosomes of strain GS2 all the data had, nevertheless, been

obtained from an 'uncoupled' GTPase assay system. So for the sake of completeness it was decided to carry out a final set of experiments involving cell-free protein synthesis to study the effects of the addition, to MJ1 ribosomes, of protein BM-L11 from the two sources and to examine the effects of micrococcin upon this process. Figure 8.5 shows that micrococcin caused a slight inhibition of polyphenylalanine synthesis catalyzed by native ribosomes from strain MJ1. Furthermore these ribosomes could be supplemented with protein BM-L11 from the wildtype with a concomitant elevation of the synthesis of polyphenylalanine, as might be expected, and this reaction could then be dramatically inhibited by micrococcin. However when these ribosomes were supplemented with the protein BM-L11 from strain GS2 the synthesis of polyphenylalanine was also enhanced but this reaction was insensitive to the action of micrococcin. In control experiments, neither protein BM-L11 from wildtype nor that from strain GS2 had any significant effect upon the levels of activity or the drug response when added to systems containing ribosomes from the wildtype (data not shown). These results were an additional bonus in confirming the importance of the state of the ribosomal protein BM-L11 in determining resistance or sensitivity to micrococcin. It also confirms the importance of this protein in protein synthesis and shows, in agreement with previous work, why the ribosomes of strain MJ1 are grossly inefficient in their functions and consequently why strain MJ1 is a sickly organism (Stark 1979; Stark and Cundliffe, 1979b; Cundliffe *et al.*, 1979).

Except with regard to the interaction with micrococcin (and as discussed in Chapter 6 with the efficiency of guanosine polyphosphate production) the ribosomal protein BM-L11 from

strain GS2 behaves like that from wildtype. Notably, when ribosomes from strain MJ1 were supplemented with purified protein from either source, similar levels of polyphenylalanine synthesis and GTP hydrolysis, dependent upon factor EF-G, were detected. This again suggests (see also Chapter 7, page 96) that this protein, albeit altered in strain GS2, cannot be radically changed. This point will be taken up again in the General Discussion.

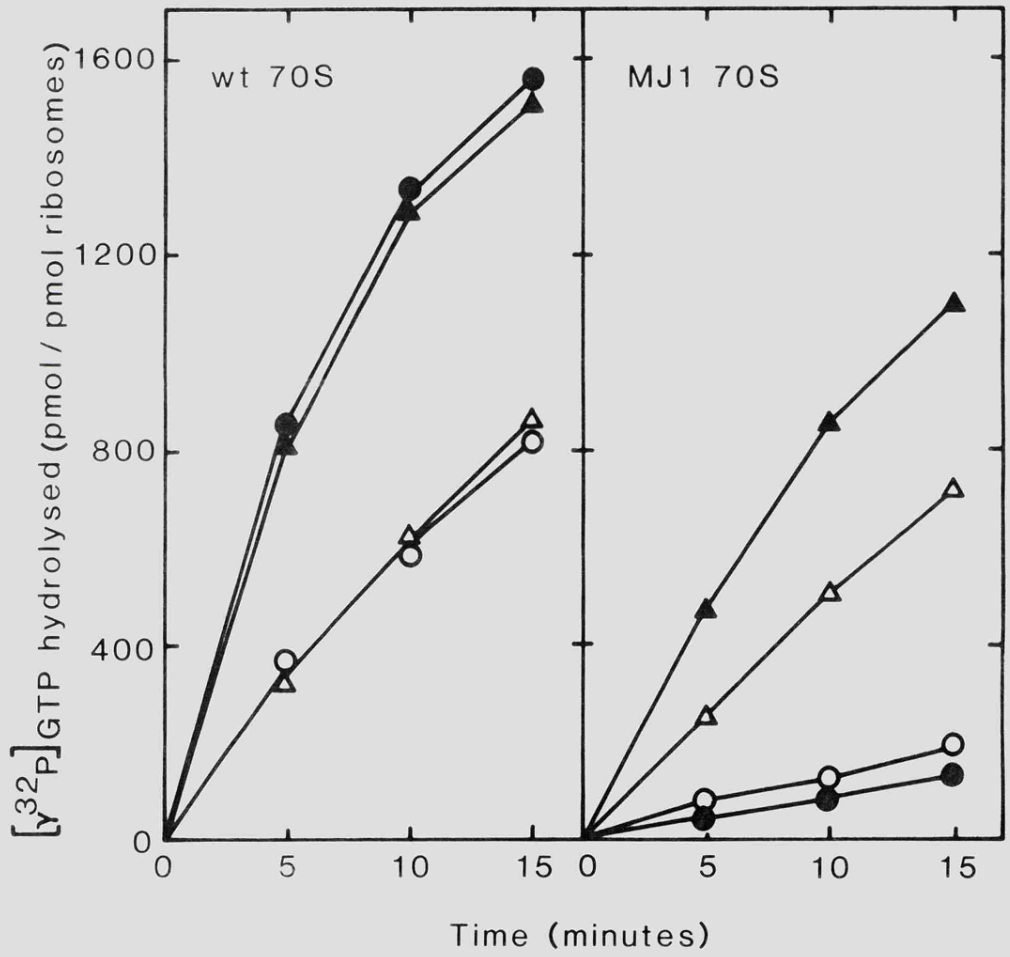
Legend to Figure 8.1

Effect of micrococcin on uncoupled GTPase activity of ribosomes from wildtype and strain MJ1 supplemented with purified ribosomal protein BM-L11 from wildtype

Ribosomes (5 pmol) were preincubated for 5 minutes at 37°C either alone or with purified protein BM-L11 (50 pmol) derived from the wildtype. Then micrococcin (500 pmol) or DMSO (1.33% $^{v}/v$ final concentration) was added and incubation continued for 10 minutes at 37°C. Assays of uncoupled GTP hydrolysis were then performed as described in Materials and Methods. For source of ribosomes see the Figure.

(○) (●) native ribosomes; (△) (▲) ribosomes plus protein BM-L11; (●,▲) micrococcin present.

Figure 8.1

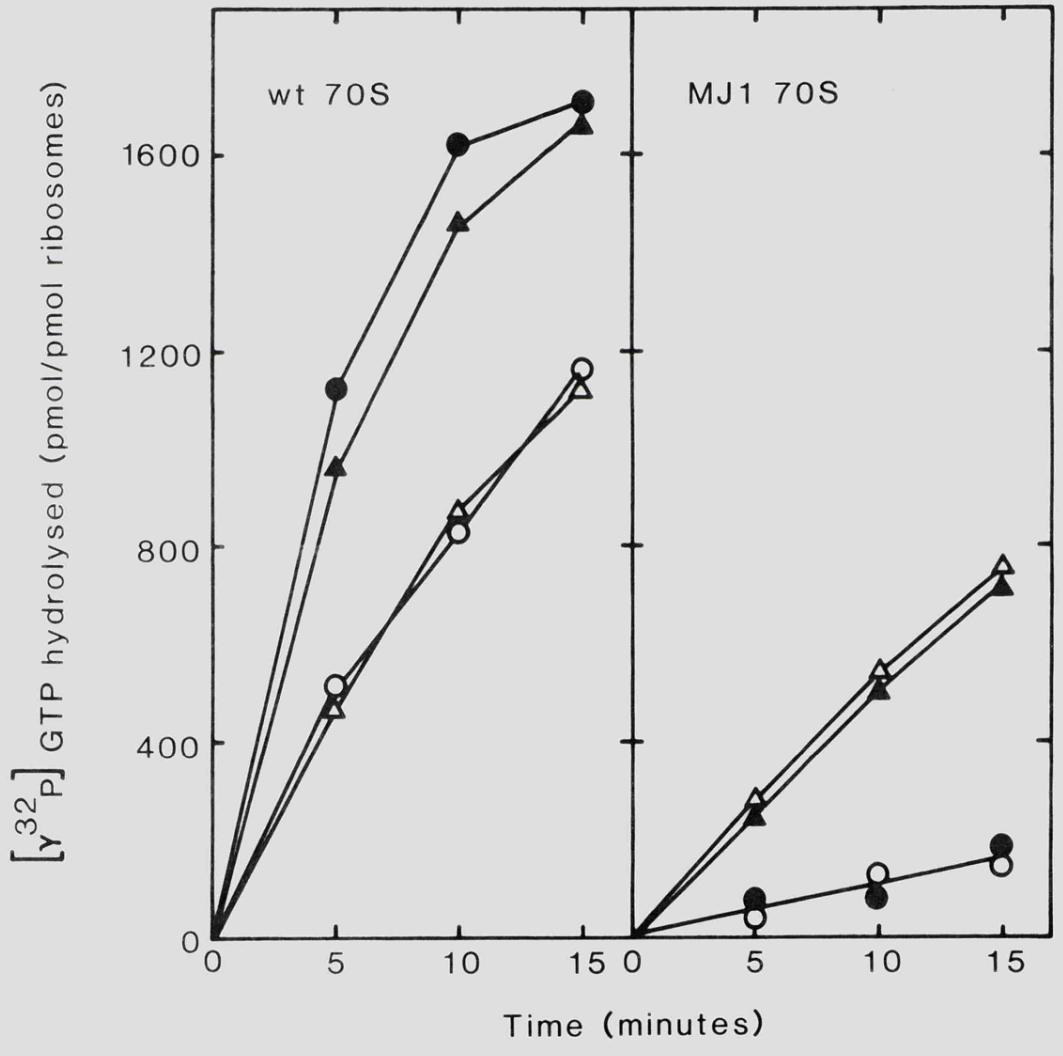

wt 70S
MJ1 70S
[γ32P]GTP hydrolysed (pmol / pmol ribosomes)
0
400
800
1200
1600
0
5
10
15
Time (minutes)

Legend to Figure 8.2

Effect of micrococcin on uncoupled GTPase activity of ribosomes from B. megaterium wildtype and strain MJ1 supplemented with purified protein BM-L11 from strain GS2

Ribosomes (5 pmol) were preincubated for 5 minutes at 37°C, either alone or with purified protein BM-L11 (50 pmol) derived from strain GS2. Then micrococcin (500 pmol) or DMSO (1.33% $^{v}/v$ final concentration) was added and incubation continued for 10 minutes at 37°C. After addition of an excess of factor EF-G and GTP assays of uncoupled GTP hydrolysis were then performed as described in Materials and Methods. For source of ribosomes see the Figure.
(O) (●) native ribosomes; (Δ) (▲) ribosomes plus protein BM-L11; (●,▲) micrococcin present.

Figure 8.2

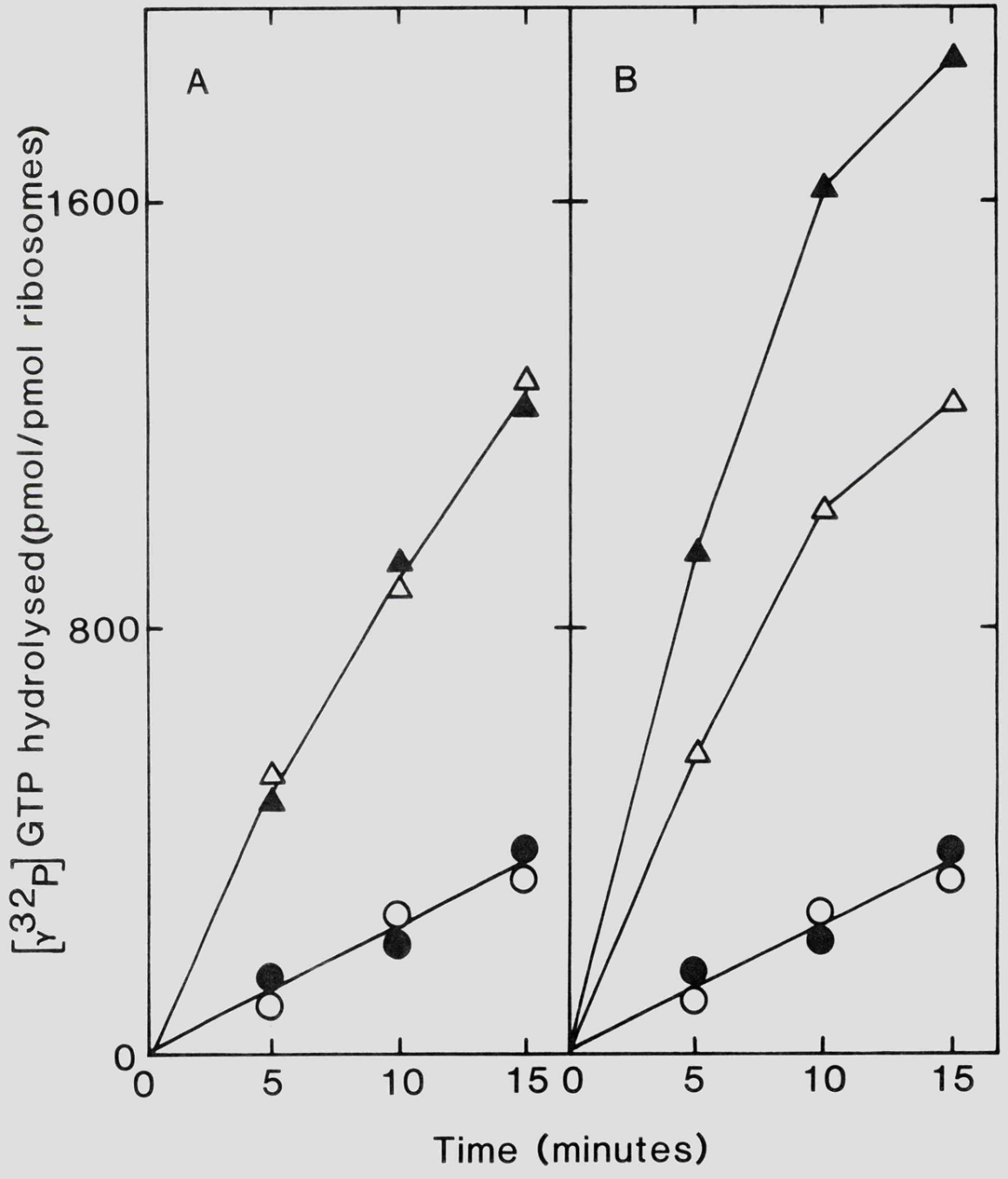

wt 70S
MJ1 70S
1600
1200
800
400
0
0
5
10
15
0
5
10
15
Time (minutes)
[γ32P] GTP hydrolysed (pmol/pmol ribosomes)

Legend to Figure 8.3

Effects of micrococcin on uncoupled GTPase activity of ribosomes from B. megaterium strain MJ1 supplemented with TP70 and protein BM-L11

Ribosomes (5 pmol) from strain MJ1 were incubated for 10 minutes at 37°C, alone or following supplementation (A) with 15 pmol equivalents of TP70 from strain GS2 or (B) with a mixture of 15 pmol equivalents of TP70 from strain GS2 plus 250 pmol of protein BM-L11 from wildtype. Then micrococcin (500 pmol, 100 fold molar excess over ribosomes) or DMSO (0.66% $^{v}/v$ final concentration) was added followed by incubation for 10 minutes at 37°C. Uncoupled GTP hydrolysis was then assayed following addition of an excess of [γ^{32}P] GTP and factor EF-G. (O) (●) control ribosomes; (Δ) (▲) ribosomes supplemented with proteins; (●,▲) presence of micrococcin.

Figure 8.3

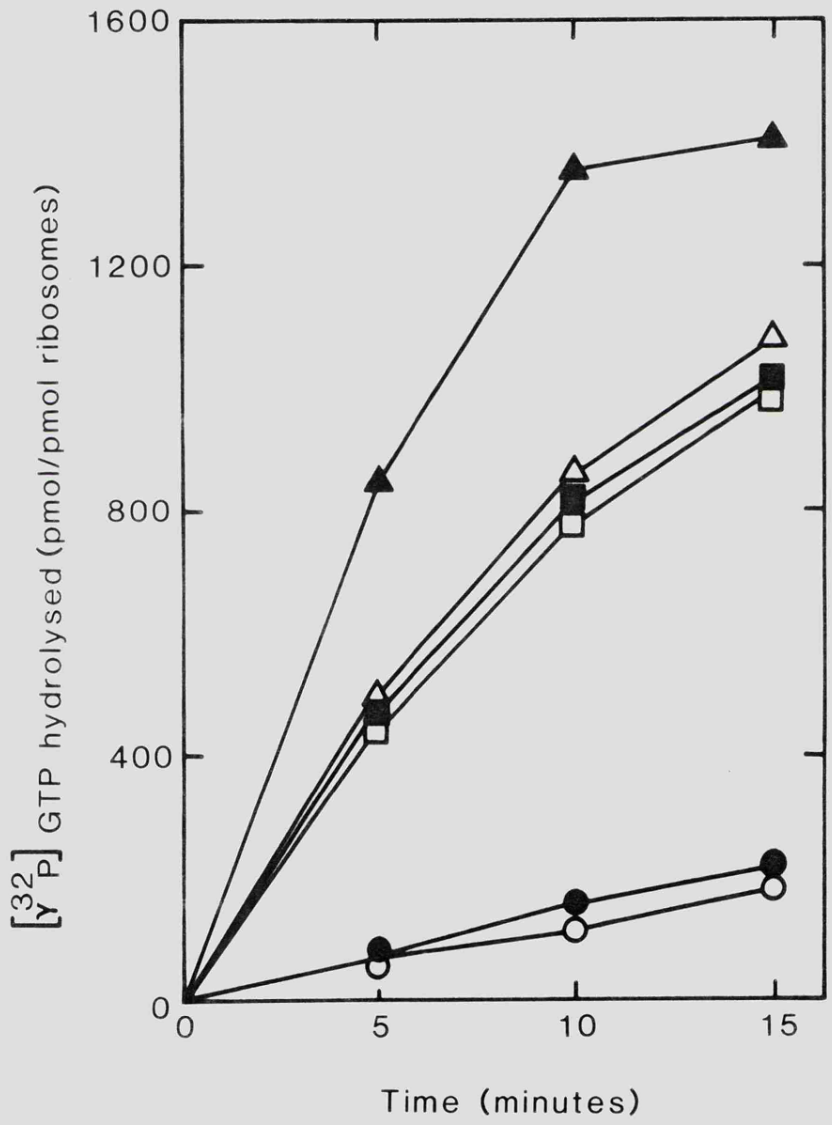

A
B
1600
800
0
0
5
10
15
0
5
10
15
[γ32P] GTP hydrolysed (pmol/pmol ribosomes)
Time (minutes)

Legend to Figure 8.4

Effect of micrococcin on uncoupled GTPase activity of ribosomes from strain MJ1 supplemented with protein BM-L11 from various sources in the presence and absence of protein BM-L11 from wildtype

Ribosomes (5 pmol) from B. megaterium strain MJ1 were incubated for 10 minutes at 37°C, alone (0) (●); with 25 pmol of ribosomal protein BM-L11 derived from strain GS2 (□) (■); or with 25 pmol ribosomal protein from strain GS2 together with 250 pmol ribosomal protein BM-L11 derived from wildtype (Δ) (▲). To some mixtures (filled symbols) micrococcin was then added (500 pmol i.e. 100 fold molar excess over ribosomes) while DMSO (1.33% $^{v}/_{v}$ final concentration) was added to the controls (open symbols). Incubation was continued for 10 minutes at 37°C before factor EF-G and [γ^{32}P] GTP were added and uncoupled GTPase activity was followed.

Figure 8.4

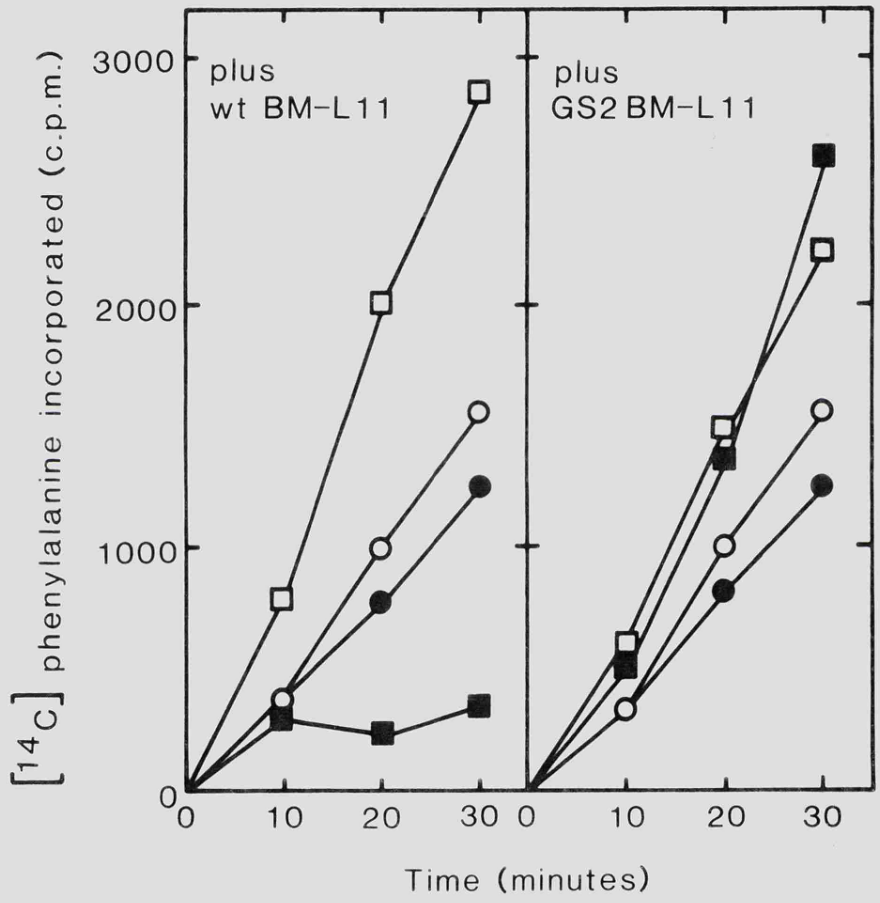

1600
1200
800
400
0
0
5
10
15
Time (minutes)
[γ 32 P] GTP hydrolysed (pmol/pmol ribosomes)

Legend to Figure 8.5

Effect of micrococcin upon cell-free protein synthesis supported by ribosomes from strain MJ1 supplemented with protein BM-L11

Ribosomes (5 pmol) from strain MJ1 were preincubated for 5 minutes at 0°C and then for 5 minutes at 37°C, either alone (O) (●) or with 25 pmol ribosomal protein BM-L11 (□) (■) derived from the wildtype (A) or from strain GS2 (B). Then micrococcin (50 pmol) or DMSO (0.5% $^{v}/v$ final concentration) was added followed by 15 µl of S100 from strain MJ1 prewarmed at 37°C. Other components necessary for synthesis of polyphenylalanine were then added as a cocktail. (●,■) micrococcin present at 10 fold molar excess over ribosomes.

Figure 8.5


plus
wt BM-L11
plus
GS2 BM-L11
3000
2000
1000
0
0
10
20
30
0
10
20
30
[14C] phenylalanine incorporated (c.p.m.)
Time (minutes)

CHAPTER 9

GENERAL DISCUSSION

CHAPTER 9

General Discussion

Protein BM-L11 is clearly altered in the ribosomes of the micrococcin-resistant strain GS2, as discussed in Chapter 8. Although it is not yet known how the protein differs from its counterpart in wildtype it is apparently not drastically altered (see Chapter 7). Notably, on SDS-polyacrylamide gels and on urea-polyacrylamide gels run at pH 4.5, the protein appeared unchanged in size, i.e. it migrated in a similar fashion to that from the wildtype. Furthermore it also eluted from an ion--exchange column under similar salt concentrations to the protein BM-L11 from wildtype (see Chapter 7). However, one potentially interesting possibility which could be raised concerning the alteration can probably be discounted. Certain ribosomal proteins from *E. coli* are methylated and ribosomal protein L11 is the one which is the most heavily modified. It contains nine methyl groups, with two residues of N-trimethyllysine and one of N-trimethylalanine (Alix and Hayes, 1974; Chang *et al*., 1974; Chang and Chang, 1975; Dognin and Wittman-Liebold, 1977). Arguing by analogy, protein BM-L11 is the homologue of protein L11 (Cundliffe *et al*., 1979) and is also methylated (Cannon and Cundliffe, 1979) and any deficiency in such modification could conceivably result in antibiotic resistance. However studies with a mutant strain of *E. coli*, deficient in the methylation of protein L11 which was isolated and designated *prm-1* (Colson and Smith, 1977) suggests otherwise. Ribosomes from strain *prm-1* together with the parental strain A19 were assayed for EF-G dependent GTP hydrolysis in the presence and absence of micrococcin. Data presented in Figure 9.1 clearly show that the ribosomes from both these strains were affected similarly by

micrococcin, i.e. both were stimulated in the GTPase reaction. Thus it would appear that the presence or absence of methyl groups on ribosomal protein L11 has no effect upon uncoupled GTP hydrolysis nor upon the interaction of the ribosomes with micrococcin. Therefore unless there is some other unexpected post translational modification of protein BM-L11, which might be different in the protein from strain GS2, it is very likely that the lesion within this strain affects the structural gene encoding the protein. It may simply be a point mutation leading to a conservative change in the amino-acid sequence of protein BM-L11. Notably, since the strain GS2 exhibits the stringent response to amino-acid starvation, this change cannot correspond exactly with any of those in relC mutants of E. coli (Parker et al., 1976). In these strains point mutations in the rplK gene, which encodes protein L11, gave rise to a relaxed phenotype. The protein had an altered electrophoretic mobility compared with that from the wildtype but in revertants it returned to normal mobility with a simultaneous reversion to the stringent phenotype. Interestingly, however, although strain GS2 is not relaxed the ribosomes from this strain could only support the in vitro synthesis of guanosine polyphosphates about half as efficiently as those from wildtype.

Assuming only a minor change has occurred in protein BM-L11 from strain GS2 it is still significant enough to lead to resistance to micrococcin and therefore it would be of interest to sequence the protein from the mutant and compare it with wildtype protein BM-L11. In this way, information might be gained as to how micrococcin interacts with ribosomes (see also below). However, arguing once more by analogy with E. coli

protein L11, it may be that protein BM-L11 is N-terminally blocked by methyl groups which would lead to difficulties of protein sequencing. In this case one way to determine the N-terminal residue, assuming the technology was available, would be mass spectrometry. Another interesting possibility for determining the amino-acid sequence indirectly would be to clone the altered gene from strain GS2 and the gene from protein BM-L11 from the wildtype into a suitable host organism and then obtain the DNA sequences of the two genes. Considering that Gram positive genes are not readily expressed in E. coli then a suitable Gram positive host organism could be chosen, possibly a streptomycete. This would involve obtaining a strain lacking a homologue of ribosomal protein L11 by selection for low level resistance to thiostrepton as was the case with strain MJ1 of B. megaterium. Shotgun cloning techniques employing multicopy Streptomyces plasmids as vectors could then be used to introduce into host protoplasts the genes encoding protein BM-L11 from B. megaterium wildtype and strain GS2. Selection of desired transformants would involve (i) appearance of fast growing colonies (mutants lacking ribosomal protein L11 grow very slowly), (ii) transformants could be distinguished from revertants on plates containing high levels of thiostrepton since Streptomyces vectors are available which carry a thiostrepton resistance (methylase) determinant (iii) transformants containing recombinant plasmids would exhibit insertional inactivation of a (pigment producing) tyrosinase gene if plasmid pIJ702 were used as vector. Once appropriate genomic inserts had been identified and isolated then the mutant gene and the wildtype allele could be subjected to standard DNA sequencing methods. Comparison of

the gene sequences from the wildtype and strain GS2 would establish the nature of the alteration in protein BM-L11 from strain GS2.

Another very important point is that the precise binding site for micrococcin has also not been established. Indeed it is not yet clear, although micrococcin apparently binds to a complex of 23S rRNA and protein L11 (Cundliffe and Thompson, 1981a), whether micrococcin has more than one binding site on the ribosome. Such information concerning the principal target for the drug and the stoichiometry of binding could only be definitively obtained if radiolabelled micrococcin were available. Nevertheless, as discussed in the Introduction, there are reasons for believing that the principal target for micrococcin may well be on 23S rRNA and if so, analogies could then be made to the case with thiostrepton (see below). Methylation of a single residue within 23S rRNA confers total resistance to the 'thio--strepton group' of antibiotics including micrococcin and presumably under such circumstances functional binding of the drugs is abolished. Moreover it has also been shown in this laboratory (Stark, unpublished observations) that, in equilibrium dialysis experiments, [^{35}S] thiostrepton can bind (albeit weakly) to 23S rRNA but not to protein L11 alone. Furthermore ribosomes lacking protein BM-L11 show partial resistance to thiostrepton (Cundliffe *et al.*, 1979; this dissertation Chapter 4). These data indicate that the principal binding site for thiostrepton is on 23S rRNA but that binding of the drug is significantly enhanced in the presence of protein (BM)-L11 possibly via a protein mediated conformational change within the RNA. One interesting observation, concerning the

action of micrococcin, is that in the absence of protein BM-L11 ribosomes are significantly more resistant to this drug than to thiostrepton (see Chapter 4). However they remain partially sensitive to micrococcin, so evidently the drug can still bind to ribosomes lacking that protein. Thus protein (BM)-L11 may be more important for directing the binding of micrococcin to 23S rRNA than it is in directing the binding of thiostrepton. Evidently changes in the structure of this protein can attenuate this effect. Alternatively an additional attachment site for micrococcin may be available on protein (BM)-L11 which might be lost or reduced in its affinity by sequence changes. One interesting set of experiments to perform when [^{35}S] micrococcin is available will be to see if there are any differences in binding of the drug to complexes formed between 23S rRNA and protein BM-L11 derived from the wildtype and with the protein from strain GS2. It will also be interesting to see if micrococcin can bind to 23S rRNA or protein BM-L11 alone, in equilibrium dialysis experiments.

Since a similar model was previously proposed for the promotion of binding of thiostrepton to 23S rRNA by protein L11 (Cundliffe et al., 1979b),it is interesting that ribosomes from strain GS2 also exhibited low level resistance to that drug (see also Chapter 4, Figure 4.3 and Chapter 5, Figure 5.3). Although thiostrepton and micrococcin attach to similar target sites it is not yet clear if these sites are in fact identical. However thiostrepton obviously binds with a greater affinity.

Prior to this present work, altered ribosomal proteins had been detected in a number of antibiotic-resistant bacteria. However relatively few cases had been reported directly linking

resistance of antibiotics to any of these altered proteins. The unequivocal means to link these two features is reconstitution analysis. The earlier examples concerned the changes in 30S ribosomal subunit proteins S12 and S5 which were responsible for resistance to streptomycin and spectinomycin, respectively (Ozaki et al., 1969; Bollen et al., 1969). The other major example is the absence of protein BM-L11 from the 50S ribosomal subunit which confers resistance to thiostrepton in B. megaterium (Cundliffe et al., 1979). Genetic experiments involving transduction analysis and cosegregation of drug resistance together with the protein alteration had rendered it highly likely that resistance to erythromycin can result from changes affecting ribosomal protein L4 (Wittman et al., 1973). By such means it was also shown that low-level resistance to gentamicin can be caused by alterations to protein L6 (Buckel et al., 1977) and that the state of protein L3 or L4 governs the ribosomal response to tiamulin (Böck et al., 1982).

A working hypothesis underlying these studies has been that at least some antibiotics might act where they bind in the ribosome (Cundliffe, 1983). Components involved in their binding sites might also be involved directly in process(es) inhibited. In such cases it would not be necessary to invoke the propagation to distant or allosteric ribosomal sites of putative conformational changes resulting from drug binding. (However, this would not deny that local perturbations of the ribosomal structure might still occur.) Such is the case with thiostrepton and micrococcin which both bind to a ribosomal site intimately associated with protein (BM)-L11 and both affect ribosomal reactions known to involve the functioning of that protein, i.e.

the manipulation of GTP molecules, hydrolysis and synthesis of guanosine polyphosphates. It may however be that the cases of the binding and action of thiostrepton and micrococcin are quite simple. Other antibiotics may well bind to ribosomal domains whose function involves a much greater degree of cooperativity between the various constituents. Within such "flexible" domains components involved in the binding of drugs might not always be the same as those which determine the consequences of such binding. Although, as stated above, the ribosomal response to streptomycin depends upon the state of protein S12 (Ozaki et al., 1969) binding of the drug can occur to core particles lacking protein S12 provided that protein S3 or S5 is present (Schreiner and Nierhaus, 1973). Similarly, resistance to erythromycin can be caused by changes in protein L4 (Wittman, 1973) or by specific methylation of 23S rRNA (Skinner et al., 1983) whereas proteins L15 and L16 are required to promote binding of the drug to ribosomal core particles (Teraoka and Nierhaus, 1978). In relation to this it has been shown, in equilibrium dialysis experiments, that protein L15 can bind erythromycin, weakly, off the ribosome and that protein L16 is probably not directly involved in binding the drug. Rather, its importance is to create the correct conformational structure in 23S rRNA to aid binding of the antibiotic (Teraoka and Nierhaus, 1978).

Regarding the mode of action of micrococcin it is not yet known how the drug acts to stimulate uncoupled GTP hydrolysis, dependent upon EF-G, when it binds to its normal target site (23S rRNA plus protein L11). Thiostrepton in contrast is a potent inhibitor of this process. It has not been unequivocally shown how such drugs with similar structures, generally similar

modes of action (see Introduction) and related ribosomal target sites can have different effects upon GTP hydrolysis. One hypothesis might be that whereas thiostrepton prevents the formation of ribosome·EF-G·guanine nucleotide complexes, micrococcin might destablize them. This could lead, at least *in vitro*, to an increased rate of turnover of GTP in the presence of micrococcin (Cundliffe and Thompson, 1981a). No major studies have been made concerning GTP hydrolysis in the presence of micrococcin in any systems actively synthesizing protein and so the effects of the drug upon this process *in vivo* remain unknown.

Notably, out of the 'thiostrepton group' of antibiotics which possess very similar structures, only micrococcin has so far been shown to stimulate EF-G dependent GTP hydrolysis. Nosiheptide inhibits this reaction although not nearly as potently as thiostrepton (Cundliffe and Thompson, 1981b). Thus it seems that the minor differences in structure of these drugs can have widely differing effects on, at least, uncoupled GTP hydrolysis. Another antibiotic which has a similar mode of action and apparently binds to a similar ribosomal binding site as thiostrepton and the related drugs but which is markedly different in structure to this group is berninamycin. This antibiotic, produced by *S. bernensis*, is also known to stimulate uncoupled GTPase activity, but not as potently as micrococcin (Thompson *et al.*, 1982b). An interesting observation from this laboratory is that an antibiotically active substance, sulfomycin, produced by *S. viridochromogenes* var. *sulfomycini* and which produces similar degradation products to berninamycin can also cause stimulation of GTP hydrolysis. Notably, however, the ribosomes from the micrococcin-resistant strain GS2 are sensitive

to this substance (N.K. Upson, unpublished observations). Moreover the sulfomycin producing organism is cross-resistant *in vivo* to thiostrepton, micrococcin and berninamycin. Thus, it seems that structurally similar (and some structurally dissimilar) drugs exert a spectrum of effects upon uncoupled GTP hydrolysis which may be aided by binding of such antibiotics to the same ribosomal domain but at slightly different sites. So far it appears that micrococcin and thiostrepton exert the extreme effects at opposite ends of this spectrum.

Finally, yet another interesting point to discuss concerns the difference in the modes of self protection of the various antibiotic producers discussed above. Those organisms which synthesize thiostrepton, nosiheptide and also berninamycin all possess similar ribosomal RNA methylases capable of rendering ribosomes resistant to these compounds. They all involve specific pentose methylation of 23S rRNA at a single site as discussed in the Introduction (Thompson and Cundliffe, 1980; Cundliffe and Thompson, 1981b; Thompson *et al.*, 1982b). Moreover, as discussed above, the action of this methylase also renders ribosomes totally resistant to micrococcin. The micrococcin producing organisms *M. varians* and *B. pumilus* (see the Introduction) on the other hand do not employ this mode of self protection. The ribosomes of such strains are known to be susceptible to these antibiotics *in vitro* (Dixon *et al.*, 1975) but it has not yet been shown how they defend themselves against their toxic products. It is interesting that the methylase responsible for resistance to the 'thiostrepton group' of antibiotics has so far only been found in actinomycetes and not in any species of micrococcus or bacillus. This in itself

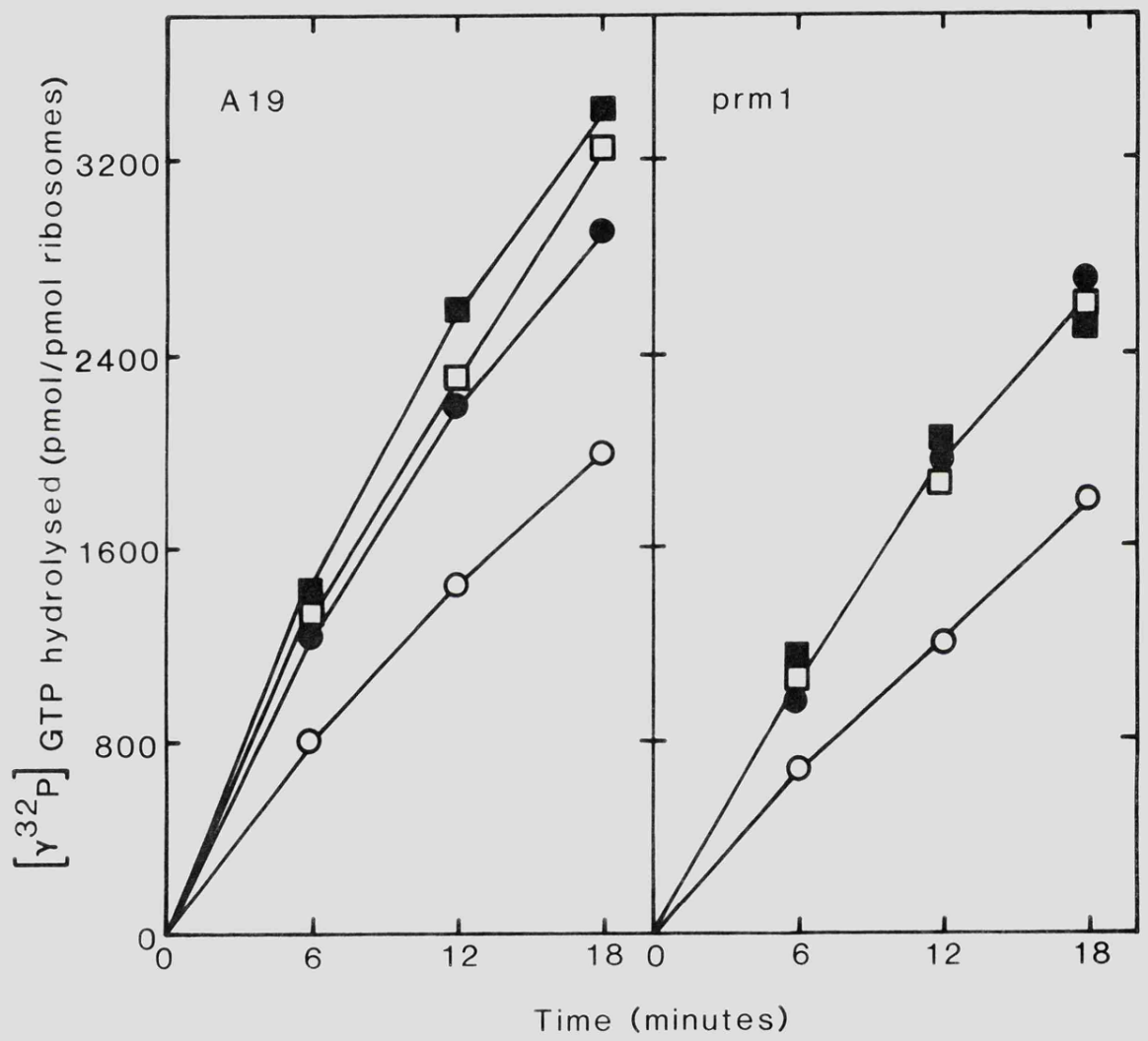
raises fascinating questions regarding the origin of such antibiotic-resistance determinants which is beyond the scope of this present work but has been the subject of a recent stimulating review (Cundliffe, 1984).

Legend to Figure 9.1

Uncoupled GTP hydrolysis dependent upon factor EF-G and ribosomes from E. coli strain A19 and strain prm1

Ribosomes (1 pmol) were preincubated for 10 minutes at 30°C with micrococcin or with DMSO (0.66% $^{v}/v$ final concentration) in control assays. Then [γ^{32}P] GTP and factor EF-G were added in excess over ribosomes in a final volume of 75 µl and assays performed as described in Materials and Methods. (O) control, no drug; (●) micrococcin 3 pmol input; (□) micrococcin 10 pmol input; (■) micrococcin 100 pmol input.

Figure 9.1


A19
prm1
[γ32P] GTP hydrolysed (pmol/pmol ribosomes)
3200
2400
1600
800
0
0
6
12
18
0
6
12
18
Time (minutes)

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Analysis of the ribosomes of a micrococcin-resistant strain of Bacillus megaterium

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Micrococcin is a modified peptide antibiotic which contains sulphur. It is an inhibitor of bacterial protein synthesis and acts by binding directly to the 50S ribosomal subunit. The drug also affects various partial reactions of protein synthesis. Of particular relevance here is the effect of micrococcin upon "uncoupled" hydrolysis of GTP catalysed jointly by the ribosome and the protein factor EF-G. Such GTPase which occurs in the absence of mRNA, tRNA and other factors normally required for protein synthesis is markedly stimulated by micrococcin. Moreover, a qualitative relationship exists between the level of stimulation of GTP hydrolysis and the level of inhibition of protein synthesis. In view of this, the ribosomal response to micrococcin in GTPase assays was used as an indicator of the levels of sensitivity.

A mutant strain of Bacillus megaterium arising spontaneously and resistant to micrococcin possesses ribosomes which contain an altered form of protein BM-L11 (the homologue of Escherichia coli protein L11). The ribosomes from this mutant were highly resistant to micrococcin.

Reconstitution analysis has revealed that the alteration to protein BM-L11 is the sole cause of resistance to micrococcin in this mutant. Ribosomes lacking protein BM-L11 were supplemented with the missing protein, purified from the wildtype or from the mutant. When the protein from the wildtype was employed, the reconstituted ribosomes exhibited wildtype characteristics, i.e. "uncoupled" GTP hydrolysis was stimulated by micrococcin. However, when protein BM-L11 from the resistant strain was employed GTP hydrolysis catalysed by the reconstituted ribosomes was not affected by the drug.

A binding site for micrococcin can be constructed in vitro solely from 23S rRNA and protein BM-L11, although the fine details of the ribosomal target site are not known. However, methylation of the 23S rRNA at a single specific-residue confers total resistance to the drug. It is therefore probable that the antibiotic binds primarily to 23S rRNA and that protein (BM)-L11 promotes such binding. Consequently it is of interest to note that alterations in this protein can lead to resistance to micrococcin.