A cluster of two serine transfer RNA genes from *Clostridium acetobutylicum* P262

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Contents

Abstract

A cloning system, using metronidazole as a screening tool and *E. coli* F19 as a selection host, was previously established to clone *C. acetobutylicum* P262 electron transport genes which may play a role in solvent metabolism in this bacterium. In theory, metronidazole would be reduced under anaerobic conditions to a cytotoxic intermediate by *C. acetobutylicum* P262 electron transport genes or reductive enzymes cloned into a recombinant plasmid. This intermediate would kill the host *E. coli* F19. One *C. acetobutylicum* P262 clone, pMET10B, was found to render the *E. coli* strain F19 sensitive to metronidazole, under anaerobic conditions. A number of subclones of the 2.56kb *C. acetobutylicum* P262 insert DNA were generated in Bluescript pKS and pSK. A range of exonuclease III deletions of this *C. acetobutylicum* P262 insert DNA were also generated which were shown to lose the metronidazole sensitivity phenotype on progressive deletion of the insert DNA. *In vitro* and *in vivo* protein transcription/translation experiments failed to reveal a protein product that was related to the metronidazole sensitivity phenotype. DNA hybridization confirmed that the insert DNA of pMET10B hybridized to *C. acetobutylicum* P262 chromosomal DNA, but not to *E. coli* chromosomal DNA.

The nucleotide sequence of a 933-bp fragment of the *C. acetobutylicum* P262 insert DNA was determined. DNA homology searches revealed two serine tRNA genes with the unmodified anticodons UGA and GCU that corresponded to the serine codons UCA and AGC, respectively. A putative promoter that had strong homology to both consensus *E. coli* promoter regions and consensus Gram positive promoter regions, was located upstream of the $tRNA^{Ser}$ genes. A 7-bp sequence showed homology to a stringent control region from

B. subtilis. Three 13-bp repeat sequences located immediately after the two $tRNA^{Ser}$ genes were identified as a *rho*-independent transcription terminator.

The position of the $tRNA^{Ser}$ genes on the exonuclease III-shortened clones indicated that they were not related to the ability of certain of the constructs to render *E. coli* F19 sensitive to metronidazole. Instead, a 16-bp sequence, that showed homology to a consensus LexA binding site from *E. coli*, appeared to be related to the metronidazole sensitivity phenotype. We speculate that this sequence may mimick an *E. coli* LexA binding site which may cause the autoregulated *lexA* structural gene to overexpress the protein. The increased expression of LexA may generate the sensitivity phenotype observed by placing an increased load on the transciption and translation machinery of the cell. In this laboratory only one of five *C. acetobutylicum* P262 clones, that conferred the metronidazole sensitivity phenotype on *E. coli* F19, was identified to contain genes related to electron transport. We therefore propose that a more specific strategy be adopted for the cloning of electron transport genes in *C. acetobutylicum* P262.

The isolation of the $tRNA^{Ser}$ genes was fortunate in that only one tRNA gene has previously been cloned from *C. acetobutylicum* P262. The stringent control element upstream of the $tRNA^{Ser}$ genes also provided the first indication that the stringent response may operate in *C. acetobutylicum* P262. The location of putative transcriptional control elements and preliminary RNA hybridization experiments indicated that the $tRNA^{Ser}$ genes may be transcribed as one transcriptional unit. This provides the first information about tRNA gene organisation in *C. acetobutylicum* P262.

Abbreviations

A	adenosine
A_x	absorbance at x nm
aa(s)	amino acids
AB	acetone-butanol
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
UK	United Kingdom
US	United States
bp(s)	base pairs
kb	kilobase pairs
C	cytidine
CTP	cytidine 5'-triphosphate
CBM	*Clostridium* basal medium
Ci	curie
CoA	coenzyme A
CsCl	cesium chloride
°C	degrees celsius
Da	daltons
kDa	kilodaltons
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside 5'-triphosphate
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
Fd	ferredoxin
Fig.	figure
g	gram(s)
G	guanosine
GTP	guanosine 5'-triphosphate
ΔG	Gibbs free-energy change
h	hour(s)
kCal	kilocalories
l	litre(s)
LB	Luria-Bertani broth
leu	leucine
log	logarithmic
m	metre(s)
MIC	minimum inhibitory concentration
min	minute(s)
mol	mole(s)

NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
OD	optical density (nm)
*ori*C	origin of replication in *E. coli*
PFOR	pyruvate ferredoxin oxidoreductase
RNA	ribonucleic acid
mRNA	messenger RNA
tRNA	transfer RNA
rpm	revolutions per minute
s	second(s)
SC	stringent control
SDS	sodium dodecyl sulfate
ser	serine
T	thymidine
thr	threonine
TTP	thymidine 5'-triphosphate
U	uridine
UTP	uridine 5'-triphosphate
UV	ultraviolet
V	volts
v/v	volume/volume
w/v	weight/volume

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Chapter One

Literature review and introduction

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Literature review and introduction

1.1 *Clostridium acetobutylicum*

Clostridium acetobutylicum is a Gram-positive, spore-forming bacterium that has been used in the past for the production of acetone and butanol via fermentation in large-scale industrial processes. In spite of the decline in the use of the AB (acetone-butanol) fermentation in the 1960's due to economic competition from chemically produced solvents, there has recently been renewed interest in the fermentation process. The rise in the petroleum price in the 1970's, the prospect of the exhaustion of the world's fossil fuel supply and the popularity of environmentally "friendly" products, all point to the feasibility of once again obtaining fuel by the microbial fermentation of renewable resources.

1.1.1 History of the acetone-butanol fermentation

Louis Pasteur was the first person to observe the production of butanol by a "*Vibrion butyrique*" in 1861. This bacterium probably belonged to the genus *Clostridium* (Morris, 1993). It was not, however, until the First World War that the study of the clostridia was initiated for two reasons. Firstly, gangrenous clostridial infection of wounds became common, and secondly, the demand for acetone in the manufacture of explosives increased. *C. acetobutylicum* was therefore used in industrial fermentations to supply the acetone requirements of the munitions industry. The history of the AB fermentation has been reviewed extensively by Jones and Woods (1986) and by Morris (1993). Both reviews have provided the basis for much of the information presented here.

The firm of Strange and Graham Ltd. in England embarked on a project in 1910 to produce synthetic rubber. Since butanol was required for the process, the possibility of producing it via microbial fermentation was investigated. Chaim Weizmann, a chemist and a self-trained microbiologist, isolated a strain of *C. acetobutylicum* that could produce good yields of butanol and acetone from starchy substrates. *C. acetobutylicum* can ferment a wide variety of substrates including starch in the form of maize-mash, sugar in molasses, and other substrates such as Jerusalem artichokes and algal biomass. With the outbreak of the First World War in 1914, the demand for acetone increased. As part of the war effort, 2000 pounds of acetone per week were fermented from maize at a plant owned by Strange and Graham Ltd. When the blockade by the Germans affected the availability of maize in the United Kingdom, the process was transferred to Canada and the United States.

After the war, the emphasis of the AB fermentation switched to the production of butanol, which was used as a solvent in nitrocellulose lacquers for the automobile industry. Attempts were made to maximise the profitability of the AB fermentation by several processes: removing the solvents, collecting the CO_2 (carbon dioxide) for sale as compressed gas or dry ice, using the H_2 (hydrogen) for the hydrogenation of vegetable oils and selling the residual biomass as animal feed. By 1927, 96 fermentation plants were in operation in Illinois and 52 in Indiana, close to the maize-growing areas of the United States. In the 1930's molasses replaced maize as the substrate of choice due to low costs. With the advent of the Second World War came the increased demand for acetone and the AB fermentation was again given high priority. After 1936, plants were built in Japan, Australia, India and South Africa. By the end of the war, two-thirds of the butanol, and one-tenth of the acetone produced in the US was via AB fermentation, with the remainder being sythesized chemically.

By 1960, however, the process had ceased to be financially viable and production in the US and Britain had virtually stopped. The chemical synthesis of acetone (from propylene) and butanol (from ethylene) was a much cheaper alternative. Also, the increase in the price of molasses due to its incorporation into animal feed meant that the costs incurred were too high. In South Africa, the production of solvents via fermentation continued long after it had been stopped in Western countries. This was due to the lack of a cheap petroleum supply and the availability of cheap molasses. In 1982, however, the plant in Germiston, South Africa followed suit and closed due to economic constraints.

The possibility of converting waste products into desirable commodities via fermentation seems increasingly attractive today, in the light of environmental awareness. Sulphite waste liquors from the paper industry and whey from the dairy industry can both be used as substrates by *C. acetobutylicum*. The bacterium can also be used in the biological conversion of sulphur gases. Gas synthesized from coal has a high sulphur content in the form of H_2S (hydrogen sulphide) and COS (carbonyl sulphide). Extraction of the sulphur is expensive, but the cost would be reduced if the gas volume were decreased. The biological conversion of these gases would result in solvent products and would reduce the volume of the sulphur gas, thereby generating income. This process is possible using a 2-stage continuous fermentation: with the conversion of CO (carbon monoxide) to acids by *Butyribacterium methylotrophicum* and the conversion of the acids to solvents by *C. acetobutylicum* (Grethlein, 1992).

The AB fermentation, in its heyday, ranked second in importance to the production of ethanol by yeast. Today, the fermentation is still important in the People's Republic of China where it generates half of the country's supply of butanol.

Although the process is not economically viable at present in the Western world, changes in the process technology or strain improvement by genetic engineering could make it financially attractive.

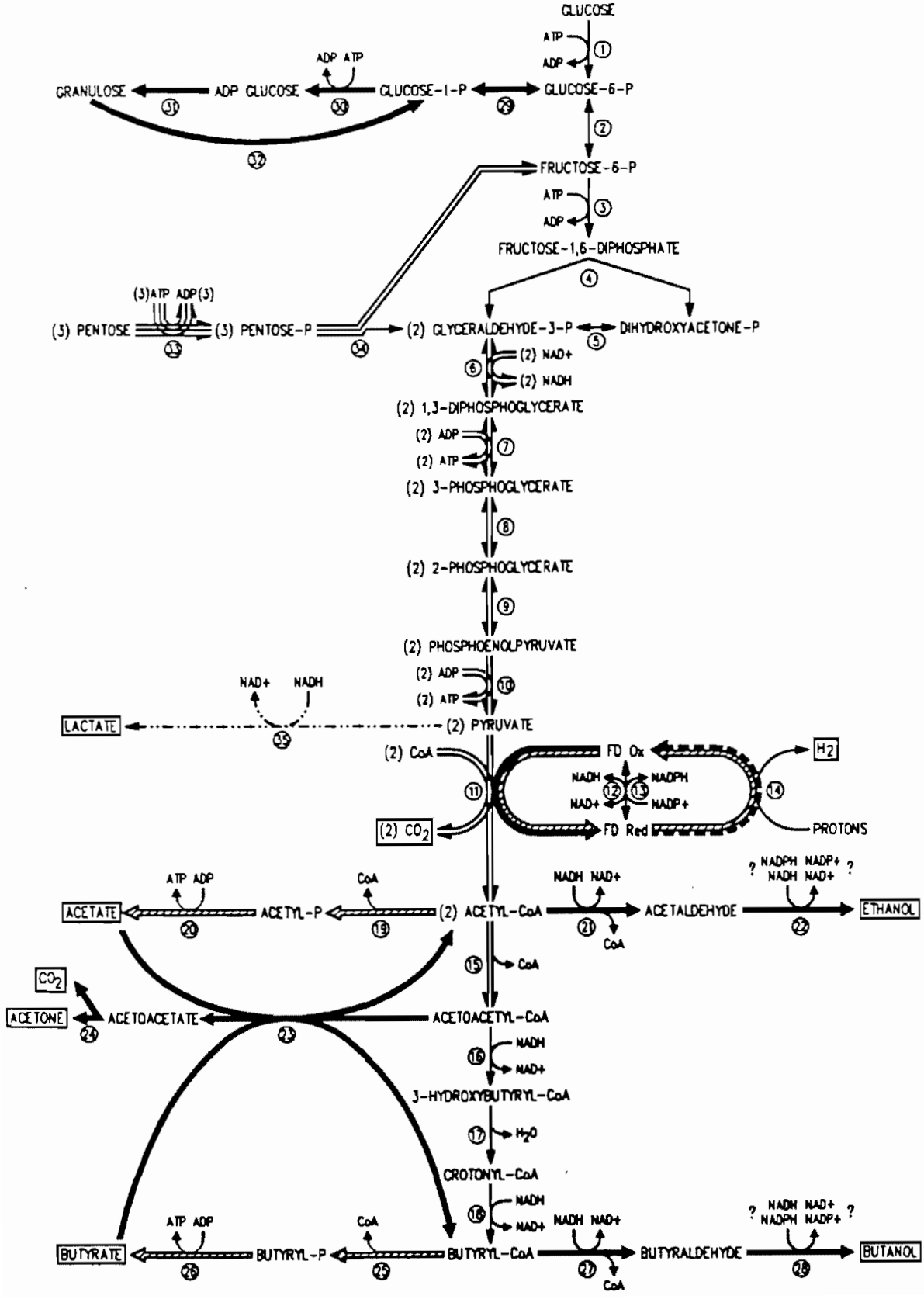
Different strains of *C. acetobutylicum* have been identified and used in the study of the genetics of the bacterium including *C. acetobutylicum* strains ATCC 824, NCIMB 8052 and DSM 1731. The *C. acetobutylicum* strain P262, which was used in this study, was identified as being a good producer of solvents and was used industrially by National Chemical Products in Germiston, South Africa. The bacterium appears to differ substantially from other *C. acetobutylicum* strains in that probes made from a number of cloned *C. acetobutylicum* ATCC 824 genes do not hybridize to DNA (deoxyribonucleic acid) from the *C. acetobutylicum* P262 strain (Wilkinson and Young, 1993). The genome of *C. acetobutylicum* P262 is small (2.85Mbp) in comparison to those of *C. acetobutylicum* strains ATCC 824 and DSM 1731 which are 4.0 and 3.5Mbp, respectively, and strain NCIMB 8052 which has a genome size of 6.5Mbp (Wilkinson and Young, 1993). The different strains have different fermentation characteristics (Young *et al.*, 1989*a*) and may differ in the regulation of their solvent metabolism.

1.1.2 The biochemistry of acid and solvent production

The biochemical pathways involved in acid and solvent production in *C. acetobutylicum* have been studied extensively during the past 20 years and are now well-formulated. The review by Jones and Woods (1986) has been used in writing this and following sections.

The growth of *C. acetobutylicum* during normal batch culture is biphasic. During the logarithmic growth phase, known as acidogenesis, the bacterium produces

acetate, butyrate, H_2 and CO_2 from an available carbohydrate source and ATP (adenosine 5'-triphosphate) is generated (Fig. 1.1).



The production of acetic and butyric acids causes a decrease in the pH of the medium. These undissociated acids are thought to inhibit cell growth by partitioning within the membrane and acting as uncouplers, leading to a collapse of the pH gradient. The concentration of acids increases until a critical breakpoint pH is reached (Jones *et al.*, 1982) which varies for different strains (Jones and Woods, 1986). For *C. acetobutylicum* P262, the breakpoint occurs at pH 5.2, which is higher than that for other strains. The decrease in the pH of the medium and an increase in undissociated butyric acid signal the end of the acidogenic phase.

During the second, stationary phase of growth, known as solventogeneis, solvents are produced. The carbohydrate source is depleted further while the acids in the medium are reassimilated and the solvents, acetone, butanol and ethanol are formed (Fig. 1.1). The shift to solventogenesis may well be an adaptation of the bacterium because acid reassimilation detoxifies the environment by raising the pH level. In a typical batch culture, the reassimilation of butyrate and acetate is via the CoA-transferase-acetoacetate decarboxylase pathway. Acid uptake is therefore coupled to acetone and CO_2 production (Hartmanis *et al.*, 1984). During solventogenesis, two sets of dehydrogenases are required to reduce acetyl-CoA to ethanol and butyryl-CoA to butanol. Ethanol production in *C. acetobutylicum* is constitutive and is not linked to the production of acetone or butanol (Bertram *et al.*, 1990). The generation of a mutant of *C. acetobutylicum* by transposon mutagenesis, that produced ethanol but was defective in the production of acetone and butanol, confirmed this (Babb *et al.*, in press). Butanol is the most toxic of the solvents produced. It exerts a chaotropic effect on the cell membrane; increasing permeability and fluidity such that the proton motive force is dissipated (Terracciano and Kashket, 1986). The isolation of butanol-resistant strains of *C. acetobutylicum*, however, did not prove to be useful for increased butanol yield, as the concentrations of solvents obtained were similar to those obtained with

industrial strains (Jones, 1993). Also, a butanol-resistant mutant isolated by Baer (1993), only produced trace amounts of butanol showing that increased tolerance was not related to increased production of the solvent.

Associated with the shift to solventogenesis, is a change in morphology (Long *et al.*, 1984). During acidogenesis, the cell is highly motile and rod-shaped. Motility decreases before the pH breakpoint is reached and a storage product, granulose, is laid down. Solventogenesis is characterised by swollen clostridial forms, that are cigar-shaped, and by the production of an extracellular slime layer (Jones *et al.*, 1982). The production of an endospore is eventually followed by the release of a resistant spore from the mother cell.

The complex, branched nature of the pathways involved in acid and solvent formation in *C. acetobutylicum* enable its adaptation to different growth conditions. However, future industrial-scale fermentations may necessitate the channelling of the pathways to one or two products, in order to increase product yield and to become economically competitive. Therefore present research needs to address the mechanisms whereby the bacterium directs and responds to the carbon and electron flow within its pathways.

1.1.3 Energy and electron flow

Bacteria that grow via anaerobic fermentation are limited by the availability of ATP. The complexity of the branched pathways involved in energy generation and the generation of end products reflects the ability of anaerobes to adapt to energy limitation. During glycolysis in *C. acetobutylicum* less ATP is generated than is required for biosynthesis and growth, but too much NADH (reduced nicotinamide adenine dinucleotide) is produced. Additional ATP is generated via the production of acetate and butyrate. Like most anaerobic bacteria that ferment substrates, one

of the main problems is the disposal of reducing power, either in the form of reduced carbon compounds (such as solvents) or in the form of hydrogen. During the conversion of pyruvate to acetyl-CoA, electrons are donated to the electron carrier, ferredoxin, which can either accept or donate electrons. The electrons are donated to the hydrogenase enzyme which adds them to protons to produce hydrogen. During acidogenesis, the hydrogenase is very active and the excess NADH that was produced during glycolysis is reoxidised through the oxidoreductase pathway resulting in increased H_2 production. Some NADH is also used up in the generation of butyrate, which is energetically less favourable to produce than acetate. Towards the end of acidogenesis ATP concentrations reach a minimum since ATP is diverted from biosynthesis to be consumed by ATPase as protons are extruded across the cell membrane to maintain the pH gradient. The pH gradient increases and the integrity of the cell membrane is maintained. However, it was noted that there is a drastic increase in the concentration of NADPH (reduced nicotinamide adenine dinucleotide phosphate) and NADH in the cell until solventogenesis is induced (Grupe and Gottschalk, 1992). During solventogenesis, H_2 and CO_2 production decreases and ATP concentrations decrease due to a reduction in acid production. The decrease in hydrogen production in the cell, alters the route of electron and carbon flow (Jones and Woods, 1989). To dispose of reducing equivalents the cell must now redirect its carbon flow to produce reduced products such as solvents. The aldehyde and alcohol dehydrogenases require NAD(P)H for alcohol production. The excess NADH generated during glycolysis of glucose to pyruvate is now utilised and electrons from reduced ferredoxin are sequestered for the reduction of NAD or NADP by the appropriate ferredoxin oxidoreductase.

The regulation of the electron flow within *C. acetobutylicum* has been reviewed by Jones and Woods (1986) and by Woods and Santangelo (1993). In short, four

proteins are responsible for most of the electron distribution; hydrogenase, ferredoxin and the NADH- and NADPH-dependent ferredoxin-oxidoreductases. Reduced ferredoxin can either transfer electrons via the hydrogenase to produce H_2 or via the ferredoxin-oxidoreductase to produce reduced pyrimidine nucleotides.

It has been shown that a number of factors can induce the shift to solventogenesis. Firstly, reducing the pH of the medium to the breakpoint pH can induce the shift (Bahl *et al.*, 1982). An increase in the intracellular concentration of undissociated butyric acid is also related to an induction of solventogenesis. Factors that prevent the hydrogenase enzyme from forming hydrogen also induce the shift. These factors include gassing with CO, which inhibits the hydrogenase (Kim *et al.*, 1984), and adding electron carrier dyes such as methyl and benzyl viologen, that prevent hydrogen formation and increase butanol production (Roa and Mutharasan, 1988).

There may be two regulators that control the metabolic switch. It has recently been proposed that a change in the internal ATP level induces acetone formation while an increase in NAD(P)H levels provides the signal to switch on butanol production (Grupe and Gottschalk, 1992). The changes that occur in energy and electron flow are complex and may form part of a regulatory signal that induces the shift from acidogenesis to solventogenesis.

The onset of solventogenesis may be regulated by a number of factors:

1]. An alternative sigma factor may direct transcription of a distinct class of promoters upstream of solvent genes (Petersen *et al.*, 1993). Two heat shock operons, *groESL* and *dnaK* that are induced in *C. acetobutylicum* during the onset of solventogenesis, however, do not appear to be recognised by alternative sigma factors. Instead, an 11-bp hairpin loop

located before the start of both operons may regulate their expression (Naberhaus and Bahl, 1992; Naberhaus *et al.*, 1992).

2]. A decrease in nucleotide levels occurs during the switch to solventogenesis (Santangelo *et al.*, 1989) which may indicate a role for small effector molecules in the regulation of metabolic pathways.

3]. The phosphorylation of proteins within a signal transduction pathway may induce the switch to solventogenesis. A heat shock protein (DnaK) that can phosphorylate a 50kDa protein in *C. acetobutylicum* may form part of such a pathway (Naberhaus *et al.*, 1992).

4]. A rare threonine tRNA (transfer RNA) gene may play a role in the translational control of solvent genes in *C. acetobutylicum.* Sauer and Dürre (1992) found that a *C. acetobutylicum* mutant, that was defective in acetone and butanol formation, had a transposon inserted 55-bp upstream of the $tRNA^{Thr}$ gene. However, levels of the $tRNA^{Thr}$ were the same during acidogenesis and solventogenesis. Therefore the regulation of genes by this tRNA may occur at the level of processing of the primary transcript of the tRNA, charging of the tRNA or by modification of the tRNA molecule.

1.1.4 The cloning of genes involved in electron transfer

In anaerobic bacteria, electrons transfer from molecules with a negative redox potential to molecules with a more positive redox potential. Metronidazole, a drug which requires reduction in order to be active against anaerobic organisms, has a relatively positive redox potential and can, therefore, accept electrons from components of the clostridial electron transfer network. It has been shown that metronidazole preferentially accepts electrons from ferredoxin and in turn, from the hydrogenase, thereby temporarily preventing the production of hydrogen in the

clostridia (O'Brien and Morris, 1972; Lockerby *et al.*, 1984; Church *et al.*, 1988). This will be discussed in detail in Section 1.2 which is devoted to metronidazole. The drug, therefore, has been used as a tool for the isolation of a flavodoxin electron transfer gene and a hydrogenase gene from *C. acetobutylicum* P262 (Santangelo *et al.*, 1991). A number of other DNA fragments from *C. acetobutylicum* also reduced the drug, implying that they may contain electron transfer genes or reductive enzymes. One such fragment, cloned into pMET10B, has formed the basis of the study presented in this thesis.

1.1.5 The cloning of solventogenesis genes

Several researchers have concentrated on the cloning of solvent production genes in *C. acetobutylicum* with a view to the future manipulation of metabolic pathways to increase solvent yields. An NADPH-dependent alcohol dehydrogenase involved in ethanol and butanol production, was cloned from *C. acetobutylicum* P262 (Youngleson *et al.*, 1989*b*). In addition, two butanol dehydrogenase genes were cloned from *C. acetobutylicum* ATTC 824. They were primarily NADH-dependent, indicating that a number of enzymes carry out the conversion of butyraldehyde to butanol (Welch *et al.*, 1989; Petersen *et al.*, 1991). Genes involved in acetone formation have also been cloned: the acetoacetyl-CoA:acetate/butyrate:CoA transferase and the acetoacetate decarboxylase (Cary *et al.*, 1990; Gerischer and Dürre, 1990; Petersen and Bennet, 1990). Other genes from the biochemical pathways involved in solvent formation that have been cloned include the thiolase (Petersen and Bennett, 1991) and the 3-hydroxybutyryl CoA dehydrogenase (Youngleson *et al.*, 1989*a*). Two genes responsible for butyrate production, the butyrate kinase and the phosphotransbutylyrase were also cloned (Cary *et al.*, 1988).

At present, techniques for the genetic transformation of *C. acetobutylicum* are still in the developmental stage. Vectors used in transformation are normally *Escherichia coli/C. acetobutylicum* or *Bacillus subtilis/C. acetobutylicum* shuttle vectors which contain *Clostridium* replication and antibiotic resistance functions. The improved regeneration of protoplasts resulted in efficient transformation of protoplasts of *C. acetobutylicum* N1-4081 (Reysett *et al.*, 1988). Electroporation of whole cells of *C. acetobutylicum* strain NCIMB 8052 was first demonstrated by Oultram *et al.* (1988). The cloned acetoacetate decarboxylase and phosphotransbutyrylase genes of *C. acetobutylicum* ATCC 824 have been successfully transformed into the homologous host by electroporation using vector pFNK1, a *B. subtilis/C. acetobutylicum* shuttle (Mermelstein *et al.*, 1992). The enzyme activities of these two genes were increased in *C. acetobutylicum* ATCC 824 and their effect on product formation is currently being investigated. This is the first example of the successful transformation of product genes back into *C. acetobutylicum*, which represents a critical step towards the future genetic manipulation of the bacterium to increase solvent yields. However, no successful electroporation of *C. acetobutylicum* strain P262 has been reported. Successful transformation of *C. acetobutylicum* has also been achieved via conjugative methods using broad-host-range enterococcal plasmids. Plasmids have been transferred to *C. acetobutylicum* from *Enterococcus faecalis* (Yu and Pearce, 1986), *E. coli* (Williams *et al.*, 1990) and *B. subtilis* (Young, 1993). Conjugative transposons such as Tn*916* from *E. faecalis* can also be used as a tool for the genetic analysis of Gram-positive organisms such as *C. acetobutylicum* (Young, 1993)

It is convenient that *E. coli* has proved to be an excellent heterologous host for cloned *C. acetobutylicum* genes as it facilitates the study of the genes at the molecular level. *C. acetobutylicum* DNA has a very low G+C content of 28% to

29% (Cummins and Johnson, 1971) and therefore, codon usage is strongly biased towards codons in which A and U predominate. Therefore, large differences occur in codon usage between *E. coli* and *C. acetobutylicum* for the amino acids arginine, leucine, threonine, proline, glycine and isoleucine (Young *et al.*, 1989). Where 4 or 6 synonymous codons are available, there is a strong preference for those codons that have U or A in the wobble position (third base). However, the alcohol dehydrogenase, endoglucanase and cellobiose genes were expressed efficiently in *E. coli* in spite of the difference in codon bias (Youngleson *et al.*, 1988; Zappe *et al.*, 1986). Gene expression may have been aided by the presence of *E. coli*-like promoters and ribosome binding sites in the A-T rich clostridial DNA. Unlike *C. acetobutylicum*, *E. coli* is able to grow either aerobically or anaerobically and this ability has been utilised in the study presented here.

1.2 Metronidazole

1.2.1 Development and use

In 1954 the pharmaceutical company, Rhône-Poulenc, decided to develop a drug that would be effective against the anaerobe *Trichomonas vaginalis* which causes human vaginitis. Previous therapy for this disease consisted of either vinegar douches (to normalise the pH of the vagina) or methyl violet impregnated tampons (Edwards, 1980). Azomycin, a 2-nitroimidazole, that was isolated from a *Streptomyces* was found to be active against the trichomonad. More than 200 chemicals, related to azomycin, were synthesized and subsequently screened for activity. Metronidazole (1-hydroxyethyl-2-methyl-5-nitroimidazole, Flagyl®) was found to display the best balance between efficacy and toxicity. The first clinical trials began in France in 1958 and since then its use has become widespread (Roe, 1977). The nitroimidazole group of drugs has a wider spectrum of activity than any other antimicrobial drug, encompassing a wide range of anaerobic Gram-positive and Gram-negative bacteria and protozoa. Today, metronidazole is still the drug of choice for the treatment of trichomoniasis of which approximately 150 million cases are reported each year (Johnson, 1993).

Following the incredible success of the drug came observations that it was useful against a variety of human diseases including *Giardia lamblia*. *G. lamblia* was first discovered by Leeuwenhoek in 1681 in his own stools (Smith and Woolfe, 1980). Today, it is the most common pathogenic internal parasite in the US and the incidence is even greater in poorer, developing countries. It can cause acute infection that results in diarrhoea, cramps and weight loss. Some patients become carriers and the disease may recur. Mild, chronic infections may go undiagnosed and untreated. Metronidazole is frequently prescribed for therapy and cure rates of 86% to 91% have been reported (Smith and Woolfe, 1980). Metronidazole is also

used to treat Vincent's disease, a gingivitis, which causes a painful acute ulcerative condition of the gums. In fact, it was the cure of Vincent's disease in a woman who was receiving metronidazole for the treatment of a *T. vaginalis* infection, that led to the realization that metronidazole acted specifically against obligate anaerobes (Shinn, 1962). The drug is also active against *Entamoeba histolytica* which causes amoebiasis, and is reported to infect 10% of the world's population (Johnson, 1993). *Gardnerella vaginalis*, which causes vaginitis is also susceptible to metronidazole, as is *Helicobacter pylori*, which is implicated in gastroduodenal ulcers (Edwards, 1980). Nitroimidazoles are routinely used to prevent post-operative sepsis which is usually caused by the Gram-negative *Bacteroides* spp. and the Gram-positive *Clostridium* spp. Other nitroimidazoles, that are based on the heterocyclic structure of metronidazole, have appeared since the inception of metronidazole in the early 1960's, (Fig. 1.2). These compounds have extended the range of activity of the 5-nitroimidazoles to include a wider range of pathogenic microorganisms.

Both metronidazole and misonidazole have been extensively used in cancer radiotherapy. When a cancerous tumour grows it tends to exclude the surrounding blood supply, causing the tumour cells to become hypoxic or micro-aerophilic (Edwards, 1980). Anticancer drugs have difficulty in penetrating the tumour due to lack of a good blood supply, and in addition penetrate poorly due to their large molecular weight. For an inoperable tumour therefore, radiotherapy is the only practical solution. The nitroimidazole drugs are effective for three reasons. Firstly, they are small molecules, and can penetrate tumour cells better than any other drugs. Secondly, they are relatively toxic to hypoxic cells because reduction of the drug occurs only in oxygen-starved environments, and thirdly, reduction of the drug within tumour cells creates a favourable concentration gradient which facilitates drug entry into tumour cells. The last two factors will be discussed in greater detail in Section 1.2.2. The nitroimidazoles cause strand breakage of DNA and act as

radiosensitisers due to the synergistic effect of the DNA damage induced by the drug and by the radiation treatment (Edwards, 1980).

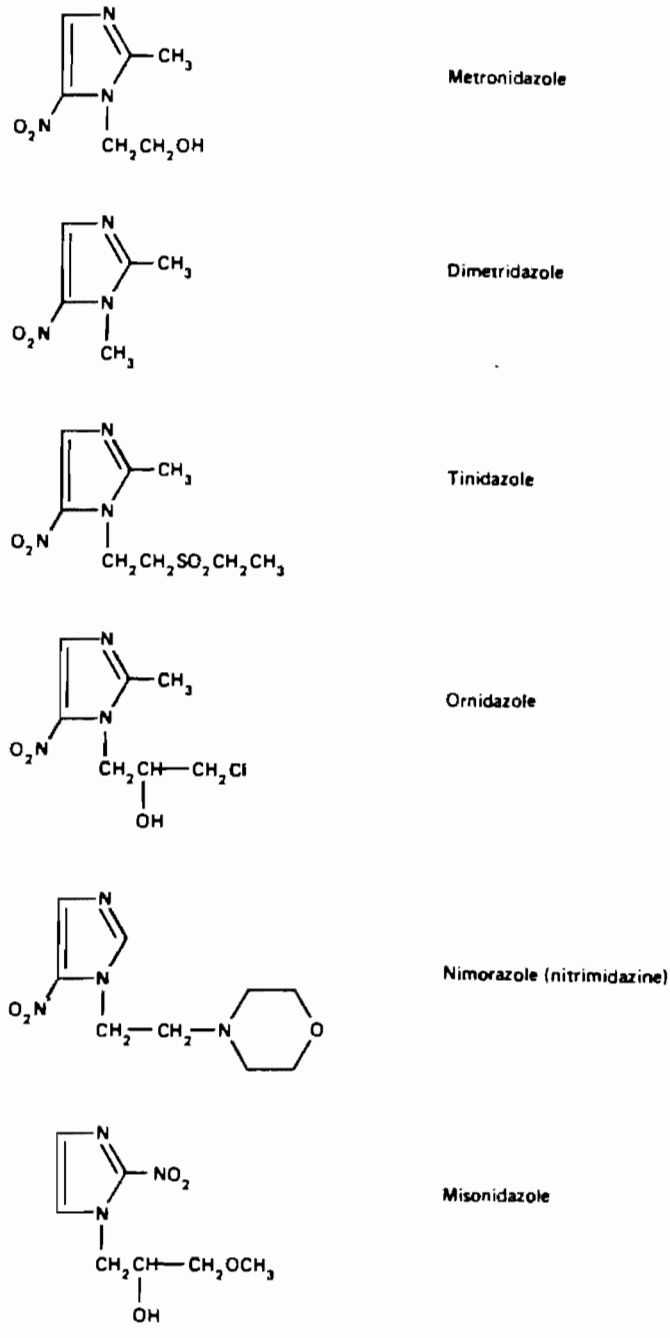


Fig. 1.2 A range of structures of nitroimidazole drugs (Edwards, 1980).

Toxicity of metronidazole is low, with common side effects being a metallic taste and a furry tongue, for doses of less than two g (gram)s per day (Edwards, 1980). At higher doses of up to 10 g per day, which are required for radiosensitisation, nausea and vomiting can occur. Metronidazole can affect the DNA of bacteria, as shown by the Ames test, and it has been shown to increase the incidence of cancer in mice (Rustia and Shubik, 1972). Therefore there has been concern that the drug or its metabolites may be mutagenic or carcinogenic to humans. However, a study of 771 women in 1979, who were given metronidazole for the treatment of trichomoniasis, showed that there was no statistically significant increase in the incidence of cancer (Beard *et al.*, 1979). Obviously, the drug should be administered only if necessary and with caution.

1.2.2 Mechanism of action

Some details about the mechanism of action of metronidazole is known, but several questions remain unanswered - such as: what actually causes damage to the cell and how? It is known that reduction of the nitro group of nitroimidazoles is required in order to kill susceptible cells. This step is the basis for the selective cytotoxicity of the drug. In anaerobic bacteria and protozoa, pathways of sufficiently low redox potential are present, which allow for reduction of the drug, whereas, in aerobic cells, pathways of higher redox potential cannot reduce the drug, thereby rendering it inactive (Johnson, 1993).

1.2.2.1 Entry into the cell

Metronidazole enters the cell via passive diffusion, although the possibility of active transport should not be completely ruled out. It was found that large quantities of [^{14}C]-metronidazole were quickly taken up from the growth medium into cells of *T. vaginalis* where it was rapidly metabolized (Ings *et al.*, 1974). The reduction of the drug inside the cell resulted in a decrease in the concentration of

the original, unchanged compound in the cell. Thus a transmembrane concentration gradient was established which promoted the uptake of metronidazole into the cell (Ings *et al.*, 1974; Müller, 1983).

1.2.2.2 Reductive Activation

Much of the following section is dealt with in a review by Edwards (1993). Theoretically, the reduction of a typical nitroaromatic compound would require the addition of six electrons ($6e^-$) to reduce a nitro group ($-NO_2$) to an amino group ($-NH_2$), equation [1], (Edwards, 1993). This classical pathway includes nitroso ($2e^-$) and hydroxylamino ($4e^-$) intermediates.

$$R\text{-}NO_2 \xrightarrow{1e^-} R\text{-}\dot{N}O_2^- \xrightarrow{1e^-} R\text{-}NO \xrightarrow{2e^-} R\text{-}NHOH \xrightarrow{2e^-} R\text{-}NH_2 \qquad [1]$$

However, the energy required for such a process is too great and also the single electron nitro radical ion undergoes rapid degradation to form a nitrite ion (NO_2-) and an imidazole radical, equation [2], which prevents further reduction.

$$R\text{-}\dot{N}O_2^- \longrightarrow \dot{R} + NO_2^- \qquad [2]$$

The actual process by which this short-lived ion causes cell death is unknown. However, it is proposed that it causes DNA damage and interacts with other cell components, such as proteins and membranes, leading to cell death (Edwards, 1993).

Reduction of metronidazole in anaerobes is thought to occur either via general reductive enzymes, or via electron carriers such as ferredoxin or flavodoxin (Edwards, 1993). A redox potential expresses, in volts, the potential for electrons to be donated or received in a reaction by a molecule that is 50% oxidised and 50%

reduced. If the reaction is only a reductive process and is not reversible, it is referred to as a reduction potential. All anaerobes posses redox mechanisms of about -430mV to -460mV, which includes the value for ferredoxin. Metronidazole, however, has a redox potential of -415mV. A redox reaction with a lower potential (more negative) than metronidazole, will donate electrons to the drug. In other words, metronidazole with a more positive reduction potential will act preferentially as an electron acceptor. In aerobes, however, the lowest redox potentials are about -324mV, which are more positive than that for metronidazole. Therefore, the drug cannot accept electrons (*ie.* be reduced) in aerobic organisms and is therefore inactive.

Even if reduction happened to occur in the presence of oxygen, damage would be minimal due to a reaction known as "futile cycling". Basically, since oxygen is the best electron acceptor in any biological system, it would abstract an electron from the nitro radical anion, forming the inert metronidazole compound and a superoxide (Edwards, 1993). A schematic diagram of all the reactions involved in metronidazole reduction in the cell is presented in Fig. 1.3.

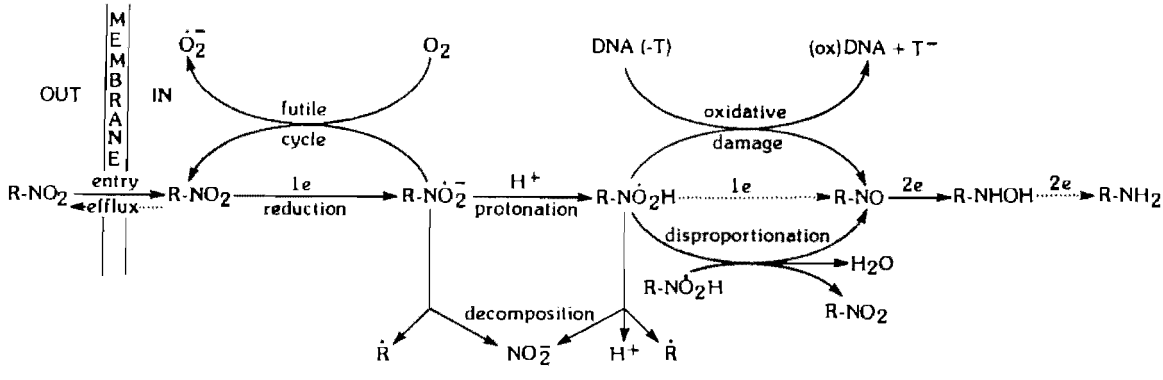


Fig. 1.3 The mechanism of action of nitroimidazoles (Edwards, 1993).

1.2.2.2.1 Reduction via the electron transfer system

It is thought that the reductive activation of metronidazole can occur via reduced electron-transport proteins in *C. acetobutylicum* and in other anaerobes. These proteins are normally involved in the oxidative fermentation of pyruvate, that is, in carbohydrate metabolism.

The first indication that metronidazole may be reduced via such a system was reported by Edwards and Mathison (1970), who noted that the addition of metronidazole to a culture of *T. vaginalis* inhibited hydrogen production. O'Brien and Morris (1972) also showed the same effect in cell extracts from *C. acetobutylicum*, while no effect on carbon dioxide production or acetyl-phosphate production was observed.

It was demonstrated *in vitro*, using *T. vaginalis* extracts, that metronidazole could be reduced by ferredoxin (Lindmark and Müller, 1976). The reduction of metronidazole by ferredoxin was also shown to occur in cell free extracts of *Clostridium pasteurianum* (Lockerby *et al.*, 1984). The hydrogenase enzyme was also found to indirectly reduce metronidazole (Church *et al.*, 1988). Purified hydrogenase I from *C. pasteurianum* caused reduction of metronidazole when coupled with electron carriers such as ferredoxin or the low redox potential electron carrier dyes such as methyl viologen. A flavodoxin from *C. acetobutylicum* was isolated and characterised based on its ability to activate metronidazole (Santangelo *et al.*, 1991). Flavodoxin is a low redox potential protein which can replace ferredoxin as an electron carrier.

The enzyme pyruvate ferredoxin oxidoreductase (PFOR) has also been implicated in the susceptibility of bacteria to metronidazole. Britz and Wilkinson (1979) found that metronidazole-resistant mutants of *Bacteroides fragilis* had decreased levels of

PFOR. Similarly, PFOR activity was not detected in cell extracts of metronidazole-resistant *Clostridium perfringens* (Sindar *et al.*, 1982). The presence of metronidazole susceptibility amongst anaerobes was also found to correlate to the activity of the PFOR enzyme (Narikawa, 1986).

Thus metronidazole acts as a type of "electron sink" drawing electrons away from the electron transport system components; directly from ferredoxin or PFOR, or indirectly from hydrogenase. Initially it was thought that the electron depletion, that resulted in the inhibition of various pathways, was the key to the mechanism of action of metronidazole (Edwards and Mathison, 1970). However, the interruption of electron flow by metronidazole cannot explain the immediate bactericidal effect of the drug (Church *et al.*, 1991), nor the resumption of hydrogen production after the reduction of all the metronidazole present (O'Brien and Morris, 1972). A later study, however, speculates that the drop in hydrogen production would not be so marked if the electron transport pathway is saturated at the time of drug addition (Lloyd and Kristensen, 1985). The authors suggest that a reduction product of metronidazole - perhaps the nitro radical anion - may directly damage some part of the electron transport pathway between pyruvate and hydrogenase. Whatever the case, it appears that a reduction product of metronidazole causes cell death.

1.2.2.2.2 Other forms of reduction

Other enzymes, that do not constitute part of the electron transfer system have been implicated in the reduction of the nitro group of metronidazole. These enzymes have broadly been referred to as nitroreductases (Chrystal *et al.*, 1980). McLafferty *et al.* (1982) thought that sensitivity to metronidazole was due to the rate of reduction of the nitro group. Similarly, enzymes in *E. coli* that are involved in the reduction of nitrates and chlorates were found to be responsible for reducing metronidazole to its active form (Yeung *et al.*, 1984). The same study showed that

mutant *E. coli* strains with reduced ability to repair DNA (due to *recA* and *uvr* lesions) were more susceptible to metronidazole. It appears that bacterial susceptibility to metronidazole may not only be related to the generation of a reactive reduction product that is cytotoxic, but is also due to the inherent sensitivity of the bacterium to the reactive product.

The authors proposed a model: metronidazole (M) is reduced to form a labile, active product (M^*) which can cause damage. Only when the rate of damage exceeds the rate of DNA repair, will it result in a lethal event. Thus two mechanisms of susceptibility to metronidazole exist, one involving an efficient reductase and the second involving an impaired DNA repair system.

It was initially thought that facultative anaerobes, such as *E. coli*, were not susceptible to metronidazole. However, Prince *et al.* (1969) first noted that under anaerobic conditions, *E. coli* was affected by the drug. A *recA* *E. coli* strain, that was impaired in its DNA repair capacity and susceptible to metronidazole, was mutated for its nitrate reductase, making it slightly less susceptible under anaerobic conditions (Santangelo *et al.*, 1991). This *E. coli* mutant F19 was considered a suitable host for the negative selection of genes that reduced metronidazole and caused cell death.

1.2.2.3 Toxic effects of metronidazole

Metronidazole is relatively inert and nontoxic (Müller, 1983). However, the reduction of the drug, as discussed in the previous section leads to the production of labile, cytotoxic intermediates that cause cellular damage.

There has been some speculation as to how metronidazole interacts with DNA to cause damage. The first indication that metronidazole targeted DNA, showed that

the drug inhibited the incorporation of [^{14}C]-labelled nucleotides into DNA and RNA in *T. vaginalis*, *ie.* it inhibited nucleic acid synthesis. Plant and Edwards (1976) also showed that metronidazole inhibited DNA synthesis, but that RNA synthesis was unaffected. They proposed that nitroimidazoles degraded DNA by a process of strand breakage.

Binding of metronidazole to DNA does not occur if the drug is unreduced (LaRusso *et al.*, 1977). Other binding studies have failed to show that significant amounts of metronidazole bind to DNA (Malliaros and Goldman, 1991). In fact, only a maximum of 3 molecules of metronidazole per 1000-bp of DNA have been shown to bind (Knox *et al.*, 1981). Binding was in proportion to the ionic strength of the buffer indicating that electrostatic charges are probably involved in the process. It is now known that the amount of damage nitroimidazoles cause, is related to the A+T content of the DNA. Therefore, damage is not due to random strand breakage, but rather involves cleavage at specific targets in the DNA (Rowley *et al.*, 1980). Misonidazole, a nitroimidazole, has been shown to specifically cleave thymidine phosphates from DNA. In addition, uridine residues were cleaved and released from RNA indicating that the nucleotide base T is the target, and not the sugar backbone of the DNA (Knox *et al.*, 1981). The relationship between DNA damage and A+T content is an additional factor therefore in the selective toxicity of nitroimidazoles. Pathogenic anaerobes that are susceptible to nitroimidazoles have high A+T contents (*Trichomonas* has 71%, *Clostridium* has 73%) whereas non-susceptible organisms have low A+T contents (*Rhodospirillum* has 38%).

It is thought that the one electron nitro radical ion causes DNA damage (Tocher and Edwards, 1990). The nitro ion becomes protonated and probably takes electrons from DNA, thus oxidising it and causing strand breaks (Edwards, 1993). A proposed mechanism of action of metronidazole, including the oxidative damage

step , is represented diagrammatically in Fig. 1.3. The disproportionation reaction occurs at high rates of reduction and basically "disables" two protonated radicals, causing a reduction in the amount of DNA damage (Zahoor *et al.*, 1987). In the process of abstracting electrons from DNA, the nitroimidazole (in this case misonidazole) reacts directly with the DNA and oxygen is transferred from the nitro group of the misonidazole radical to the DNA sugar damage product (Kappen *et al.*, 1989).

Other forms of damage due to metronidazole have been observed. Ninety-nine percent of *C. pasteurianum* cells were killed within five minutes of drug addition at 10mg/l. However, the cytotoxicity of the reductive products of metronidazole cannot account for such rapid killing. Therefore the authors proposed that cellular lysis may be triggered on metronidazole addition (Church *et al.*, 1991). Levett (1991) also showed that rapid killing of vegetative *Clostridium difficile* cells occurred over a wide range of metronidazole concentrations greater than the minimum inhibitory concentration.

Metronidazole may also alter the surface properties of bacteria. Metronidazole decreased the cell surface charge of *B. fragilis* and made the cell surface hydrophobic, which may facilitate the engulfment of the cell by phagocytes (Cavalcanti *et al.*, 1991). Also, the cells became filamentous and showed a significant loss of cytoplasm. Elongation of *E. coli* upon addition of metronidazole has also been observed (Jackson *et al.*, 1984). DNA damage in the cell would lead to the activation of the DNA repair genes. The SOS repair system has approximately 20 genes associated with it and it is known that nitroimidazoles induce DNA repair (Widdick and Edwards, 1991). DNA repair mutants that are susceptible to UV (ultraviolet) light, are also susceptible to metronidazole, which

suggests that both agents cause similar lesions in bacterial DNA (Yeung *et al.*, 1984).

1.2.2.4 End products

Nontoxic end products such as acetamide and 2-hydroxyethyl oxamic acid are formed following the reduction of metronidazole. These innocuous end products diffuse out of the cell. An additional advantage of the drug, therefore is that the reactive and toxic species are generated inside the cell, which may reduce its toxicity to humans.

1.3 Transfer RNA

The translation of the nucleotide sequence of a gene into the amino acid sequence of a protein is one of the most fundamental processes in living cells. tRNA (transfer RNA) links the two sequences by adapting the nucleotide triplets of a DNA sequence into a sequential aa (amino acid) sequence. Firstly, the tRNA is required to interact specifically with an aminoacyl-tRNA synthetase that charges the tRNA with its cognate aa. Secondly, a specific interaction in the ribosome is required, where the anticodon interacts with the codon triplets of the mRNA at one end, and the amino acids are incorporated into the growing polypeptide chain at the other end, during translation. Detailed information on all aspects of tRNAs and translation are included in Watson *et al.*, (1987) and Lewin, (1987). Therefore only background and details that are relevant to the discovery of two tRNA genes on plasmid pMET10B (Section 3.3.1) are included in this Section.

1.3.1 Structure of tRNA

Transfer RNAs comprise a family of structurally similar molecules. This is because they must all be able to fit into the A, P and E sites of the ribosome and must be able to be recognised by the appropriate translation factors. However, groups of tRNAs that are charged with the same aa (synonymous tRNAs) must be recognised as being different from other groups so that the aminoacylation step can be absolutely specific.

The primary sequences of hundreds of tRNA genes and tRNA molecules are now known from DNA and RNA sequencing information (Sprinzl *et al.*, 1989). Most tRNA sequences can be depicted as a cloverleaf structure (Fig. 1.4) which is maintained by base-pairing between complementary sequences in the stems. The four arms are named according to their functions (Lewin, 1987). The acceptor arm normally ends in the unpaired sequence -CCA which becomes aminoacylated. The

D arm contains the modified base dihydrouridine (ψ) and the anticodon arm contains the anticodon triplet in the centre of the anticodon loop. tRNAs are classed into 2 groups on the basis of an extra arm which is a variable loop: Class I tRNAs include all molecules with a short extra arm of 4 to 5 nucleotides and represent about 75% of all tRNAs (Lewin, 1987), while Class II tRNAs constitute a smaller group of molecules with a larger variable loop of 13 to 21 nucleotides. Some mitochondrial tRNAs, however, do not follow the cloverleaf form and have unusual structures due to the lack of the D or TψC arms.

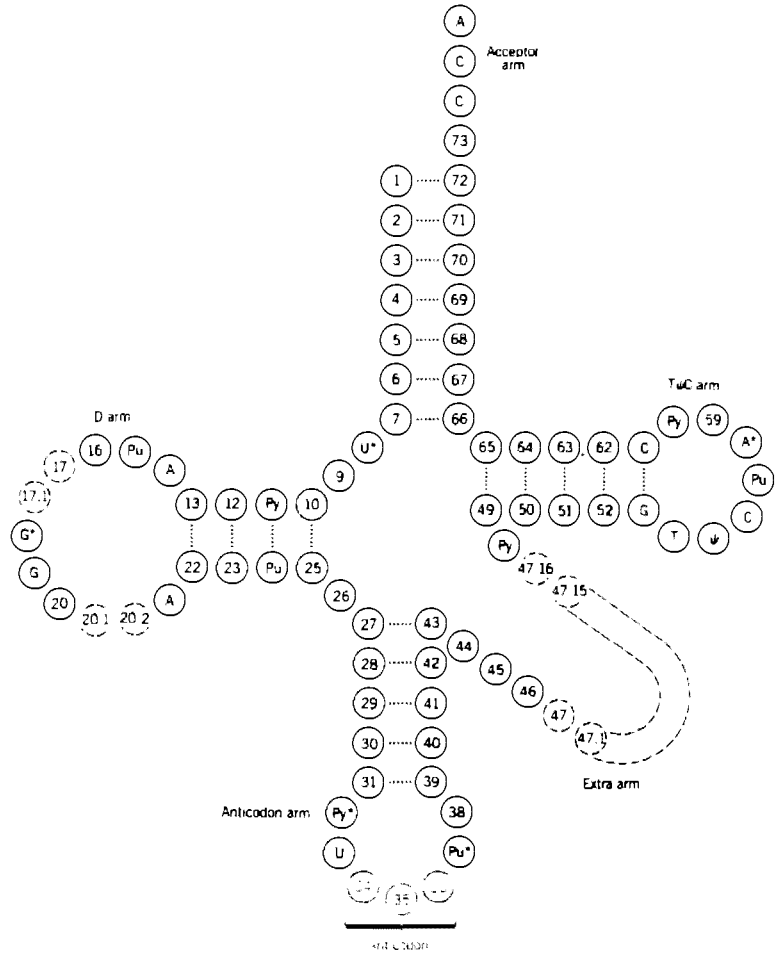


Fig. 1.4 The typical cloverleaf structure of a tRNA molecule. From Lewin (1987).

In *E. coli*, all the tRNA genes sequenced thus far (31 genes) encode the 3' -CCA terminus. In the Gram-positive *B. subtilis*, however, 24% of the tRNA genes sequenced do not encode the -CCA terminus (Komine *et al.*, 1990; Vold, 1985). Most of the clusters of tRNAs found in *B. subtilis* contain a tRNA gene that lacks the -CCA, but the same gene may encode the terminus when it occurs elsewhere on the chromosome, in another cluster. It is not yet clear if tRNA genes lacking the -CCA end can be expressed as functional molecules, but it is thought that the terminus can be added post-transcriptionally (Vold, 1985).

1.3.2 The chromosomal distribution of tRNA genes

An exhaustive survey of tRNA genes on the *E. coli* K12 chromosome was undertaken due to the availability of an ordered clone library of the whole *E. coli* genome (Komine *et al.*, 1990). Seventy-nine tRNA genes were identified by fingerprinting analyses of which 31 were sequenced at the DNA level. A comprehensive map of the tRNA genes on the *E. coli* chromosome indicated that the genes are scattered over the entire genome in 40 different transcription units. A maximum of 7 tRNA genes were contained in one such unit (Komine *et al.*, 1990).

The tRNA gene organisation in *B. subtilis* differs from that found in *E. coli*, because the genes are clustered in large transcriptional units and are closely linked to rRNA (ribosomal RNA) operons. Until gene organisation in other prokaryotes is investigated one can only speculate as to whether this type of organisation is typical of Gram-positive organisms or specific for *B. subtilis* (Vold, 1985). Ten *Eco*RI fragments of the *B. subtilis* chromosome were shown to hybridize to labelled tRNA (Wawrousek and Hansen, 1983). Seven of these fragments containing tRNA gene clusters have been sequenced and they have been found to contain a minimum of 2 or a maximum of 21 tRNA genes in each cluster. All but one are associated with rRNA operons and are named after the preceding rRNA gene set (Vold, 1985).

One cluster of 4 tRNA genes (*trnY*) is not associated with rRNA genes (Yamada *et al.*, 1983). At least two other *Eco*RI fragments that are unmapped contain at least 7 tRNA genes, but no rRNA genes (Rudner *et al.*, 1993).

In both *E. coli* and *B. subtilis*, many of the tRNA genes are represented more than once. This duplication of genes may occur in different operons, as in *B. subtilis*, or in the same operon, as in *E. coli*. It appears that the gene copy number for a single tRNA species does not exceed four (Komine *et al.*, 1990). *Pseudomonas aeruginosa* also contains duplicate copies of the same tRNA gene in one operon (Bally *et al.*, 1993).

It had been suggested that multiple copies of tRNA genes may be necessary to maintain the correct amount of tRNA in a cell. However, the differences in gene copy number between major and minor tRNA species cannot account for the large differences observed in the abundance of major and minor tRNAs (Emillson *et al.*, 1993). The location of tRNA genes relative to the *ori*C was thought to affect the tRNA gene dosage because the chromosomal location of rRNA operons has been shown to cause their differential expression (Condon *et al.*, 1992). tRNA genes that were close to the *ori*C and replicated early, therefore, would have an increased copy number relative to late genes. Although some tRNAs that recognise major codons are situated near the *ori*C (Komine *et al.*, 1990), others were found to be located on the opposite side of the chromosome (Emillson *et al.*, 1993). Therefore gene dosage effects and chromosomal location do not seem to affect tRNA abundance.

Amino acids that are represented by more than one synonymous codon are not represented by all the codons equally. Instead a coding bias operates in most organisms. It is known that in highly expressed genes there is a bias towards the selection of major codons which may be as much as 100-fold higher than the

selection of other minor codons (Andersson and Kurland, 1990). Experiments have revealed that the occurrence of major codons in *E. coli* mRNA (messenger RNA) is related to the relative abundance of their cognate tRNAs (Ikemura *et al.*, 1981). This bias in codon usage and in the tRNA profile of synonymous codons is thought to maximize growth rates by the efficient translation of the mRNA. Evolution would select for the use of major codons in rapidly expressed genes, but not for major codons in genes expressed at lower levels, thus causing the appearance of minor codons. In *E. coli*, the accumulation of tRNA species was tested. In accordance with the growth rate theory, tRNAs for major codons increased in abundance as the growth rate increased. Three minor tRNAs, however, did not obey the rules: they also increased in abundance as the growth rate increased (Emillson *et al.*, 1993). Either the proposed model is incorrect, or perhaps the tRNAs may be involved in processes other than translation, for example, in regulation.

1.3.3 Transfer RNAs and development

In a number of cases it has been proposed that a particular tRNA may play a role in the regulation of development of certain bacteria. This will now be discussed with reference to *Streptomyces coelicolor* and *C. acetobutylicum.* Lastly, the possible role of tRNAs in the differentiation of *B. subtilis* will be discussed.

In *S. coelicolor*, *bldA* mutants are defective in antibiotic production and in the development of aerial hyphae and spores, while being unimpaired in vegetative growth (Lawlor *et al.*, 1987). Five *bldA* mutations were all found to be due to single base changes within a $tRNA^{Leu}$ TTA that possesses the anticodon UAA. *Streptomyces* spp. have an unusually high G+C base composition (typically 73%) and therefore, this codon is rare in *Streptomyces* genes. Its rarity is biased though, as most of the genes that contain this codon specify regulatory or resistance proteins

associated with antibiotic production (Leskiw *et al.*, 1991). It has been shown that processing of this primary tRNA transcript in young cultures is inefficient, but that processed $tRNA^{Leu}$ TTA's increase in abundance as the culture aged (Leskiw *et al.*, 1993). This temporal regulation of $tRNA^{Leu}$ TTA may point to a regulatory role for the molecule in differentiation and antibiotic production.

An analagous situation has been described for a $tRNA^{Thr}$ ACG in *C. acetobutylicum* (Sauer and Dürre, 1992). A transposon-induced insertion near this tRNA gene resulted in a mutant defective in acetone and butanol formation. Solvent production in *C. acetobutylicum*, like antibiotic production in *Streptomyces*, is associated with the onset of secondary metabolism and is strictly regulated. The ACG codon is also rare and is found exclusively in genes expressed at the end of the exponential growth phase, or in genes involved in the uptake and metabolism of alternative or minor nitrogen and carbon sources (Sauer and Dürre, 1992). However, levels of $tRNA^{Thr}$ ACG during acidogenesis and solventogenesis were not found to be different. But, the authors proposed that if temporal regulation of the processing of the primary transcript occurred, it may provide a mechanism whereby this rare tRNA may induce a switch to solventogenesis.

In *B. subtilis*, sporulation represents a second stage in the cellular development of the bacterium. It has been suggested that tRNA gene clustering in *B. subtilis* might be related to the role tRNAs play in development (Rudner *et al.*, 1993). During spore germination and outgrowth, large amounts of tRNA and rRNA are needed due to the rapid activation of the translation machinery. The clustering of the genes may enable their coordinate transcription and processing. The regulatory role that tRNAs may play in the sporulation process has also been investigated. Conflicting reports as to whether the amounts of certain tRNAs change during the different stages of development, have been published (Vold, 1985; Henner and Steinberg,

1979). Rather, it is thought that the alteration might be at the level of post-transcriptional modification of the anticodon loop (Vold, 1978). Therefore, in *S. coelicolor*, *C. acetobutylicum* and *B. subtilis*, tRNAs may play a role in regulating the onset of sporulation and secondary metabolism.

1.3.4 Other functions for tRNAs

Other functions for tRNAs have also been described. In *E. coli*, a serine tRNA has been shown to be involved either in the biosynthesis of certain proteins, or in the regulation of cell division (Tamura *et al.*, 1984). In *E. coli*, tRNAs are also known to be involved in regulating the expression of the *trp*, *his*, *phe* and *ilvGMEDA* biosynthetic operons (Landick and Yanofsky, 1987). In the *trp* operon, charged $tRNA^{Trp}$ acts as an effector molecule that signals the presence of adequate supplies of a particular aa. However, when a ribosome pauses at one of two tryptophan codons in the mRNA leader peptide coding region, due to the low availability of charged $tRNA^{Trp}$, a transcription antiterminator is formed and the biosynthetic operon is transcribed. In *B. subtilis*, tRNA sythetase genes are regulated by a common transcription antitermination mechanism (Grundy and Henkin, 1993). Yet, each gene responds individually to the low availability of the cognate aa. The authors propose that uncharged $tRNA^{Tyr}$ acts as a positive regulator for the transcription of *tyrS*, the tyrosyl-tRNA synthetase gene. The uncharged tRNA could interact with the leader mRNA, which contains one tyrosine codon. This interaction could alter the terminator and cause readthrough of the *tyrS* gene. Therefore, both charged and uncharged tRNAs have been shown to act as effector molecules that affect transcription by interaction with mRNA.

1.4 Aims and overview of this thesis

C. acetobutylicum P262 is an industrially important bacterium that has been used in the past for the production of solvents via the fermentation of molasses. During the past 30 years in Europe and in the US and during the last 10 years in South Africa, no industrial fermentation of solvents using *C. acetobutylicum* has taken place due to economic constraints. However, the future genetic manipulation of genes that may be involved in solvent metabolism in *C. acetobutylicum* may improve the commercial viability of the process by increasing solvent yields. To this end, a system using the drug metronidazole was developed for the cloning of *C. acetobutylicum* electron transport genes that may play a role in its solvent-producing pathways. This was based on the observation that the electron distribution and flow in the cell is largely mediated by electron transport genes which also largely control the carbon flow *ie.* the ratios of the different end-products produced by the branched fermentation pathways. The aim of the study reported in this thesis was to characterize pMET10B, a clone containing *C. acetobutylicum* P262 DNA, which conferred a metronidazole sensitivity phenotype on an *E. coli* mutant strain, in the hope of identifying genes involved in the electron transport pathways. The theory behind the cloning system using metronidazole as a tool, involved the reduction of metronidazole by electron transport genes encoded by recombinant *C. acetobutylicum* P262 DNA, thus causing sensitivity of the host *E. coli* to the reduced, toxic intermediates of the drug. The proposed strategy of this thesis involved subcloning of pMET10B and protein and physiological studies of pMET10B in an attempt to identify the region responsible for the metronidazole sensitivity. Exonuclease III shortening of DNA constructs would enable the precise location of the metronidazole sensitivity region responsible for the phenotype. Nucleotide sequencing would enable the further characterization of the metronidazole sensitive area and may reveal elements responsible for the phenotype.

Chapter Two

The molecular characterization of plasmid pMET10B

Chapter Two

The molecular characterization of plasmid pMET10B

2.1 Introduction

The drug metronidazole has been used as a tool for the isolation of electron transport genes that may influence the production of solvents in *C. acetobutylicum* (Santangelo *et al.*, 1991). Metronidazole (Flagyl®) is active against a wide range of anaerobic organisms and is thought to exert its effect via the reduction of its nitro group resulting in an activated, cytotoxic compound (Edwards, 1993). Metronidazole is also active against the facultative anaerobe, *E. coli*, and is especially toxic to those strains that are mutated in their DNA repair capacity (Yeung *et al.*, 1984). *Escherichia coli* CC118, a *recA* strain, was mutated via transposon mutagenesis to be more resistant to metronidazole (Santangelo *et al.*, 1991). This *E. coli* F19 mutant was thought to be more resistant to metronidazole due to a decrease in its nitroreductase activity which would result in decreased concentrations of the reduced, cytotoxic drug inside the bacterium. The mutant was considered to be a suitable host for the cloning of electron transfer genes which would theoretically reduce and activate metronidazole, rendering the mutant more sensitive to the drug. Twenty-five recombinant plasmids that contained *C. acetobutylicum* P262 DNA cloned into pEcoR251 were found to make *E. coli* F19 sensitive to metronidazole under anaerobic conditions, presumably due to the reductive ability of the *C. acetobutylicum* insert DNA. One such clone, pMET13A, was found to encode a flavodoxin gene and a hydrogenase gene, both of which are involved in electron transfer systems in *C. acetobutylicum* (Santangelo *et al.*, 1991). A second clone, pMET10B, was therefore characterized in the hope of identifying further such genes. Metronidazole sensitivity assays using the *E. coli* mutant F19 were used to localize the region responsible for the metronidazole sensitivity

phenotype on different subclones and exonuclease III-shortened clones. DNA hybridization and protein analysis were also carried out to further characterize the clone.

2.2 Materials and methods

Standard materials and methods are recorded and referenced in the Appendix. All restriction enzymes were obtained from Boehringer Mannheim (South Africa), Johannesburg, except for *Apo*I which was obtained from New England Biolabs, Beverly, Massachusetts.

2.1.1 Bacterial strains and plasmids

Clostridium acetobutylicum strain P262 has been described by Allcock *et al.* (1982) and was used for industrial fermentation by National Chemical Products, Germiston, South Africa. *E. coli* strains used for all manipulations and for metronidazole sensitivity tests are presented in Table 2.1. Plasmid pMET10B contains *C. acetobutylicum* P262 DNA cloned into the *Bgl*II restriction endonuclease site of the cloning vector pEcoR251 (Fig. 2.1 A) and was obtained from J. Santangelo (Santangelo *et al.*, 1991). The plasmids Bluescript pKS and pSK (Stratagene, San Diego, California) were used for exonuclease III shortening and nucleotide sequencing (Section 3.3.1). The plasmids contain a large polylinker with 26 unique restriction enzyme recognition sites in the N-terminal portion of the *lacz* gene (Bluescript Instruction Manual, Stratagene). Insertion into the *lacz* gene results in a white *E. coli* colony, while no insertion into the polylinker enables the normal production of the N-terminal LacZ polypeptide, which complements the LacZΔM15 polypeptide produced from the appropriate bacterial strain resulting in a blue colony when plated on YT plates containing X-gal (Appendix). The

polylinker in these vectors also consists of unique 3' restriction sites on the outside edge of the polylinker and unique 5' sites and blunt sites internally. This enables the generation of nested deletions of the insert DNA following exonuclease III treatment of digested plasmid DNA, as discussed in Section 2.2.2.

E. coli strain	Genotype	Reference or Origin
JM105	*thi, ppsL, endA, sbcB*15 *hspR*4, Δ*(lac-proAB)* *[F', traD*36, *proAB, lacI*^{q}*ZΔM*15]	Yanish-Perron *et al.*, 1985
JM109	*recA*1, *endA*1, *gyrA*96, *thi, hsdR*17 *supE*44, *relA*1, λ$^-$, Δ*(lac-proAB)* *[F', traD*36, *proAB, lacI*^{q}*ZΔM*15]	Yanish-Perron *et al.*, 1985
AB1157	*rac*$^-$, *ara, argE*, Δ*(gpt-proA)*62, *galK, hisG, kdgK, lacY*1, *leuB*6, *mgl*51, *mtl*1 *qsr, rfbD1, rpsL, sup*E44, *thi*1, *thr*1 *tsx*33, *xyl*5	ATCC 29055
CC118	*recA1, araD*139, Δ*(ara-leu)*7697 Δ*lacX*74, *galE, galK, thi, rpsE* *rpoB, argEam*	Manoil and Beckwith, 1985
F19	*ntr, phoA* del20, otherwise as CC118	Santangelo *et al.*, 1991

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

The plasmids, pKS and pSK, also contain a T7 promoter which can be used for *in vivo* transcription/translation of the cloned insert DNA (see Section 2.2.4.2). Plasmid pGP1-2, used in the T7 RNA polymerase expression system was a gift from G.U. Dachs (Microbiology Dept., UCT).

2.2.2 DNA manipulation of pMET10B

Plasmid DNA was routinely prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981). Both small-scale isolation (miniprep) and large-scale isolation (maxiprep) of DNA are detailed in the Appendix. A 1.86kb *Hin*dIII/*Pst*I *C. acetobutylicum* restriction DNA fragment from pMET10B was subcloned into the *Hin*dIII/*Pst*I restriction endonuclease sites of Bluescript pKS and pSK (Fig. 2.1 B). These subclones were subsequently shortened using restriction exonuclease III according to the method of Henikoff (1984). Subclones were digested to completion with endonuclease *Sac*I that generates a 3' overhang which is not susceptible to the enzyme exonuclease III, and endonuclease *BamH*I that generates a 5' overhang which is susceptible to digestion by the enzyme. Exonuclease III digestion produced a range of unidirectionally-shortened nested deletions for phenotype testing and nucleotide sequencing (Fig. 2.1 C).

2.2.3 Metronidazole sensitivity assays

An aerobically grown overnight culture of *E. coli* F19 or *E. coli* CC118 was diluted to approximately 10^3 to 10^4 cells per ml in sterile water. Subsequent steps were performed in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio) under stringent anaerobic conditions in an oxygen-free atmosphere of N_2, CO_2 and H_2 (85 : 10 : 5). Aliquots of 100µl of the dilutions were plated onto prereduced YT (yeast tryptone) plates (Sambrook *et al.*, 1989) containing 0.2% (w/v) $NaNO_3$ and

0.1% (w/v) glucose, 100μg/ml ampicillin and different concentrations of metronidazole. The plates were then incubated anaerobically at 34 °C for 2 days and the MIC (minimum inhibitory concentration) of metronidazole was determined. The MIC was defined as the lowest concentration of metronidazole (μg/ml) that completely inhibited growth of *E. coli* after 2 days at 34 °C.

2.2.4 Protein analysis

2.2.4.1 Cell-free coupled transcription-translation system

The Promega prokaryotic DNA-directed translation kit (No. L4500) was used according to the manufacturer's instructions. Plasmid DNA from a range of exonuclease III-shortened clones was used in an attempt to correlate protein expression with the metronidazole sensitivity phenotype observed. [^{35}S]-methionine-labelled proteins were separated on a discontinuous SDS-page system (Laemmli, 1970) and viewed by autoradiography.

2.2.4.2 T7 RNA polymerase expression system

The method was adapted from Tabor and Richardson (1985) and Scholz *et al.* (1989). Two compatible plasmids were used in this expression system. The first plasmid was the kanamycin-resistant pGP1-2 which expressed the T7 RNA polymerase under the control of the inducible λP_L promoter and the heat sensitive cI*857* repressor. The second plasmid, Bluescript pSK or pKS, contained the cloned insert to be expressed under control of the T7 RNA polymerase promoter. Exclusive expression of the insert occurred after heat induction of the T7 RNA polymerase, while transcription by the *E. coli* RNA polymerase was shut off by the addition of rifampicin. [^{35}S]-methionine was used to pulse-label the plasmid-encoded proteins. Strains *E. coli* F19 or CC118 were not suitable for this

experiment since they are *rpoB* (or *rif*), (Bachman, 1987) and therefore their RNA polymerase cannot be shut down by the addition of rifampicin. *E. coli* AB1157, however, is sensitive to rifampicin and was therefore suitable. Experimental details are contained in the Appendix.

2.2.5 Growth curve analysis

A standard growth curve was determined for *E. coli* JM105 transformed with an exonuclease III-shortened clone (pKS2) that displayed the metronidazole sensitivity phenotype in *E. coli* F19, compared to a clone with larger deletions (pKS4) that had lost the ability to make *E. coli* F19 sensitive to metronidazole. This was in order to confirm that the observed sensitivity was not due to a growth deficiency that the insert DNA had conferred on *E. coli*. The growth of these *E. coli* strains in LB (Luria-Bertani broth), (Sambrook *et al.*, 1989) containing ampicillin (100 μg/ml) was monitored over an 11 h period. At time intervals, the OD_{600} was recorded and diluted cells were plated onto YT agar containing 100 μg/ml ampicillin. Results generated are the average of three such experiments.

2.2.6 DNA hybridization

In order to confirm the origin of the *C. acetobutylicum* P262 insert DNA of clone pMET10B, DNA hybridization was performed according to Southern (1975). Chromosomal DNA from *E. coli* JM105 and *C. acetobutylicum* P262 were a gift from F. Lin (Department of Microbiology, UCT). The *C. acetobutylicum* P262 chromosomal DNA was prepared according to the method of Zappe *et al.* (1986) which compensates for the large amount of nucleases produced by the bacterium. *E. coli* JM105 chromosomal DNA was prepared according to standard methods (Sambrook *et al.*, 1989). All DNA was digested to completion with the appropriate restriction endonucleases. The DNA fragments were separated on a 0.8% agarose

gel and transferred by capillary blotting onto Hybond N^+ (Amersham International, Amersham, UK) according to the manufacturer's instructions.

The *C. acetobutylicum* P262 insert DNA of pKS1, was purified by restriction endonuclease digestion, gel electrophoresis, electroelution of the DNA fragment and phenol-cleaning, to prepare it for use as a probe. The DNA fragment was non-radioactively labelled with digoxigenin using the random-priming method. DNA labelling and hybridization were according to the manufacturer's instructions that accompanied the Nonradioactive DNA Labelling and Detection Kit (No. 1093 657, Boehringer Mannheim (South Africa), Johannesburg). Detection of the hybridized probe was via chemiluminescence using the AMPPD® substrate (No. 1357 328, Boehringer Mannheim (SA)). All practical details concerning nonradioactive hybridization are contained in the Appendix.

2.3 Results

2.3.1 DNA manipulation of pMET10B

Subcloning of the *C. acetobutylicum* P262 insert DNA from pMET10B into Bluescript pKS and pSK was required for nucleotide sequencing. Initial attempts at subcloning were unsuccessful; for instance, subcloning the entire 2.56kb *Bgl*II/*Bgl*II insert into the *BamH*I restriction endonuclease site of Bluescript pKS resulted in a subclone that had lost the metronidazole sensitivity phenotype as determined by metronidazole sensitivity assays. Restriction endonuclease digestion and gel electrophoresis of this subclone showed that the *C. acetobutylicum* P262 insert DNA had one of two *Sty*I restriction endonuclease sites deleted and the insert DNA deleted by 100-bp. The rearrangement of the insert DNA was thought to be related to the loss of the metronidazole sensitivity phenotype. However, a 1.86kb *Hin*dIII/*Pst*I restriction endonuclease fragment of *C. acetobutylicum* P262 insert DNA from pMET10B was

stably subcloned into the *Hin*dIII/*Pst*I restriction sites of Bluescript pKS and pSK to yield subclones pKS10B and pSK10B, respectively (Fig. 2.1 A and B). Both subclones rendered *E. coli* F19 sensitive to metronidazole (see Section 2.3.2). These subclones were shortened using exonuclease III to generate a range of nested deletions, some of which were used in metronidazole sensitivity tests and for nucleotide sequencing (Fig. 2.1 C).

2.3.2 Metronidazole sensitivity assays

Metronidazole sensitivity assays were routinely performed to test the metronidazole MIC (minimum inhibitory concentration) of *E. coli* F19 transformed with the various DNA constructs. Results were recorded as the average of at least three experiments. The MIC, under conditions of anaerobic respiration, for strain *E. coli* F19 was previously shown to be 35 μg/ml, while the MIC of strain *E. coli* CC118 was 20 μg/ml (Santangelo *et al.*, 1991). *E. coli* F19 showed an increase in sensitivity to metronidazole (MIC = 15 μg/ml) when transformed with pKS10B and pSK10B, compared to a control pKS plasmid (MIC = 35 μg/ml). This indicated that the *C. acetobutylicum* P262 locus responsible for the phenotype had been subcloned into another vector without any loss of activity. Exonuclease III-shortened clones of pKS10B and pSK10B were also tested for their ability to render *E. coli* F19 sensitive to metronidazole (Fig. 2.1 C). Exonuclease III-shortened clones of the pKS10B subclone (pKS1 to pKS5) showed a concomitant loss of activity as the insert DNA was progressively deleted. Exonuclease III-shortened clones of the pSK10B subclone (pSK1 to pSK4) showed a partial loss of activity on progressive deletion of the insert DNA.

Metronidazole sensitivity assays were also carried out using the *E. coli* F19 parental strain *E. coli* CC118, in order to check that the sensitivity phenotype in *E. coli* F19 was not due to a side effect of the transposon-mutagenesis of *E. coli* CC118. The

MIC of *E. coli* CC118 plated on metronidazole was lower, as expected, due to an increase in the nitroreductase activity of the strain. *E. coli* CC118 was still sensitive to metronidazole (MIC = 10 μg/ml) when transformed with pKS2, an exonuclease III-shortened clone that conferred the phenotype on *E. coli* F19 , compared with a pKS control (MIC = 20 μg/ml).

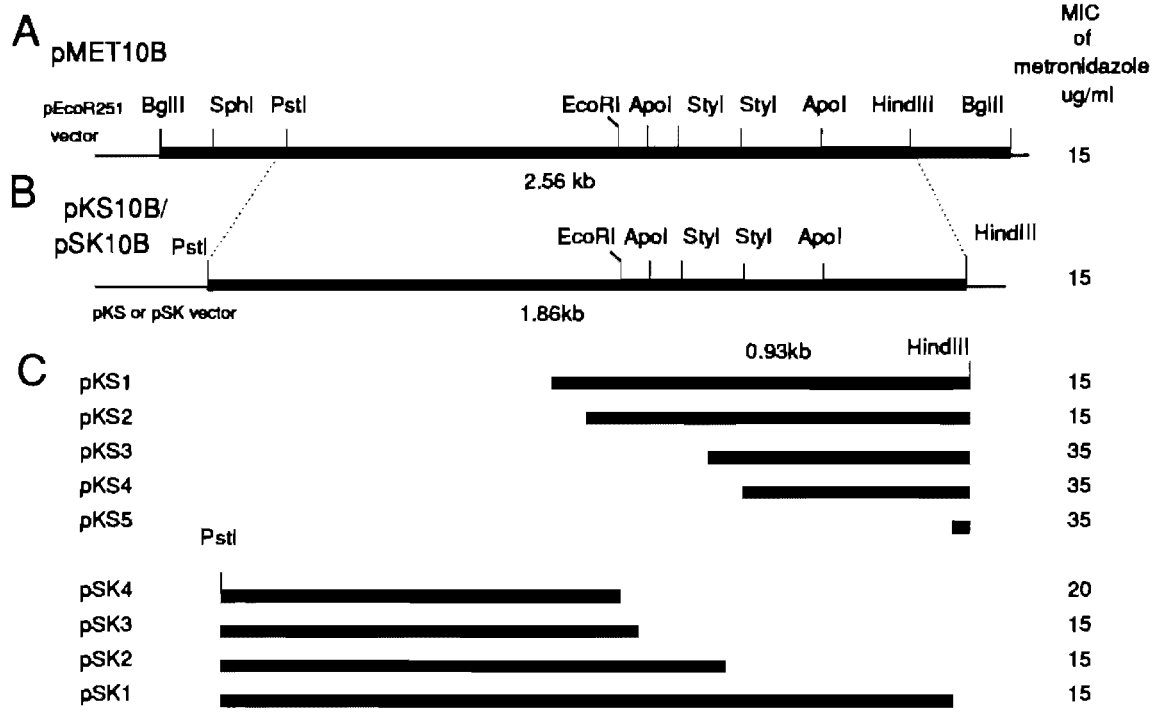


Fig. 2.1 Schematic diagram of a selection of the DNA constructs and the exonuclease III-shortened clones generated in this study. The corresponding MIC (minimum inhibitory concentration) of metronidazole for each construct in *E. coli* F19 is shown. Thick lines correspond to *C. acetobutylicum* P262 DNA and thin lines correspond to vector DNA.
A. Diagram of pMET10B which contains 2.56kb of *C. acetobutylicum* P262 insert DNA cloned into the *Bgl*II restriction site of pEcoR251.
B. Diagram of a 1.86kb *Hin*dIII/*Pst*I restriction fragment of *C. acetobutylicum* P262 insert DNA from pMET10B cloned into the *Hin*dIII/*Pst*I restriction sites of pKS and pSK, generating pKS10B and pSK10B, respectively.
C. Diagram of a range of exonuclease III-shortened clones generated from pKS10B and pSK10B.

2.3.3 Protein analysis

In vitro cell-free transcription/translation experiments were carried out using a range of exonuclease III-shortened clones to establish whether a protein was produced from the *C. acetobutylicum* P262 insert DNA of pKS1. First, the pSK10B exonuclease III-shortened clones were used in a transcription/translation experiment (Fig. 2.2). Lane 1 contained proteins expressed from pSK4 that did not confer the metronidazole sensitivity phenotype on *E. coli* F19. Lane 3 represented the pSK control and lanes 2, 4 and 5 represented proteins from pSK3, pSK2 and pSK1, respectively, all of which increased the sensitivity of *E. coli* F19 to metronidazole. No difference in the protein banding pattern could be detected between lane 1 and lanes 2, 4 and 5. This indicated that the production of a specific protein could not be correlated with the metronidazole sensitivity phenotype conferred by these constructs.

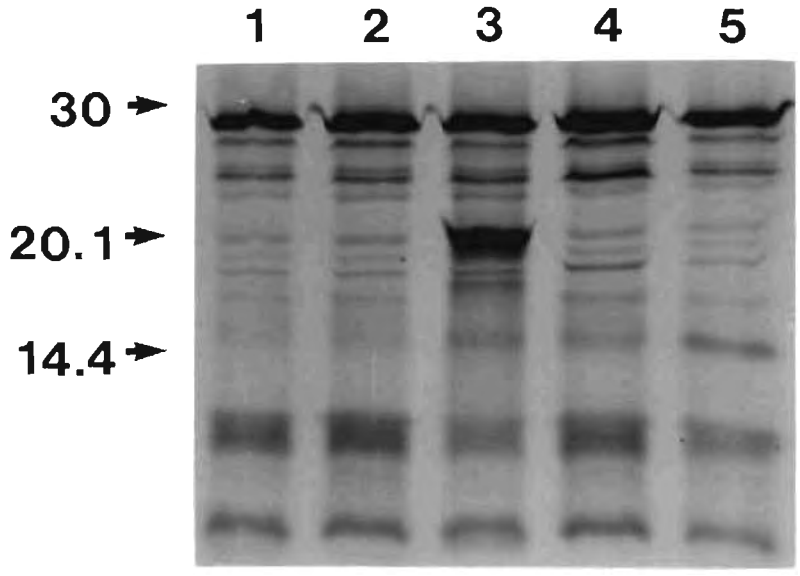


Fig. 2.2 *In vitro* transcription/translation experiment of the pSK constructs. Plasmid pSK4, that does not confer the metronidazole sensitivity phenotype on *E. coli* F19, was used for protein production in lane 1. Lanes 2, 4 and 5 correspond to constructs pSK3, pSK2 and pSK1, respectively, all of which confer the metronidazole sensitivity phenotype on *E. coli* F19. Proteins from the control plasmid pSK are shown in lane 3. Numbering indicates the positions and the sizes of molecular weight markers in kDa.

Four of the pKS10B exonuclease III-shortened clones were used in a similar experiment (Fig. 2.3). Lane 1 contained proteins produced from the control plasmid pKS. Lane 2 contained proteins expressed from the pKS2 construct which made *E. coli* F19 sensitive to metronidazole. Lanes 3 to 5 contained proteins expressed from pKS3, pKS4 and pKS5, respectively, all of which had lost the ability to confer the metronidazole sensitivity phenotype. An overproduced protein band with an apparent molecular weight of approximately 15 kDa was visible in lanes 2 and 3. However, it appeared to be unrelated to the phenotype since it was produced from pKS2, which conferred the phenotype and from pKS3 which had lost the ability to confer the phenotype on *E. coli* F19. To conclude, the pKS exonuclease III-shortened clones did not produce a protein band that correlated to the ability of the pKS constructs to confer the metronidazole sensitivity phenotype on *E. coli* F19. Although the prominent protein band in lane 2 of Fig. 2.3 was unrelated to the sensitivity phenotype, we speculated that it was the product of a fusion protein coded for partially by the pKS vector DNA and partially by the *C. acetobutylicum* P262 insert DNA. An ATG start codon located after the *lac* promoter in pKS (Stratagene Bluescript Instruction Manual) may have represented the site of translation initiation on the corresponding mRNA and a stop codon located in the *C. acetobutylicum* P262 insert DNA may have caused the termination of translation. Nucleotide sequencing (see Section 3.2.3) showed that a suitable stop codon was located at positions 670-672 in the insert DNA on the non-coding strand of pKS1. The vector would have contributed 92 nucleotides and the insert DNA 264 nucleotides to produce a fusion transcript of 356 nucleotides in length. A putative fusion protein would have been approximately 118 amino acids in length which corresponded to an apparent molecular weight of 13.4 kDa. This theoretical fusion protein may correspond to the overproduced protein band seen in Fig. 2.3, since the smallest exonuclease III-shortened clone, pKS5, had the stop codon deleted and did not overproduce the protein.

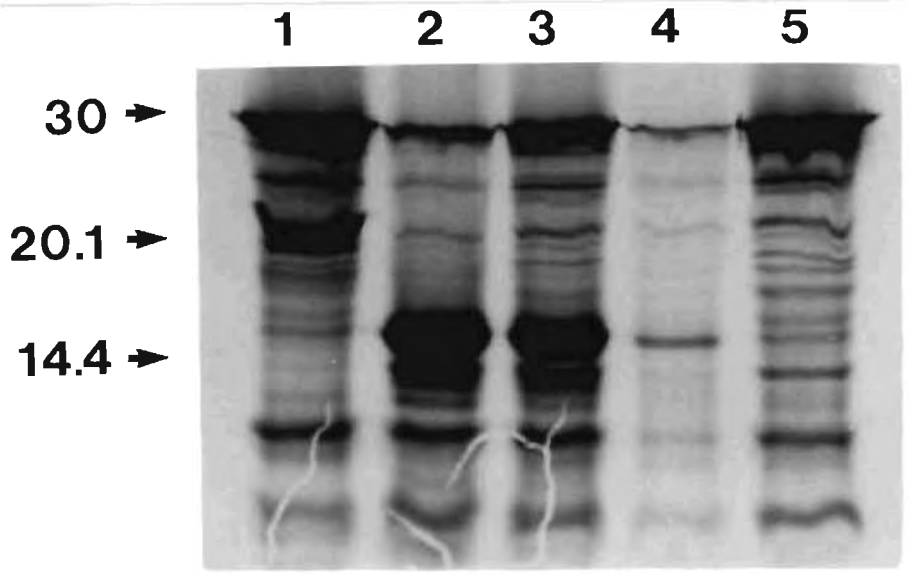


Fig. 2.3 *In vitro* transcription/translation experiment of the pKS constructs. Proteins from the control plasmid, pKS are shown in lane 1. Plasmid pKS2 that rendered *E. coli* F19 sensitive to metronidazole was used for protein production in lane 2. Proteins produced from constructs pKS3, pKS4 and pKS5 that did not confer the phenotype are shown in lanes 3, 4 and 5, respectively. Numbering indicates the positions and sizes of molecular weight markers in kDa.

Thus, no protein band was observed from both *in vitro* transcription/translation experiments that corresponded to the ability of the metronidazole sensitivity phenotype to be conferred on *E. coli* F19 by the *C. acetobutylicum* P262 DNA.

A second *in vivo* protein expression system was used that employed T7 RNA polymerase for the exclusive transcription of insert DNA (Tabor and Richardson, 1985). This method was previously reported to reveal a protein product after initial *in vitro* transcription/translation experiments had failed to do so (Wehnert *et al.*, 1992). The unshortened subclone pKS10B was transformed into *E. coli* AB1157 with pGP1-2 that expresses the T7 RNA polymerase (see Section 2.2.4.2). The control bacterium was transformed with Bluescript pKS. Fig. 2.4 illustrates that no

differences in protein expression were detected for the plasmid pKS10B (lane 2) that conferred the metronidazole sensitivity phenotype on *E. coli* F19, compared to the control plasmid pKS (lane 1).

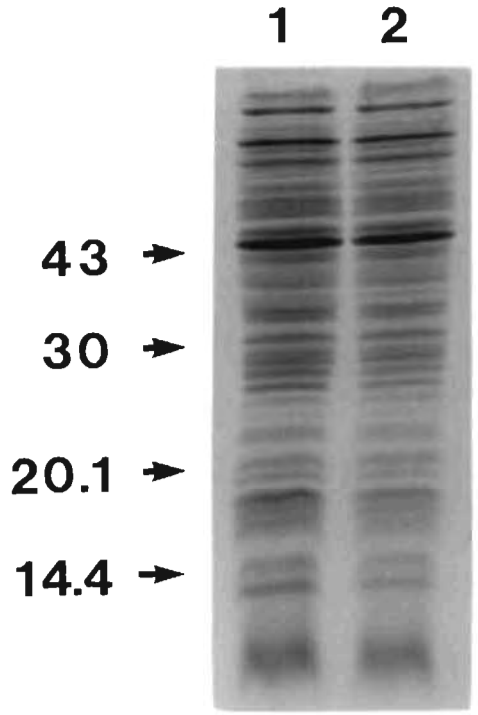


Fig. 2.4 T7 promoter-directed expression of plasmid proteins in *E. coli* AB1157. Lane 1 shows proteins produced from the control plasmid pKS. Lane 2 shows proteins produced from the subclone pKS10B that rendered *E. coli* F19 sensitive to metronidazole. Numbers show the positions of molecular weight markers in kDa.

2.3.4 Growth curve analysis

A growth curve of *E. coli* JM105 transformed with pKS2 that rendered *E. coli* F19 susceptible to metronidazole, and *E. coli* JM105 transformed with pKS4 that had lost the ability to make the bacterium susceptible, was determined over an 11 h period (Fig. 2.5 A). Both *E. coli* (pKS2) and *E. coli* (pKS4) followed a standard growth curve (Fig. 2.5 A) and entered stationary phase at approximately the same optical density reading of A_{600}= 1.3. *E. coli* (pKS2) had a doubling time of 50 min while *E. coli* (pSK4) had a doubling time of 44 min. The only observed difference was that *E. coli* (pKS2) had a longer lag time of 1 h, but the growth characteristics of *E. coli* (pKS2) were that of a healthy culture and the construct did not cause any substantial alteration in the growth curve under normal conditions. A graph of viable bacterial cell number versus time (Fig. 2.5 B) showed that both cultures reached a viable cell count of greater than 10^8 cells per ml, but that *E. coli* (pKS2) took longer to reach this cell density. It can also be seen that the experimental culture contained 10-fold fewer viable cells than the control culture at the 11 h time point in the growth curve. The reduced viability of the *E. coli* cells was due to the presence of the additional insert DNA that pKS2 contained. This will be discussed further in Section 2.4.

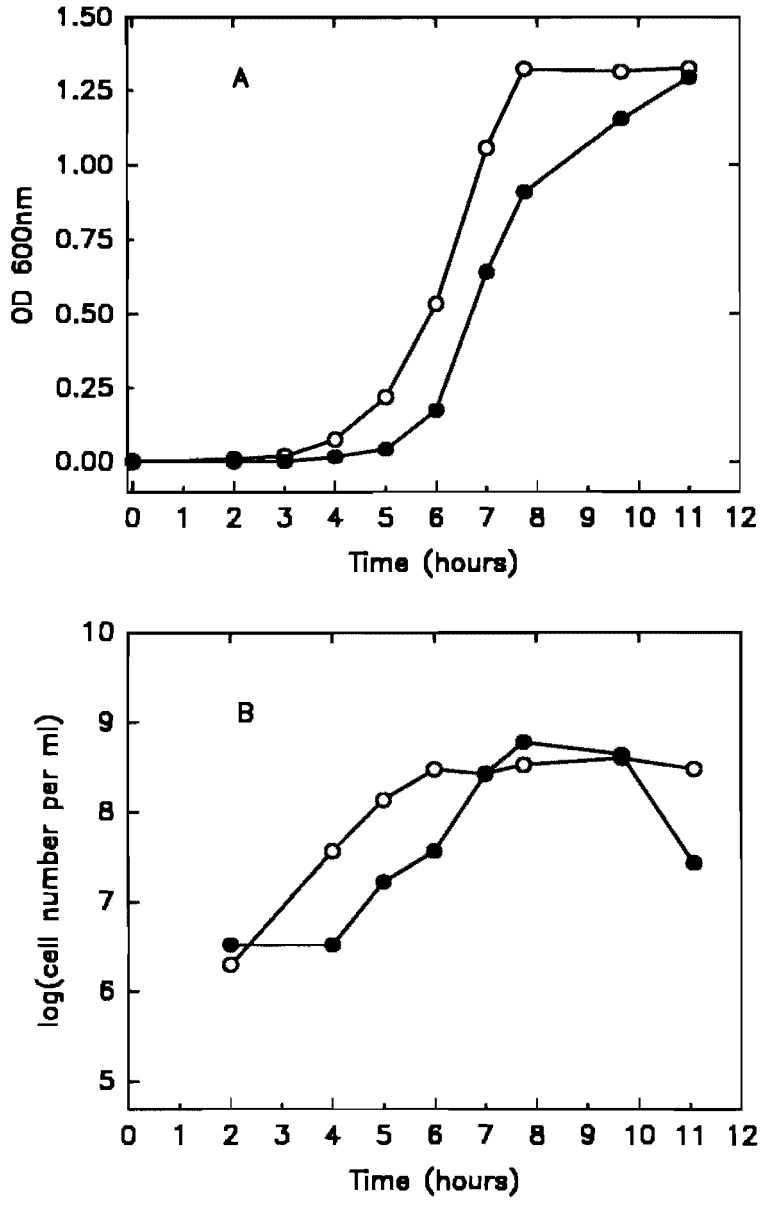


Fig. 2.5 Standard growth curves of two *E. coli* JM105 cultures. Lines with open circles denote the *E. coli* control transformed with pKS4 that had lost the ability to make *E. coli* F19 sensitive to metronidazole. Filled circles represent *E. coli* transformed with pKS2 that rendered *E. coli* F19 sensitive to metronidazole. Results show the average of three experiments.
A. Graph of the absorbance (OD 600nm) versus time (h).
B. Graph of the log of the viable *E. coli* JM105 cell numbers versus time (h).

2.3.5 DNA hybridization

The origin of the insert DNA of pMET10B was confirmed by DNA hybridization (Fig. 2.6).

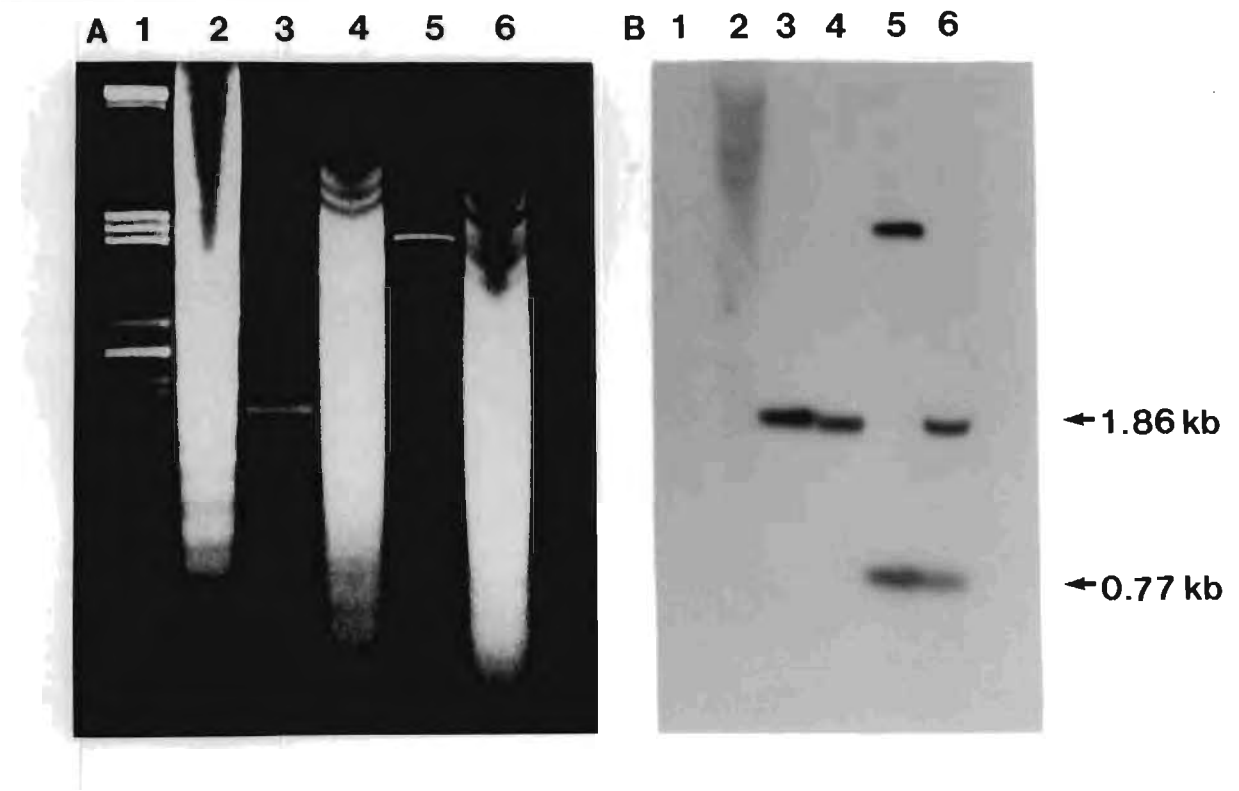


Fig. 2.6 Gel electrophoresis and corresponding DNA hybridization to confirm the origin of the insert DNA of pKS10B.
A. Gel electrophoresis of *E. coli* chromosomal DNA (lane 2) and *C. acetobutylicum* P262 chromosomal DNA (lane 4) digested with *Hin*dIII/*Pst*I endonucleases. Lane 6 contained *C. acetobutylicum* P262 chromosomal DNA digested with *Hin*dIII/*Eco*RI endonucleases. Lanes 3 and 5 contained pMET10B DNA, digested to completion with *Hin*dIII/*Pst*I (lane 3) and *Hin*dIII/*Eco*RI (lane 5) endonucleases. Lane 1 contained lambda DNA digested with *Pst*I as a DNA size marker. Sizes of the lambda DNA markers in kb are detailed in the Appendix.
B. Corresponding autoradiograph of the hybridization of a nonradioactively-labelled 1.86kb *Hin*dIII/*Pst*I DNA probe from pKS10B to **A.** Arrows and numbers in kb indicate the positions and sizes of the common bands between pMET10B (lanes 3 and 5) and *C. acetobutylicum* P262 (lanes 4 and 6).

The nonradioactively-labelled DNA fragment hybridized to pMET10B (lane 3) digested with *Hin*dIII/*Pst*I endonucleases (Fig. 2.6). No specific hybridization between the probe and *Hin*dIII/*Pst*I restriction endonuclease-digested *E. coli* JM105 chromosomal DNA occurred. A small amount of non-specific hybridization was observed in the top of lane 2, presumably due to the high concentration of *E. coli* chromosomal DNA. The probe hybridized to a corresponding 1.86kb *C. acetobutylicum* P262 *Hin*dIII/*Pst*I-digested chromosomal DNA band in lane 4 thus confirming the origin of the pKS10B insert DNA. An internal 0.77kb *Hin*dIII/*Eco*RI restriction endonuclease band was common between the pMET10B digest (lane 5) and the *C. acetobutylicum* P262 digest (lane 6). The top 4.5kb band of *Hin*dIII/*Eco*RI-digested pMET10B DNA (lane 5) corresponded to a hybrid *Eco*RI/*Eco*RI band consisting of pEcoR251 vector DNA and *C. acetobutylicum* P262 insert DNA.

2.4 Discussion

Subcloning and exonuclease III shortening of the *C. acetobutylicum* P262 insert DNA of pMET10B enabled the further characterization of the DNA by *in vitro* and *in vivo* transcription/translation experiments, metronidazole sensitivity testing and DNA hybridization. Metronidazole sensitivity tests showed that progressive exonuclease III deletions of the insert DNA were associated with the complete loss of the phenotype for the pKS shortened constructs and with the partial loss of the phenotype for the pSK shortened constructs.

SDS-polyacrylamide gels of the *in vitro* transcription/translation experiment products did not reveal a specific protein band that was related to the metronidazole sensitivity phenotype that the relevant exonuclease III-shortened clones conferred on *E. coli* F19. *In vivo* transcription/translation experiments also failed to reveal

Chapter Three

Nucleotide sequencing and RNA studies

any protein that was associated with the ability to confer the metronidazole sensitivity phenotype on *E. coli* CC118 and *E. coli* F19.

The growth curves of *E. coli* JM105 (pKS2) and *E. coli* JM105 (pKS4) were determined in LB without the addition of metronidazole to confirm that the metronidazole sensitivity was not due to a growth deficiency conferred on *E. coli* F19 by the *C. acetobutylicum* P262 insert DNA. Both *E. coli* JM105, transformed with pKS2 that conferred the metronidazole sensitivity phenotype on the *E. coli* mutant F19, and *E. coli* JM105 transformed with a deleted plasmid pKS4, that did not confer the metronidazole sensitivity phenotype, followed a standard growth curve with *E. coli* (pKS2) showing an increased lag time of 1 hour. However, this lag time would not manifest itself in a metronidazole sensitivity plate assay after 2 days growth at 34 °C. The graph of bacterial cell numbers versus time showed that the numbers of viable cells in *E. coli* (pKS2) was reduced at 11 h compared to *E.coli* (pKS4). This was important since the metronidazole sensitivity phenotype observed was based on cell numbers. However, in the metronidazole sensitivity assays, the number of *E. coli* F19 cells, transformed with different constructs, was standardized to be the same on all plates containing no metronidazole. Thus, *E. coli* cell death at higher concentrations of metronidazole, was related to the transformed plasmid DNA and not to the plating of reduced cell numbers. Thus the observed metronidazole sensitivity phenotype was considered to be real and not artifactual.

Finally, DNA hybridization established that the insert DNA of pKS10B hybridized to pMET10B and to the chromosomal DNA of *C. acetobutylicum*, but not to *E. coli* chromosomal DNA. The origin of the 1.86kb *Hin*dIII/*Pst*I insert DNA of pKS10B was therefore confirmed to be from *C. acetobutylicum* P262.

It was considered that nucleotide sequencing of the *C. acetobutylicum* P262 insert DNA of pKS1 would confirm the absence of an open reading frame, thus explaining the lack of an observable protein product linked to the metronidazole sensitivity phenotype. A protein may in fact have been produced from the pSK1 *C. acetobutylicum* P262 insert DNA, but may have been masked by other proteins present in the SDS-polyacrylamide gels. However, if no protein was responsible for the metronidazole sensitivity phenotype, an RNA or other sequence motifs at the DNA level might have been responsible. Nucleotide sequencing of the pSK1 *C. acetobutylicum* P262 insert DNA was therefore undertaken.

Chapter Three

Nucleotide sequencing and RNA studies

3.1 Introduction

The *C. acetobutylicum* P262 insert DNA of pKS1, that originated from pMET10B, was shown in Section 2.3.2 to transform *E. coli* F19 to a metronidazole sensitive phenotype. No protein product from the insert DNA, however, could be associated with this phenotype. This Chapter documents the nucleotide sequencing strategy used to characterize the DNA to try to identify the elements responsible for the sensitivity phenotype. RNA isolation and hybridization are reported in the second half of the Chapter (Section 3.3.2 and 3.3.3).

3.2 Materials and methods

All details of materials and methods are contained in the Appendix.

3.2.1 Bacterial strains and plasmids

Bacterial strains used were the same as those reported in Fig. 2.1 in Section 2.1.1.

3.2.2 DNA preparation

DNA for nucleotide sequencing and general use was prepared according to Ish-Horowicz and Burke (1981). The maxiprep method was used (see Appendix) followed by cesium chloride gradient centrifugation in order to generate a pure preparation which was necessary for good resolution on sequencing gels.

3.2.3 Nucleotide sequencing

The nucleotide sequence of both strands of the *C. acetobutylicum* P262 insert DNA of exonuclease III-shortened clone pKS1 was determined by an adaptation of the dideoxynucleotide triphosphate chain termination method of Sanger *et al.* (1977). The Sequenase® Version 2.0 DNA Sequencing Kit was used (No. 70700, United States Biochemical, Cleveland, Ohio, USA) which incorporates the Sequenase® enzyme originally described by Tabor and Richardson (1987). Two oligonucleotide primers that were designed to be complementary to an internal region of the insert DNA were used for nucleotide sequencing and were synthesized by Prof D. Botes in the Biochemistry Department, UCT. The M13 forward and reverse sequencing primers from the kit were also used. Extensive compressions were located in some areas of the DNA template and in order to resolve them, Klenow polymerase was added to the sequencing termination reactions (see Appendix).

3.2.4 Sequence analysis

The sequencing data was analyzed using the sequence analysis programs of the Wisconsin University Genetics Computer Group Inc. (Devereux *et al.*, 1984) on a VAX-6000-330 computer. The GenBank, EMBL, SWISS-Protein and PIR databases were searched directly for DNA and amino acid homology. Databases were also accessed using the BLAST network service (Basic local alignment search tool) through the NCBI (National Centre for Biotechnology Information), Maryland, USA, (Altschul *et al.*, 1990). The centre was accessed via electronic mail to the NCBI BLAST E-Mail Server (blast@ncbi.nlm.nih.gov). The BLOCKS E-Mail searcher (blocks@howard.fhcrc.org) was also used to detect any sequence homologies between the query sequence and the current database of protein blocks at the NCBI. The BLOCKS E-Mail searcher is based on the PATMAT searching tool and can search protein or DNA sequences (Wallace and Henikoff, 1992). The

tRNA Search program was also used to analyze the sequence information (Dr. P. Dürre, Institute for Microbiology, Göttingen, Germany, pers. comm.).

3.2.5 tRNA preparation and visualization

C. acetobutylicum P262 was grown under anaerobic conditions in *Clostridium* basal medium (O'Brien and Morris, 1972). *E. coli* JM105 was grown in LB containing 100 μg/ml ampicillin. RNA was isolated from both *C. acetobutylicum* P262 and *E. coli* JM105 using hot phenol, according to the method of Aiba *et al.* (1981). RNA was separated on a 2.5% denaturing agarose gel (Fourney *et al.*, 1988) and was subsequently vacuum-blotted onto Hybond N^+ (Amersham Int., Amersham, UK) for 45 min using 0.05M NaOH. RNA markers were obtained from Gibco Brl, Maryland, USA.

3.2.6 RNA hybridization

The 1.86kb *Hin*dIII/*Pst*I *C. acetobutylicum* P262 DNA restriction endonuclease fragment from pMET10B was used as a probe for RNA hybridization. The gel-purified DNA fragment was further purified using the Geneclean Kit (BIO 101, La Jolla, California, USA). The DNA fragment was random-primed with [α-^{32}P]CTP (3000 Ci/mmol) using the Random Primed DNA Labelling Kit (No. 1004 760, Boehringer Mannheim (SA)). Hybridization and detection were according to the instructions of Amersham Int., Amersham, UK. Methods for both tRNA preparation and RNA hybridization are contained in the Appendix.

3.3 Results

3.3.1 Nucleotide sequencing and sequence analysis of pKS1

The pKS and pSK exonuclease III-shortened clones and two oligonucleotide primers were used for nucleotide sequencing of the 933-bp *C. acetobutylicum* P262 insert DNA of pKS1. Homology searches of 100-bp sections of nucleotide sequence at the DNA level revealed two genes coding for two transfer RNA-like structures. The putative tRNAs contained the unmodified anticodons UGA and GCU that corresponded to the serine codons UCA and AGC respectively (Fig. 3.1). The tRNA Search program also confirmed that the DNA encoded two tRNA genes (Dr. P. Dürre, Institute for Microbiology, Göttingen, Germany, pers. comm.). Both tRNA genes were 91-bp in length and were separated by a 34-bp spacer. They were transcribed from the same strand and both encoded the 3' CCA terminus of the acceptor arm. Both tRNAs were typical of class II tRNAs as they conformed to the predicted cloverleaf structure (Fig. 3.2) and each included a large variable extra arm of 15-bp and 14-bp for $tRNA^{Ser}$ AGC and $tRNA^{Ser}$ UCA, respectively. Analysis of upsteam sequence revealed a region with very strong homology to both consensus *E. coli* and consensus Gram-positive bacterial -35 and -10 Pribnow box promoter regions (Fig 3.1). In addition, a 7-bp region showed homolgy to a consensus SC (stringent control) sequence GT(C/T)G(C/T)(T/Pu) from *B. subtilis* (Ogasawara *et al.*, 1983). Analysis of the downstream sequence revealed a 13-bp inverted repeat sequence, located immediatedly after the second tRNA gene, that could form a putative *rho*-independent transcription termination stem structure (ΔG = -27kCal/mol), (Rosenburg and Court, 1979). A third 13-bp sequence, 22-bp downstream of the putative terminator showed extensive homology to the putative terminator structure and could possibly form a stem structure (with 4 mismatches) with one of the strands of the terminator (ΔG = -20kCal/mol).

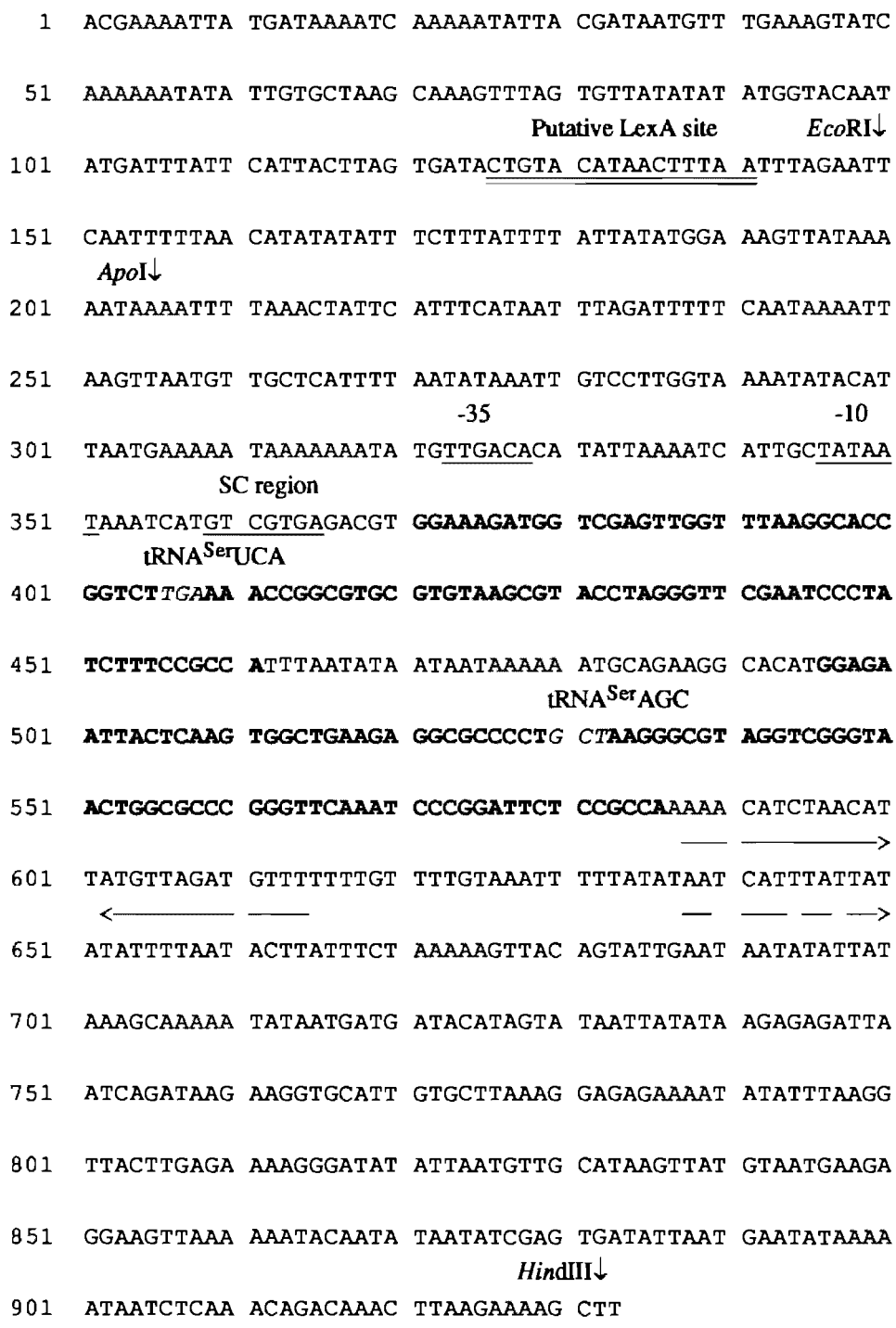


Fig. 3.1 DNA nucleotide sequence of the non-coding strand of the 933-bp *C. acetobutylicum* P262 insert DNA of pKS1 encoding the two serine tRNA genes. The tRNA genes are shown in bold with the anticodons in italics. The -35 and -10 promoter regions and the stringent control (SC) sequence are underlined. The 13-bp inverted repeat sequences that may form a terminator structure are underlined by arrows, with the mismatched repeat sequence indicated by a multiply dashed arrow. The position of a putative LexA binding sequence is indicated by a double underline. The nucleotide sequence depicted here has been submitted to the GenBank sequence database and has been accorded accession number L24102.

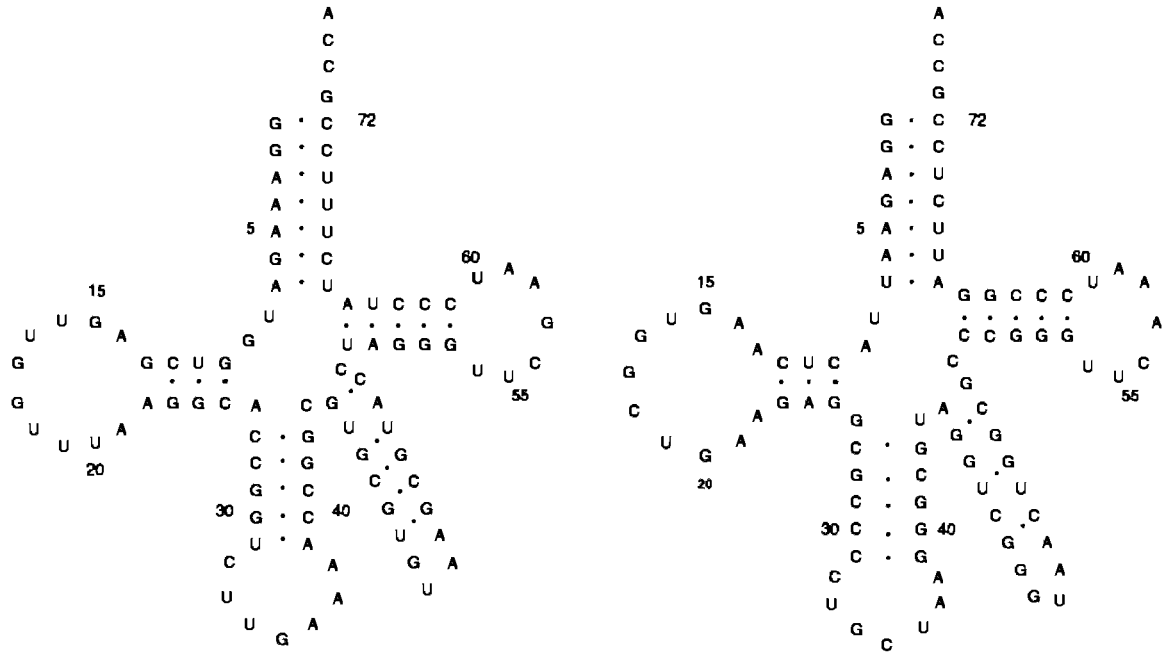


Fig. 3.2 The predicted secondary structure of two serine tRNA molecules from *C. acetobutylicum* P262. The primary sequence of the tRNA molecules was obtained from the insert DNA sequence of pKS1 in Fig. 3.1. The structures predicted are according to the tRNA Search program (Dr P. Dürre, pers. comm.). Numbering is according to standard methods (Sprinzl *et al.*, 1989).

It was initially assumed that the two tRNA genes were related to the metronidazole susceptibility phenotype conferred by pKS1 on *E. coli* F19. However, the position of the tRNA genes on the various exonuclease III-shortened clones did not appear to be related to the ability of the constructs to make *E. coli* F19 susceptible to metronidazole (Fig. 3.3). In an attempt to confirm this, further subcloning of pKS1 was carried out and further *E. coli* F19 metronidazole susceptibility assays were conducted. Subclone B (Fig. 3.3) was generated by the ligation of a 0.79kb *Eco*RI/*Hin*dIII restriction endonuclease *C. acetobutylicum* P262 insert DNA fragment from pKS10B, containing the two tRNA genes, into the *Eco*RI/*Hin*dIII restriction sites of pKS. Subclone A (Fig. 3.3) was generated by the ligation of a 0.43kb *Apo*I endonuclease-digested DNA fragment of the *C. acetobutylicum* P262 insert DNA into the compatible *Eco*RI restriction site of Bluescript pKS. Both subclones did not make *E. coli* F19 more susceptible to metronidazole than a

control plasmid pKS, indicating conclusively that the tRNA genes were not responsible for the phenotype.

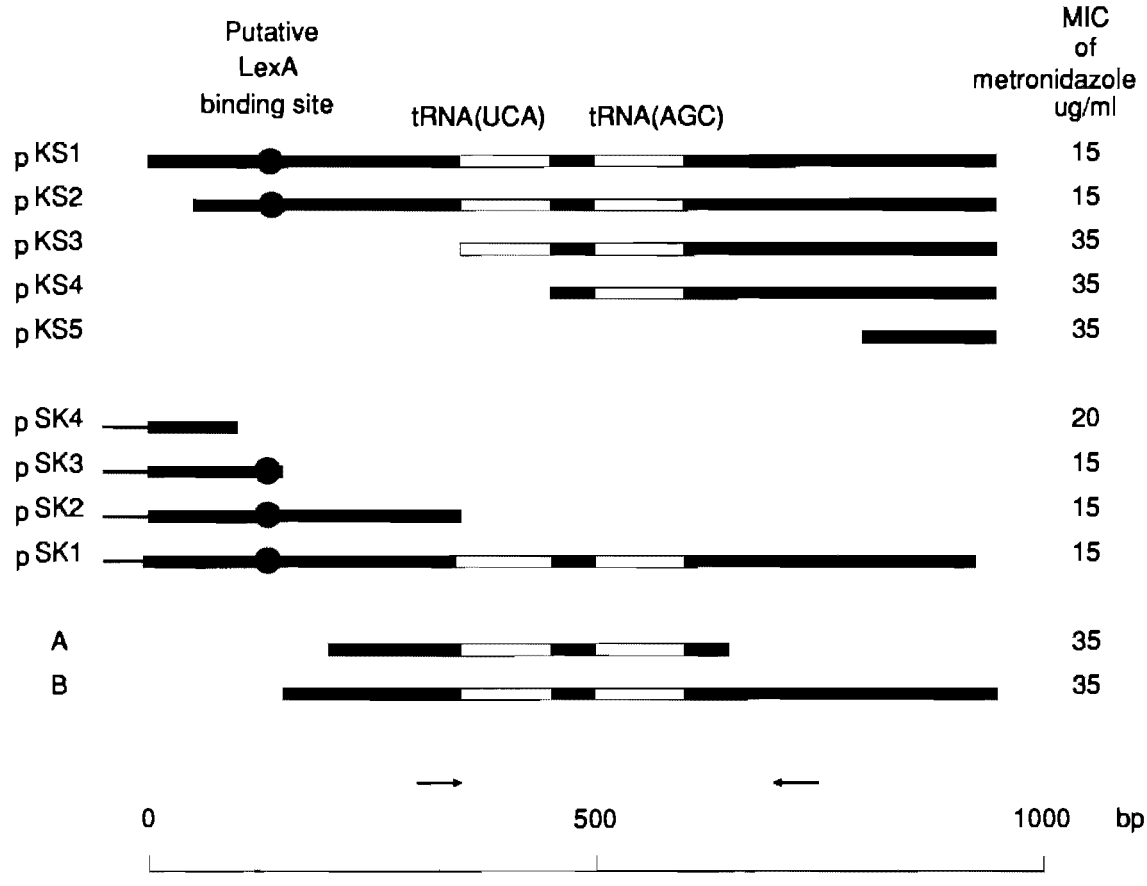


Fig. 3.3 Schematic diagram of a range of *C. acetobutylicum* P262 DNA inserts generated in Bluescript pKS and pSK and the relative positions of the two tRNA genes. The extent of the *C. acetobutylicum* P262 inserts of the subclones A and B are shown. Thick lines indicate *C. acetobutylicum* P262 DNA for which the nucleotide sequence was determined, while thin lines indicate the unsequenced DNA region. Vector DNA is not indicated. The tRNA genes are depicted as blank boxes and the oligonucleotide primers used for nucleotide sequencing are shown as arrows. The presence of the putative LexA binding sequence is indicated by a filled circle and its relationship to the phenotype (minimum inhibitory concentration of metronidazole) in *E. coli* F19 is shown.

Coincidentally, it was noted that a sequence was present that had homology to an *E. coli* LexA binding site. This sequence was present upstream of the tRNA genes on all the constructs that conferred the metronidazole sensitivity phenotype, but was absent from those that did not (Fig. 3.3). LexA is a repressor protein that regulates many of the *E. coli* SOS genes that are involved in bacterial DNA repair. Whether LexA in fact binds to this *C. acetobutylicum* P262 sequence in *E. coli* F19 is unknown since the sequence has 6 mismatches out of a 16-bp consensus sequence as shown in Fig. 3.4 (Wertman and Mount, 1985). However, the nucleotide sequences of the different *E. coli* LexA binding sites vary considerably, indicating that a range of nucleotides can be accommodated by the protein.

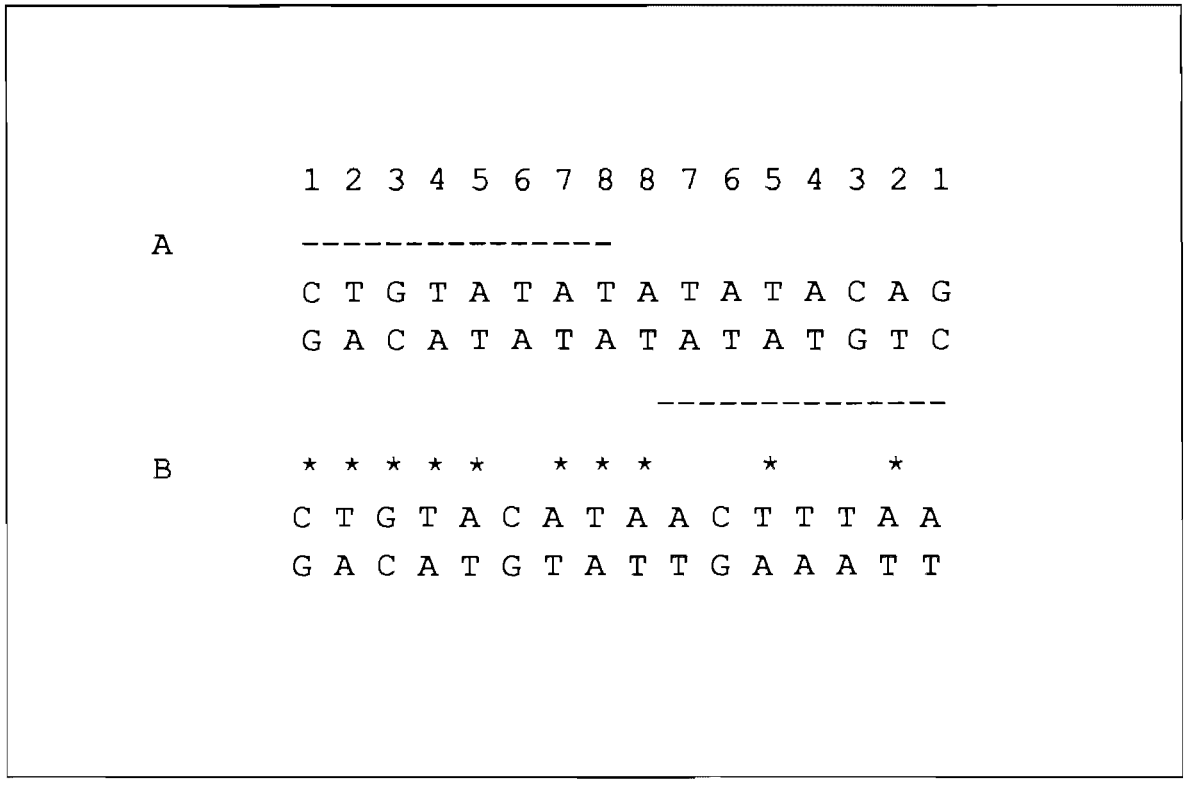


Fig. 3.4 Nucleotide sequence alignment of a consensus LexA binding site from *E. coli* (A), with a putative LexA binding sequence on the *C. acetobutylicum* P262 insert DNA of pKS1 (B). Horizontal dashes indicate nucleotides that exhibit dyad symmetry in the consensus site while asterisks indicate identical nucleotides between the consensus site and the *C. acetobutylicum* P262 sequence. Numbering represents the position of nucleotides within the consensus operator half-site (for details see text), (Wertman and Mount, 1985).

3.3.2 tRNA preparation and visualization

Plasmid-encoded tRNA was isolated together with endogenous bacterial tRNA as part of a bulk RNA preparation from *C. acetobutylicum* P262 and *E. coli* JM105. The endogenous and plasmid-encoded tRNA species could be visualized by electrophoresis on a denaturing 2.5% agarose gel (Fig. 3.5 A.) which separated the tRNA from the bulk RNA. Lane 1 contains RNA isolated from *E. coli* JM105 transformed with pKS2, encoding the two tRNA genes. Lane 2 contains RNA isolated from *E. coli* cells transformed with the plasmid pKS5 in which both tRNA genes had been deleted, as a negative control. Total RNA isolated from *C. acetobutylicum* P262 is shown in lane 3. The transfer RNA was observed as a band immediately below the 5S rRNA band. The 23S rRNA and 16S rRNA bands were visible higher up in the gel. RNA markers were visible as six faint bands (lane 4) which corresponded in increasing size to 0.24kb, 1.4kb, 2.4kb, 4.4kb and a top band which appeared as a doublet of 7.5kb and 9.5kb.

3.3.3 RNA Hybridization

Northern hybridization was carried out to confirm whether it was possible to distinguish between the plasmid-encoded serine tRNAs and the endogenous *E. coli* JM105 tRNAs. Northern hybridization also confirmed that the two serine tRNA molecules were produced in *C. acetobutylicum* P262. The 0.43kb *C. acetobutylicum* P262 *Apo*I-digested DNA fragment that encoded the two tRNA genes, was used as a probe. Hybridization was observed in lane 1 (Fig. 3.5 B) to tRNA produced from *E. coli* containing the plasmid pKS2 encoding the tRNA genes. No hybridization was observed in lane 2 to tRNA produced from *E. coli* containing the deletion plasmid pKS5. This indicated that the hybridization observed was to plasmid-encoded tRNA since no signal was observed in lane 2. Stronger hybridization was observed to tRNA produced from *C. acetobutylicum* P262 in lane 3, than to tRNA produced from *E. coli* in lane 1. A very faint band

was observed in both lanes 1 and 3, just above 0.24kb and may correspond to the position of the primary transcript which would be approximately 220 nucleotides in length.

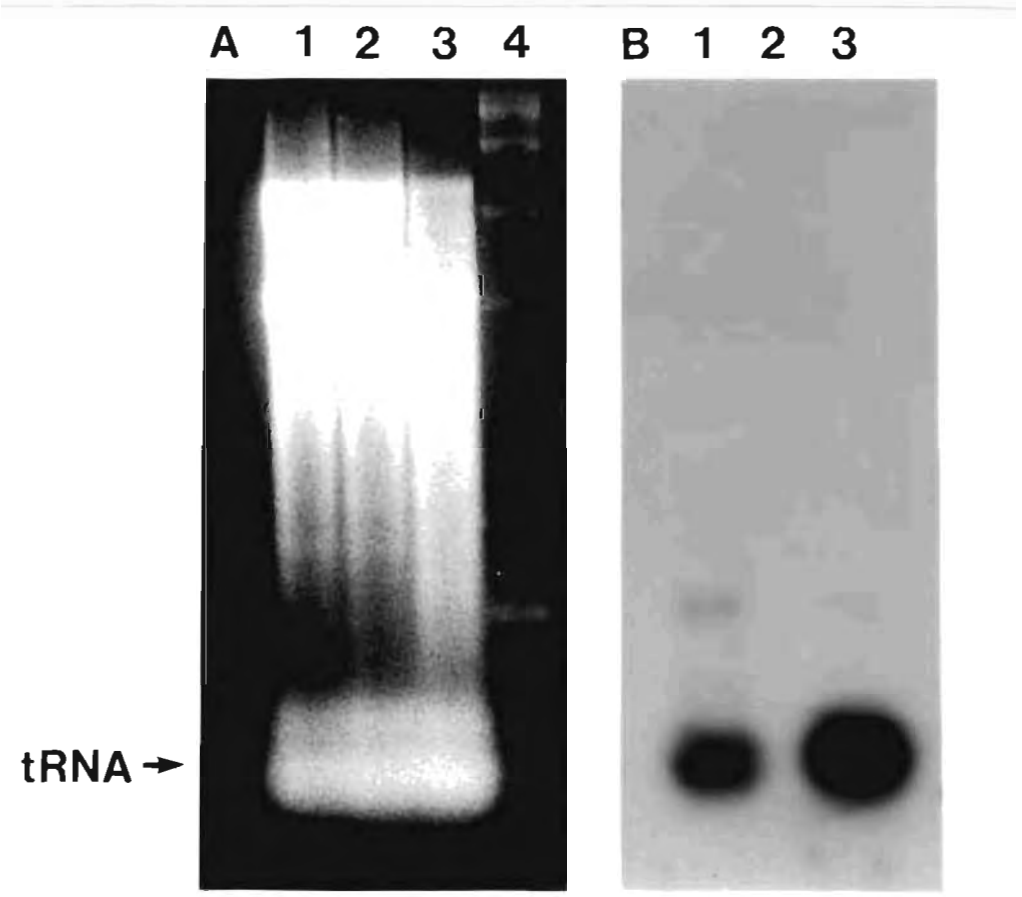


Fig. 3.5 Northern analysis of tRNA produced from *E. coli* JM105 and *C. acetobutylicum* P262.
A. Denaturing gel electrophoresis of total RNA produced from *E. coli* transformed with pKS2 encoding the two tRNA genes (lane 1) , transformed with pKS5 which had the two tRNA genes deleted (lane 2). Lane 3 shows total RNA produced from *C. acetobutylicum*. Lane 4 shows RNA markers (see text for sizes).
B. Northern hybridization of RNA in **A** to the [α-^{32}P]-labelled *Apo*I *C. acetobutylicum* P262 DNA restriction fragment containing the two tRNA genes (depicted as A in Fig. 3.3) as a probe.

3.4 Discussion

The two serine tRNA genes from *C. acetobutylicum* P262 showed high homology to the corresponding serine tRNA genes from other bacterial species, presumably due to the large number of nucleotides that are conserved in these molecules (Lewin, 1987). $tRNA^{Ser}AGC$ showed 70.2% identity to the *E. coli* $tRNA^{Ser}AGC$ and 89% identity to the *B. subtilis* $tRNA^{Ser}AGC$ (Sprinzl *et al.*, 1989; Green and Vold, 1983). Likewise, the $tRNA^{Ser}UCA$ showed 76.9% identity to the synonymous tRNA from *E. coli* and 69.9% identity to the synonymous *B. subtilis* tRNA. In addition, $tRNA^{Ser}$ UCA shared 67.4% identity with the corresponding tRNA in *Streptomyces griseus*, illustrating the sequence similarity between species (Bar-nir, 1992).

The organization of the genes suggested that they were transcribed as one transcriptional unit; they were preceded by a consensus promoter sequence and followed by three repeated sequences that may have formed a putative transcription terminator. The putative promoter region showed strong consensus to both typical *E. coli* promoters and Gram-positive vegetative promoter regions.

Graves and Rabinowitz (1986) reported that a cluster of A residues, conserved at positions -45 to -41 in more than 50% of the Gram-positive promoters analyzed, was thought to represent an extended promoter consensus sequence . An identical sequence (TA_AAAAA) was present upstream of the promoter sequence identified in this study. Fig. 3.6 shows the alignment of this promoter with other consensus *E. coli*, *Clostridium* and Gram-positive promoter regions. No unique recognition sequences upstream of *Clostridium* operons have as yet been identified to be recognized by alternate sigma factors. It is likely, though, that this $tRNA^{Ser}$ operon is routinely transcribed by *C. acetobutylicum* P262 and the consensus-like nature of

the putative promoter may reflect the continued requirement of these molecules by the bacterium.

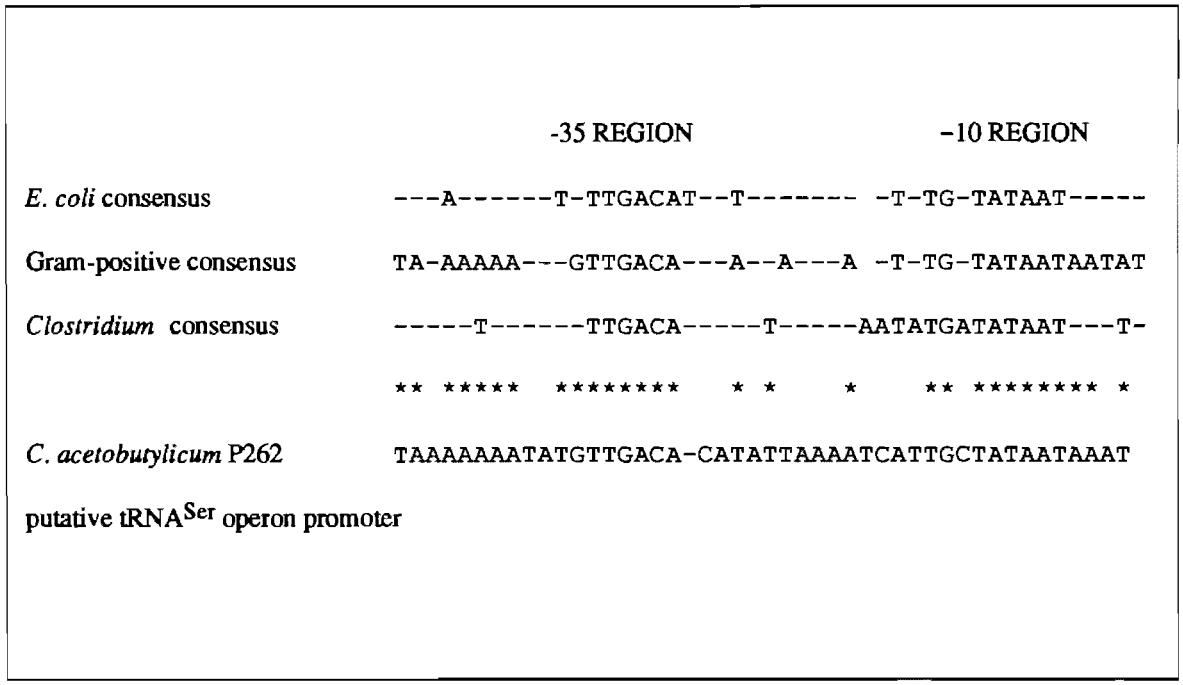


Fig. 3.6 Alignment of the putative *C.acetobutylicum* P262 $tRNA^{Ser}$ operon promoter with consensus promoter sequences from *E. coli*, *Clostridium* and Gram-positive bacteria. Asterisks indicate identical nucleotides. Adapted from Gerischer and Dürre (1992).

An additional control element, the SC (stringent control) or discriminator sequence, has been identified upstream of a number of RNA genes in Gram-positive and Gram-negative bacteria. SC sequences are thought to negatively regulate a number of components of the translation machinery, such as some rRNAs and tRNAs, under conditions of amino acid starvation (Cashel and Rudd, 1987). A consensus SC region was first identified in *E. coli* to be CggC-CC, where it lies immediately 3' to the Pribnow box of promoters negatively regulated during the stringent response (Travers, 1980). In Gram-positive *B. subtilis*, however, the consensus sequence is different; GT(C/T)G(C/T)(T/Pu) and its position corresponds to the putative transcription initiation site (Ogasawara *et al.*, 1983). The 7-bp region reported in

this study showed homology to the *B. subtilis* SC sequence and was situated 7-bp after the -10 Pribnow box region, similar to the SC sequence position in that bacterium. The presence of the SC sequence may indicate that this putative *C. acetobutylicum* P262 serine tRNA operon is subject to stringent control. An SC sequence that was identified in *P. aeruginosa* was of the *E. coli*-type (Bally *et al.*, 1992) indicating that the currently reported examples of control elements indicate differences between Gram-positive and Gram-negative organisms. The only other report of a tRNA gene from *C. acetobutylicum* P262 showed no obvious promoter or SC region (Sauer and Dürre, 1992). This is therefore, the first example of a *C. acetobutylicum* P262 tRNA gene cluster with a consensus promoter and stringent control region, indicating that the stringent response probably operates in this bacterium.

The termination of transcription in *E. coli* may be factor dependent (*eg. rho*-dependent) with these terminators exhibiting no obvious sequence similarities (Roberts, 1969). A factor-independent terminator was identified in this study and consisted of a typical GC-rich region that exhibited dyad symmetry followed by a string of T's. A third repeat sequence was also identified, which could base-pair with the second repeat sequence located after the tRNA genes. A similar situation was observed in *E. coli*, where a third 11-bp sequence could base-pair with one of the strands of the putative terminator located after the $tRNA^{Ser}$ UCA gene (Tamura *et al.*, 1984).

The two tRNA genes have an unusually high percentage of G + C for *Clostridium* spp. *C. acetobutylicum* is characterized by a low G + C content of 28% to 29% (Cummins and Johnson, 1971). The $tRNA^{Ser}$ AGC gene is 60% G + C, while the $tRNA^{Ser}$ UCA gene is 53% G + C. This high G + C content is presumably required for the stability of the tRNA molecule. However, the G + C composition for the

entire 933-bp sequenced region is 27%. The codon recognized by the first tRNA in the operon, AGC, is used only moderately by *C. acetobutylicum* P262 (12% of the time). The second tRNA recognizes the codon UCA, which is used the most frequently out of all the synonymous serine codons (41% of the time), (J. Santangelo, PhD Thesis, UCT, 1991).

Since the tRNAs in this study were sequenced at the DNA level, but not at the RNA level, the predicted codon-anticodon recognition is based on the unmodified anticodon sequence. The influence of modification on the codon-anticodon interaction is not well understood but it is known to affect the nature of the recognition. Indeed, Wahab *et al.* (1993) showed that a 7-fold increase in $tRNA^{Leu}$ levels in *E. coli* caused at least 90% of the tRNA to be hypomodified. This hypomodification may have altered the codon-anticodon recognition which may explain the reduced growth rates they observed.

It is likely that the uridine in the first position of the anticodon of *C. acetobutylicum* P262 $tRNA^{Ser}$ UCA is post-transcriptionally modified since nearly all uridines are modified at this position, except for a few cases (Sprinzl *et al.*, 1989). NMR studies have examined the effect of a modified uridine at this position on the mechanism of codon recognition (Yokoyama *et al.*, 1985). It appears that two types of modified uridine prevail; xm^5s^2U or xo^5U (Fig. 3.7). In *B. subtilis*, 5-methoxyuridine (mo^5U) has been detected in $tRNA^{Val}$, $tRNA^{Thr}$ and $tRNA^{Ala}$, whereas in *E. coli*, modification has been of the cmo^5U and mnm^5s^2U type. The modified uridine xm^5s^2U, maintains the ribose ring in its usual form and can form a stable base pair with adenine only. However, xo^5U has a flexible ribose ring and can base pair with uridine and guanine as well as adenine. If we assume that the modification in *C. acetobutylicum* P262 is of the same type as that in *B. subtilis* (mo^5U), then the UGA anticodon of $tRNA^{Ser}$ UCA can also recognise codons UCU (used 21% of the

time in *C. acetobutylicum* P262, (J. Santangelo, PhD Thesis, UCT, 1991)) and UCG (used 2.4% of the time).

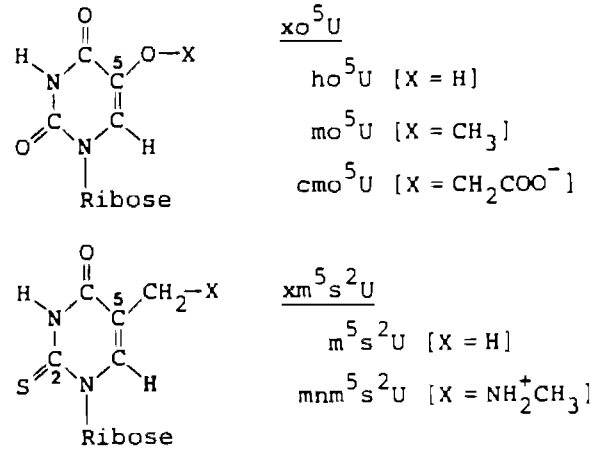


Fig. 3.7 Chemical structure of modified uridines (Yokoyama *et al.*, 1985)

Similarly a wobble, as proposed by Crick (1966), in the guanine of the first position of the anticodon of $tRNA^{Ser}AGC$ means that this tRNA can also recognise codon AGU as well as AGC. AGU is used 22% of the time out of the group of synonymous serine codons in *C. acetobutylicum* P262 (J. Santangelo, PhD Thesis, UCT, 1991). In theory therefore, up to 5 synonymous serine codons, out of a total of 6 could be recognised by the two serine tRNAs present on the *C. acetobutylicum* P262 insert DNA of pSK1. One can speculate that *C. acetobutylicum* P262 may limit the number of tRNA genes by preferring anticodons capable of reading more than one codon. However, it is possible that duplicate copies of these tRNA genes are present on the *C. acetobutylicum* P262 chromosome, as in the case of some *B. subtilis* tRNA genes (Vold, 1985).

The position of the two tRNA genes on the exonuclease III-shortened clones indicated that they were not related to the ability of certain constructs to render *E. coli* F19 sensitive to metronidazole. Two other subclones, A and B, were generated in an attempt to prove this. The biggest subclone, subclone B, contained 787-bp of *C. acetobutylicum* P262 DNA which included the two tRNA genes of the 933-bp sequenced region of the insert DNA of pKS1. Yet, it did not render *E. coli* F19 sensitive to metronidazole. Therefore the 146-bp region, upstream of the *Eco*RI site in pKS1, was examined again. As before, no open reading frame could be detected and homology searches using BLAST and BLOCKS revealed no relevant homology to known protein or DNA sequences in the existing databases. However, on searching the DNA sequence visually, a 16-bp sequence that showed homology to an *E. coli* LexA binding site was noted, 230-bp upstream of the tRNA genes.

LexA is a small repressor protein (22 300 Da) that represses a number of unlinked genes of the SOS regulon, that are involved in the repair of DNA damage in the cell. The nucleotide sequences of a number of LexA repressor sites are known and on the basis of 25 sites, a 16-bp consensus sequence was proposed (Wertman and Mount, 1985). This sequence has a central axis of symmetry which divides the site into an operator half site. This is based on the idea that a dimer repressor binds symmetrically to the operator site about this axis, making identical contacts with the nucleotides present in the site if the second monomer is swung through 180° relative to the binding position of the first (Fig. 3.4). The terminal trinucleotide CTG is the most conserved, but homology among the central five nucleotides is also conserved between sites. The sequence observed in this study on pKS1, has an almost perfect consensus sequence for the first 8 nucleotides. The second half-site, however, has only 3 out of 8 consensus nucleotides. There are six deviations from the consensus in the *C. acetobutylicum* P262 sequence which may be sufficient for binding since

one of the *umuDC* operators has eight deviations and still binds the repressor (Wertman and Mount, 1985). The presence of this *C. acetobutylicum* P262 sequence, with homology to *E. coli* LexA binding sites, could perhaps be responsible for the metronidazole sensitivity phenotype observed in *E. coli* F19. This will be discussed in the General Discussion and Conclusion in Chapter Four.

Lastly, the RNA hybridization experiment proved that the relevant tRNA molecules were transcribed from *C. acetobutylicum* P262 DNA, both in the parent organism and in the heterologous host, *E. coli* JM105. Thus the Gram-negative *E. coli* RNA polymerase recognized and transcribed the Gram-positive *C. acetobutylicum* promoter. This is not unexpected since a number of Gram-positive genes have been expressed successfully in *E. coli* and the problem lies rather in the expression of *E. coli* genes in Gram-positive bacteria (Graves and Rabinowitz, 1986). Northern blotting showed that no hybridization of a DNA probe encoding the two tRNA genes, occurred to tRNA produced from the control *E. coli*, which contained a plasmid that had been deleted for both tRNA genes. This was unexpected since the two endogenous *E. coli* $tRNA^{Ser}$ molecules showed more than 70% identity to their *C. acetobutylicum* P262 counterparts. A lowering of the stringency conditions may result in cross-hybridization. The tRNA from *C. acetobutylicum* P262 also produced a stronger hybridization signal than the tRNA from *E. coli*. This may have been due to unequal amounts of tRNA loaded in the agarose gel, however, it is not possible to detect this visually on the gel photograph. An alternative explanation is that the proportion of the two serine tRNA species relative to the total amount of tRNA produced, may have been larger in *C. acetobutylicum* P262. This may have been due to the increased recognition of the *C. acetobutylicum* P262 endogenous promoter in the parent organism relative to its recognition in *E. coli*.

Chapter Four

General discussion and conclusion

The work presented in this thesis has provided further information about the genetics of *C. acetobutylicum* P262. An improved understanding of the genetics of this bacterium is vital for the future genetic manipulation of pathways involved in solvent metabolism. This work has given insight into *C. acetobutylicum* P262 serine tRNA genes and has raised interesting questions regarding a putative LexA sequence that was found upstream of the two tRNA genes on pKS1. The putative LexA sequence will be discussed with reference to how it may have generated the metronidazole sensitivity phenotype in *E. coli* F19. Other genes that have been isolated and characterized in this laboratory using the metronidazole system and the *E. coli* F19 mutant will also be mentioned. From this information, suggestions will be made concerning future research in *C. acetobutylicum* P262.

LexA is a repressor protein that during normal cell growth in *E. coli*, represses a set of about 20 SOS genes. The SOS regulatory system represents about 0.5% of all *E. coli* genes and controls the response of the cell to DNA damage or DNA replication inhibition (Little, 1993). The SOS system includes the *uvrABCD* genes for excision repair and the *recBCDEF* genes for recombinational repair, some of which are under control of the LexA and RecA proteins. In a normal cell, LexA differentially represses a number of these repair genes, including its own structural gene by binding to a range of operator/promoter sequences. It binds tightly to the *recA* gene, while binding less tightly to its own operator and the *uvrA* operator so that a basal level of certain repair proteins is maintained in the cell (Walker, 1984). It is known that the addition of metronidazole to an *E. coli* culture causes DNA damage to the bacterium (Jackson, 1984) that would induce the SOS response in a similar way to UV (ultraviolet) light or other mutagens. The DNA damage is

thought to induce a signal that results in the reversible activation of the RecA protein to a coprotease that increases the rate of cleavage of the LexA repressor (Walker, 1987). Upon cleavage the repressor is inactivated and the SOS genes are expressed. LexA can in fact cleave itself at high pH and RecA stimulates the self-cleavage process, by acting as a coprotease (Little, 1993). It is likely that self-cleavage of LexA occurs continuously at a slow rate and that the interaction with activated RecA increases the rate of cleavage dramatically. Cleavage of the repressor then results in the increased expression of the SOS genes and the damaged DNA is repaired thus removing the inducing signal. RecA is, therefore, no longer induced to act as a coprotease and it reverts to its original inactivated state. LexA protein is continuously synthesized throughout the above process and binds to a range of operators/promoters of the SOS genes once again before finally repressing its own structural gene.

The *E. coli* strains F19 and CC118 used in this study were *recA* so the above situation is altered, but to what extent is unknown. The MIC of metronidazole for strains *E. coli* F19 and *E. coli* CC118 that are mutated in their DNA repair genes is much lower that that for parent strains (Jackson, 1984) but the bacteria still manage to survive exposure to DNA damage. When pKS1 was present in an *E. coli* F19 cell, however, the sensitivity to metronidazole was further increased, as discussed in Section 2.3.2 and 3.3.1. We can only speculate at this stage as to the mechanism of this increased sensitivity, because many details of the repair process are unknown.

One possibility is that the 16-bp sequence upstream of the two tRNA genes mimicked an *E. coli* LexA binding site that binds any available LexA repressor protein. One might expect that the sequestration of the LexA repressor would lead to a derepression of the repair genes under LexA control and a resistance phenotype. This is incompatible with the findings of this study which showed that

the *E. coli* F19 mutant was sensitive to the drug when transformed with pKS1. However, the sensitivity phenotype may have been caused by the continuous overproduction of the LexA protein by the cell. If available LexA protein was sequestered from some of the SOS gene operators by the 16-bp *C. acetobutylicum* P262 sequence, it is likely that the *lexA* structural gene continued production of the protein because a certain concentration of LexA repressor was probably required in the cell before autorepression of the *lexA* structural gene occurred. Thus, the presence of multicopy plasmids bearing a number of artifactual LexA binding sites would have required the cell to produce abnormally increased amounts of the repressor protein. The increased expression of such a protein may have disadvantaged the cell by titrating cell machinery normally required for the transcription and translation of other essential genes. Such a load on the cell may have caused the metronidazole sensitivity phenotype observed in this study.

In order to test this hypothesis, the binding of LexA to such a sequence would first need to be demonstrated in a gel retardation experiment. A synthetic oligonucleotide should preferably be used which includes the 16-bp putative LexA sequence. This would eliminate the possibility of other nonspecific binding events. The oligonucleotide should be labelled at the 5' end using [α-^{32}P]CTP and T4 polynucleotide kinase and then incubated for 30 min with varying concentrations of LexA protein (from 5nM to 200nM). Binding could then be detected as retardation of the oligonucleotide after polyacrylamide gel electrophoresis and autoradiography.

A number of other *C. acetobutylicum* P262 genes have been isolated and characterized in this laboratory using the *E. coli* F19 mutant and the metronidazole system. These include a catabolite repressor of alpha-amylase (S. Davison, pers. comm.), a putative glutamate synthase small subunit - *gltD* (H. Stutz, pers. comm.)

and a *spoIID* gene (K. Hancock, pers, comm.) as well as the flavodoxin and hydrogenase genes first isolated using the system (Santangelo *et al.*, 1991). In terms of an explanation for the genes isolated, the electron transport genes (flavodoxin and hydrogenase) agree with the theory: an *E. coli* F19 cell transformed with a recombinant plasmid encoding electron transport genes, will contain increased amounts of the reduced, toxic intermediates of metronidazole and will be rendered more sensitive to the drug than *E. coli* F19 containing a control plasmid. However, other genes not involved in electron transport in *C. acetobutylicum* P262 have been isolated using this system. The putative *gltD* subunit of glutamate synthase, although not part of the electron transfer system, may donate electrons to the large subunit of the enzyme which may account for its reductive properties in *E. coli* F19. However, the reasons as to why a catabolite repressor and a *spoIID* gene from *C. acetobutylicum* P262 should be isolated on the basis of a metronidazole sensitivity phenotype, are unknown at this stage. Thus, although some success has been achieved in the cloning of electron transport genes from *C. acetobutylicum* P262 with the metronidazole system, it has been limited. In the future, the establishment of alternative methods for the cloning of such genes may prove to be more profitable.

In the study presented in this thesis, two serine tRNA genes were located adjacent to a region responsible for a metronidazole sensitivity phenotype in *E. coli* F19. Their isolation was fortunate as they represent only the second example of tRNA genes that have been isolated from *C. acetobutylicum* P262. A sequence that had homology to a consensus stringent control sequence from *B. subtilis* was identified upstream of the putative $tRNA^{Ser}$ operon, which provides the first indication that the stringent response may operate in *C. acetobutylicum* P262. The organisation of the genes and transcriptional control elements, such as a putative promoter and terminator, suggests that the genes may be transcribed as one unit. Northern

hybridization experiments showed a faint band of approximately 240 nucleotides that may have corresponded to the position of a putative primary transcript. However, primer extension experiments and S1-nuclease mapping data would be needed to confirm this. The work presented in this thesis represents the first information available about the organisation of clustered tRNA genes on the *C. acetobutylicum* P262 chromosome. Further analysis and sequencing of tRNA genes would be needed to confirm whether clustering of tRNA genes is common on the *C. acetobutylicum* P262 chromosome. Such clustering would enable the coordinate transcription and processing of *C. acetobutylicum* P262 tRNAs which may facilitate the rapid activation of the translation machinery during spore germination and outgrowth in this bacterium.

Appendix

Appendix

A1. Methods

A1.1 Large scale isolation of plasmid DNA (maxiprep)

DNA isolations were according to Ish-Horowicz and Burke (1981).

1. A 200ml culture was grown overnight with shaking at 37°C in the presence of 100 µg/ml ampicillin.
2. The cells were harvested in a GSA centrifuge tube at 5000 rpm for 5 min.
3. The pellet was resuspended in 4 ml of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0), transferred to a SS34 centrifuge tube and left for 5 min at room temperature.
4. 8 ml of Solution II (0.2 M NaOH, 1% (w/v) SDS) was added. The tube was rolled gently to mix the solutions and was incubated on ice for 5 min.
5. 6 ml of Solution III (5M potassium acetate (KOAc), pH 4.8) was added, the tube was mixed well and incubated for 5 min on ice.
6. The debris was spun down at 15 000 rpm for 15 min and the supernatant decanted into a new SS34 tube.
7. An equal volume (about 20 ml) of isopropanol was added and the tube was centrifuged at 15 000 rpm for 15 min.
8. The pellet was washed with 70% ethanol, the tube was inverted to drain it and the pellet was resuspended in 4.2 ml Tris-EDTA (TE) buffer (pH 8.0). 5.15 g of cesium chloride (CsCl) was added and dissolved and 200 µl of ethidium bromide (EtBr) (10 mg/ml stock) was added.
9. The tube was centrifuged at 13 000 rpm for 15 min to precipitate any remaining protein debris.
10. The refractive index of the solution was adjusted to 1.394.

11. The sample was sealed in a Beckman Quickseal ultracentrifuge tube (5 ml) and centrifuged for a minimum of 6 h at 55 000 rpm in a Beckman Vti 65 rotor at 15 °C.
12. The plasmid band was extracted in the smallest possible volume from the side of the tube using a 1 ml syringe and a 1.2 mm guage needle under long wave UV light (350 nm).
13. The EtBr was extracted at least three times using equal volumes of salt-saturated isopropanol.
14. The DNA solution was divided into 166 µl quantities. Two volumes (333 µl) of water and two total volumes (1 ml) of absolute ethanol were added and the microcentrifuge tubes were spun at 10 000 g in a microcentrifuge for 10 min at room temperature.
14. The pellet was resuspended in 450 µl of TE, 50 µl of 3M sodium acetate (NaAc, pH 5.2) and 1 ml of absolute ethanol. The solution was centrifuged as above and the pellet was washed with 70 % ethanol and resuspended in 100 µl TE.
15. The concentration of DNA was determined spectrophotometrically by measuring the A_{260} between 210 nm and 310 nm of a 1 in a 100 dilution. The relationship A_{260} = 1 for 50 µg/ml of double-stranded DNA was used.

A1.2 Small scale isolation of plasmid DNA (miniprep)

The method is a scaled down version of the above maxiprep method.

1. YT broth containing 100 µg/ml ampicillin was pipetted into Eppendorf microcentrifuge tubes which were then inoculated and grown at 37 °C with vigorous shaking overnight.
2. Cells were harvested by centrifugation at 10 000 g for 1 min.
3. 200 µl of Solution I was added. The tube was vortexed until the pellet was resuspended.

4. 400 µl of Solution II was added, the tube vortexed and incubated for 5 min.
5. 300 µl of Solution II was added, the tube vortexed and after 5 min, the cellular debris was precipitated at 10 000 g for 5 min.
6. The supernatant (750 µl) was removed to a new Eppendorf, 750 µl of isopropanol was added, mixed and spun at 10 000 g for 15 min.
7. The DNA pellet was resuspended in 450 µl TE, to which 50 µl of 3M NaAc and 1 ml of absolute ethanol were added and was spun for 15 min.
8. The pellet was washed with 70% ethanol and resuspended in 20 µl TE.

A1.3 Restriction endonuclease digestion of DNA

Restriction digests were performed according to Sambrook *et al.* (1989). Restriction digests of miniprepped DNA typically contained 2-4 µl of DNA, 1 unit of endonuclease per 100 ng of DNA and 2 µl of the appropriate restriction endonuclease buffer (A2.6) in a total volume of 20 µl. Restriction digests of large quantities of DNA were performed in large (100-200 µl) volumes and then precipitated or electrophoresed on agarose gels for DNA fragment purification. For restriction of *C. acetobutylicum* P262 and *E. coli* JM105 chromosomal DNA, single digests of approximately 10 µg of DNA were performed in 100 µl volumes for 1.5 h. The appropriate digests were mixed, after checking for complete digestion, additional endonuclease was added and the digestion was allowed to proceed for an additional 1.5 h. The enzyme concentration never exceeded 10% of the digest volume and star activity in some endonucleases was taken into account by reducing the incubation time.

A1.4 Phenol cleaning of DNA solutions

DNA restriction digests were phenol cleaned to remove restriction endonucleases before commencing restriction with a second endonuclease requiring different

restriction buffers or conditions. Phenol cleaning was also used to prepare electroeluted DNA fragments for subsequent ligation reactions.

1. The DNA-containing solution was made up to 450 µl with TE in order to increase the working volume so as to minimise any DNA loss.
2. One tenth volume (50 µl) of TE-buffered phenol was added and the tube was vortexed. One volume (500 µl) of chloroform:isoamylalcohol (24:1) was added, the tube vortexed again and spun in a microcentrifuge for 5 s.
3. The top aqueous layer was carefully removed (without any of the protein interface) and extracted twice with equal volumes of chloroform:isoamylalcohol (24:1).
4. The DNA was precipitated by adding 50 µl NaAc to 450 µl of the DNA-containing solution. 1 ml of ethanol was added and microfuged for 10 min at room temperature. The pellet was washed with 70% ethanol and resuspended in TE buffer.

A1.5 Agarose gel electrophoresis of DNA fragments

Agarose gel electrophoresis was carried out using horizontal submerged gels (Sambrook *et al.*, 1989). Sigma Type II agarose was used (0.8%) in Tris-acetate EDTA (TAE) buffer. Electrophoresis loading buffer, containing the dye bromophenol blue, was added to the DNA samples (1/5 volume of the volume to be loaded) before they were loaded into the wells of agarose gels. Long gels were run at 100V constant voltage for about 5 h or overnight at 30V. Slide gels (10 ml agarose in total) were routinely used to check whether DNA digests had cut to completion and were run at 50V for 30 min. Gels contained 25 µl of EtBr (10 mg/ml stock) in 500 ml of agarose (0.5 µg/ml) so that DNA bands could be visualized using a 254 nm transilluminator. If DNA fragments were to be isolated from the agarose gel for subsequent ligation reactions, the 310 nm transilluminator was only used for a few seconds. Gel photography was carried out using a Polaroid

CU-5 Land camera fitted with a red filter. Polaroid type 667 film (ASA 3 000) was used with an exposure time of 1-2 s at f4.7. Standard DNA fragments were obtained by digesting lambda (λ) DNA with *Pst*I restriction endonuclease. The fragment sizes corresponded in kb in descending order to 14; 11.5; 5.08; 4.75; 4.5; 2.84; 2.56; 2.45; 2.14; 1.99; 1.7; 1.16; 1.09; 0.81; 0.51; 0.47; 0.45; 0.34; 0.26; 0.25.

A1.6 Purification of DNA fragments

The Geneclean Kit (Bio 101, La Jolla, California, USA) was used according to the manufacturer's instructions to isolate DNA fragments from agarose gel slices.

DNA fragments were also electroeluted from agarose gel slices:

1. The appropriate agarose gel slice (0.8% agarose) was cut out of the gel under long wave UV light (310 nm).
2. The slice was inserted into an 8 cm length of dialysis tubing, clamped at one end with a plastic dialysis clip.
3. 0.5 ml of Tris-acetate EDTA (TAE) buffer was pipetted into the tubing, air bubbles were squeezed out using gloved fingers and the other end of the tubing was clamped.
4. The tubing was floated in TAE in an electrophoresis tank, secured with masking tape to the sides of the tank. The gel slice was placed so that the DNA could move from the slice into a region of buffer in the tubing under the influence of the electric field.
5. The slice was electrophoresed at 100V for 30 min and the direction of the current reversed for 5 s to release any DNA adhered to the tubing. The TAE buffer inside the tubing was removed into an Eppendorf and the DNA solution was phenol cleaned in order to remove contaminating nucleases.

A1.7 DNA ligation reactions

Ligation reactions were allowed to proceed overnight at room temperature. Ligation reaction volumes were usually 20 µl containing 2 µl of ligation buffer with ATP and 1 unit of T4 ligase.

A1.8 Exonuclease III shortening of insert DNA

1. 12 µg of plasmid DNA was double digested to completion with the appropriate restriction endonucleases generating a 3' overhang which is susceptible to the exonuclease III enzyme and a 5' overhang adjacent to vector sequence which is resistant to the enzyme.
2. The DNA was precipitated and resuspended in 100 µl of exonuclease III buffer.
3. Eleven Eppendorf tubes containing 25 µl per tube of freshly prepared S1 mix were placed on ice.
4. The tube containing the DNA mixture was equilibrated at 37 °C in a water bath and at T = 0 a 9 µl sample was removed and placed in the first of the 11 tubes as an undigested control sample.
5. The shortening reaction was started by the addition of 300 U of exonuclease III to the DNA tube, which was mixed well.
6. While at 37 °C, samples (9 µl) were removed from the DNA tube at 20 s intervals and were added to the S1 tubes and mixed well by vigorous pipetting.
7. The Eppendorf tubes containing the S1 mixes were raised to room temperature and incubated for 30 min.
8. The S1 nuclease was stopped by the addition of 3.5 µl of S1 stop and by placing the tubes at 70°C for 10 min.

9. Whether digestion had in fact occurred was confirmed by the electrophoresis of 5 µl amounts from every second tube on a slide gel. A smearing of the DNA indicated that digestion of the linearized DNA had occurred.
10. 3.4 µl of Klenow mix and 1 µl of klenow enzyme were added to each tube and left for 5 mins

at room temperature to blunt the ends of the DNA fragments.

11. 1 µl of dNTP's (0.125 mM) were added to each tube and left for 15 min at room temperature to "polish" the ends.
12. Half of the sample from each tube was added to 60 µl of ligation mix (ligation buffer, T4 ligase and sterile water) and ligation was allowed to proceed for at least 3 h. Competent *E. coli* cells were transformed followed by minipreparation of plasmid DNA to find the appropriate shortened plasmids.

A1.9 Preparation and transformation of competent *E. coli* cells

The method of Chung and Miller (1988) was used for the transformation of *E. coli* cells:

1. A 1/100 dilution (0.2 ml) of an overnight culture was made into 20 mls of LB and was grown with shaking at 37 °C until the cells reached mid-log phase at an A_{600} of 0.4-0.6 units (in approximately 3 h).
2. The cells were poured into an SS34 centrifuge tube on ice and were collected by centrifugation at 5 000 rpm for 5 min.
3. The pellet was carefully resuspended in 1/10 volume (2 ml) of ice-cold transformation and storage buffer (TSB buffer) and was incubated on ice for a minimum of 30 min.
4. 100 ng of DNA was added to 100 µl of cells in an Eppendorf tube on ice and was left to transform for 30 min.

5. TSB solution (0.5 ml) containing 20 mM glucose was added to each tube and the plasmid-borne antibiotic resistance gene was allowed to express at 37 °C for 30 min. For routine transformation of plasmid DNA expressing the ampicillin gene, no expression at 37 °C was required.
6. 100 µl amounts were plated onto YT plates containing 100 µg/ml ampicillin or the relevant antibiotic.
7. Surplus cells were stored at -70°C and when needed were thawed slowly on ice for routine plasmid transformations (not ligation transformations).

A1.10 Alkali DNA blotting

1. The DNA fragments to be blotted were separated by 0.8% agarose gel electrophoresis in TAE buffer overnight.
2. The DNA was depurinated by soaking the gel in 0.25 M hydrochloric acid (HCl) for 15 to 25 min with gentle shaking or until the bromophenol blue turns yellow.
3. The gel was briefly rinsed in distilled water and then soaked in a solution of 0.5 M NaOH/1.5 M NaCl for 30 min with gentle agitation.
4. A capillary transfer system was set up. The gel was placed on a clean glass plate on top of which was placed the wetted nylon Hybond N^+ membrane. All bubbles were excluded by rolling a glass pipette over the top of the membrane. Three sheets of Whatmann 3MM filter paper were wetted in the 0.5 M NaOH/1.5 M NaCl solution and were placed on top of the membrane, taking care to cut off any overlapping pieces.
5. A thick layer of paper towels were placed on top and weighted and alkali capillary transfer was allowed to proceed overnight without drying out the membrane.
6. The nylon membrane was removed and rinsed in 5×SSC for 5 s to remove any adhering agarose.

7. The membrane was then ready for prehybridization.

A1.11 Preparation of DNA probe and southern hybridization

The method followed was according to the instructions of the manufacturer of the DNA Labeling and Detection Kit Nonradioactive (Boehringer Mannheim (SA)).

A1.11.1 DNA labeling

1. 1 µg of linearized DNA was purified by phenol/chloroform extraction and ethanol precipitation and resuspended in a maximum volume of 5 µl.
2. The DNA was denatured by heating in a water bath at 95 °C for 10 min and then cooling rapidly in an ice/NaCl bath.
3. The following was added to a tube on ice:
   - 1 µg denatured DNA in 5 µl
   - 2 µl hexanucleotide mixture (vial 5 from the kit)
   - 2 µl dNTP labeling mixture (vial 6)
   - 5 µl sterile distilled water (made up to 19 µl)
   - 1 µl klenow enzyme.
4. The tube was incubated for 3 h at 37 °C, incubation overnight can increase the incorporation of label.
5. The reaction was stopped by the addition of 2 µl of EDTA (0.2M at pH 8.0)
6. The labelled DNA was precipitated with 2.5 µl LiCl (4M) and 75 µl of ethanol at -20 °C which was well mixed and left for 30 min at -70 °C.
7. The DNA was centrifuged in a microcentrifuge for 15 min and the pellet was washed with cold 70% ethanol.
8. The pellet was resuspended in TE and added to 20 ml of prehybridization solution in a plastic tube

A1.11.2 Hybridization

1. The membrane was allowed to prehybridize for a minimum of 3 h in prehybridization buffer in a plastic tupperware at 65 °C with gentle shaking and the buffer was then poured off.
2. The probe (in 20 ml volume) was denatured by boiling for 10 min in its plastic tube in a water bath and was then placed straight into an ice slurry.
3. The membrane was placed in a hybridization bag sealed on three sides and the 20 ml of hybridization buffer was added, all the bubbles were squeezed out and the bag sealed.
4. Hybridization was allowed to proceed overnight at 65 °C.
5. The probe was poured back into the 20 ml plastic tube and was frozen at -20 °C for further use.
6. The membrane was given two 5 min washes in 2×SSC/0.1% SDS.
7. The membrane was then rinsed in a small volume of 0.1×SSC/0.1% SDS and then washed twice for 15 min at 65 °C in the same solution.

A1.11.3 Chemiluminescent detection

All volumes are for a membrane size of 100 cm^2

1. The membrane was washed briefly (1-5 min) in wash buffer.
2. It was incubated for 30 min in 100 mls of buffer 2 (which can then be kept at 4 °C for subsequent use).
3. The anti-digoxigenin-AP, Fab fragments were diluted 1:6666.6 in buffer 2 (3 µl in 20 mls) and kept at 4 °C for up to one week.
4. The membrane was incubated with 20 mls of the above for 30 min in a plastic tupperware with gentle agitation.
5. Unbound conjugate was removed by 3 × 10 min washes with 100 mls wash buffer.
6. The membrane was equilibrated for 3 min in buffer 3.

7. The AMPPD® stock solution (10 mg/ml) was diluted 1:200 (200 µl in 40 ml) in buffer 3, which can then be stored in the dark at 4 °C and reused at least 5 times.
8. The membrane was incubated with enough of the above solution to just wet the membrane. The liquid was continually moved over the membrane surface by tilting the tupperware container.
9. The membrane was blotted in between two sheets of blotting paper to remove all wet spots, but not to completely dry it.
10. The damp membrane was sealed in a hybridization bag and was exposed to XAR-5 autoradiographic film for 5-15 min.

A1.12 Protein analysis

A1.12.1 Cell-free transcription/translation

The Promega prokaryotic DNA-directed translation kit was used according to the manufacturer's instructions except that one third of the recommended reaction volumes was used.

1. 3 µg of plasmid DNA suspended in a total volume of 4.7 µl was placed in an Eppendorf tube to which the following was added:
   6.7 µl pre-mix minus methionine
   1 µl ^{35}S-methionine
   5 µl *E. coli* S30 extract.
2. The tubes were incubated in a heating block at 37 °C for 1 h, after which 17 µl of sample treatment buffer was added.
3. The samples were denatured in a water bath by heating to 95 °C for 3 min and were loaded onto a 15% SDS-PAGE gel.

A1.12.2 T7-promoter directed expression system

The method was according to Tabor and Richardson (1985) and Scholz *et al.* (1989).

1. *E. coli* AB1157 cultures, transformed with pGP1-2 and the exonuclease III-shortened clones, were grown overnight in YT in the presence of ampicillin (100 µg/ml) and kanamycin (50 µg/ml).
2. The stationary phase cultures were diluted 1/50 into supplemented M9 medium, containing ampicillin and kanamycin, and grown at 30 °C to early logarithmic phase (A_{600} = 0.2-0.4).
3. 5 ml of each culture was pipetted into a sterilin tube and heated in a water bath at 42 °C for 20 min to induce the T7 polymerase.
4. 40 µl of rifampicin stock (25 mg/ml) was added to a final concentration of 200 µg/ml and the cells were grown for 10 min at 42 °C.
5. The cells were then grown for 20 min at 30 °C.
6. In a 30 °C water bath in the radioactive area, 1 µl of ^{35}S-methionine (10 µCi) was added and the cells were pulse-labeled for 5 min.
7. The tubes were spun at 9 000 rpm for 5 min and the pellet was resuspended in 30 µl of sample treatment buffer and transferred to an Eppendorf.
8. The samples were denatured by heating them to 95 °C for 5 min and 10 µl was then loaded onto a 15% SDS-PAGE gel.

A1.13 Nucleotide sequencing

Nucleotide sequencing was according to the dideoxynucleotide triphosphate chain temination method of Sanger *et al.* (1977). The Sequenase® Version 2.0 DNA Sequencing Kit was used according to the manufacturer's instructions (United States Biochemical) which uses the T7 DNA polymerase (Sequenase®) enzyme.

A1.13.1 Primer annealing reaction

1. 10 µg of freshly maxiprepped DNA in TE buffer was diluted to 18 µl with sterile distilled water.
2. 2 µl of 2M NaOH was added and the tube was incubated at 37 °C for 30 min.
3. The tube was placed on ice immediately and 4 µl of 3M NaAc and 150 µl of absolute ethanol at -20 °C was added.
4. The tube was placed at -70 °C for 15 min and then microcentrifuged for 20 min at 4 °C.
5. 500 µl of 70% ethanol was added and the tube was centrifuged again so that the pellet was spun through the ethanol wash. The supernatant was carefully poured off.
6. The DNA pellet was resuspended in 7 µl of sterile distilled water to which 2 µl of Reaction buffer was added and 1 µl of primer (2 pmol).
7. The tubes were incubated in a heating block at 40 °C for 30 min and were tapped occasionally to mix the contents.
8. The tubes were placed in a beaker of water at 40 °C and were allowed to cool slowly to room temperature. The beaker could also be placed in the fridge overnight.

A1.13.2 Sequencing reactions

1. Termination tubes were first prepared: 1.5 µl of termination mix for one dNTP and 1.0 µl of extension mix was mixed in a single tube, 0.25 µl of DMSO was added (the quantities were scaled up for the number of templates) and then aliquoted in 2.5 µl quantities into termination tubes marked A, C, G or T. These tubes could be stored at 4 °C for a few h.

2. The labeling mix was prepared on ice and the amount per template was:

   1.0 µl DTT

   1.7 µl DMSO

   0.125 µl Pyrophosphatase buffer

   2 µl Labeling mix minus dATP

   1.63 µl Enzyme Dilution Buffer (scaled down if the amount of ^{35}S-dATP was increased).

   0.25 µl T7 polymerase (kept at -20°C)

   0.5 µl ^{35}S-dATP

3. The template tubes were placed in a water bath at 10-18 °C and 8 µl of the labeling mix was added to each tube for exactly 4 min.
4. The termination tubes (A, C, G, T) were prewarmed in a heating block to 48 °C for 30 s.
5. 3.8 µl from the template tube was aliquoted into each of the four termination tubes, mixed by pipetting and incubated for exactly 10 min at 48 °C.
6. 1 µl (0.25 U/µl) of klenow enzyme was added and mixed into each tube and the incubation continued for 4 min.
7. 5 µl of stop solution was added to each tube. Prior to electrophoresis, the samples were denatured at 95 °C for 3 min and then placed on ice.
8. 3-4 µl amounts were run on 6% polyacrylamide gels for 2 to 6 h at 96 Watts using TBE buffer.
9. The gel was dried at 80 °C onto Whatmann 3MM blotting paper using a Dual Temperature Slab Gel Dryer (Hoefer Scientific Instruments, San Francisco, USA) for 60 min and then exposed to XAR-5 autoradiographic film for a minimum of 16 h.

A1.14 RNA work

Care was taken to avoid contamination with ribonucleases:

1. Glassware and pipettes were baked at 200 °C for 4 h. Glassware was also rinsed out with chloroform (in the fume hood) and then rinsed in RNase-free water instead of baking.
2. All plasticware (gel trays, plastic lids) was soaked in a 5% solution of hypochlorite (Jik) for 30 min and then rinsed at least three times with RNase-free water.
3. All water used was Millipore-Q water and was therefore RNase-free.
4. Gloves were used at all times to reduce RNase levels.
5. Chemicals were used off the shelf and tips were not prepared specially.

A1.14.1 RNA isolation from *E. coli* JM105 and *C. acetobutylicum* P262

The method used was according to Aiba *et al.* (1981).

1. An *E. coli* overnight culture was diluted 1:125 (200 µl in 25 ml) in YT and grown for about 3 h until $A_{650} = 0.4\text{-}0.5$.
2. *C. acetobutylicum* spore stock (50 µl) was heat shocked at 70 °C for 3 min and placed straight onto ice. 10 µl was inoculated in the anaerobic box into a hungate tube containing *Clostridium* basal medium (CBM). This tube was placed at 37 °C with shaking for 11 h until an $A_{650} = 0.05$ was reached and then placed on ice overnight. 100 µl of this culture was used to inoculate two 10 ml hungate tubes which were grown for 12 h at 37 °C until $A_{650} = 0.5$.
3. Both cultures were now treated in the same way: 25 ml of culture was poured over about 30 ml of ice in an SS34 centrifuge tube and pelleted at 5 000 rpm for 8 min at 4 °C.

4. The pellet was resuspended in 125 µl of ice-cold 0.3M sucrose/0.01M sodium acetate (pH 4.5) and transferred to a microfuge tube.
5. 125 µl of 0.01M sodium acetate (pH 4.5)/2% SDS was added to the tube. The tube was heated in a 65 °C water bath for 1.5 min.
6. 250 µl of hot phenol (heated to 65 °C in a water bath in a fume hood) was added to the sample, vortexed and incubated for 3 min in the water bath.
7. The tube was placed in -70 °C ethanol in a plastic beaker until frozen and then microfuged for 5 min.
8. The aqueous layer was decanted and extracted with hot phenol two more times.
9. 30 µl of 3M NaAc (1/10 volume) and 900 µl of absolute ethanol (3 volumes) were added to precipitate the RNA. The tube can be stored at -70 °C overnight at this stage.
10. After precipitation for at least 5 min at -70 °C, the tube was spun at 4 °C for 10 min, the pellet washed with 70% ethanol and respun for 10 min across the tube.
11. The pellet was resuspended in 180 µl of Millipore-Q water and 20 µl of 10× DNase buffer:

    200 mM NaAc (pH 4.5)
    100 mM $MgCl_2$
    100 mM NaCl
12. 30U of RNase-free DNase was added and DNA digestion was allowed to proceed for 30 min at room temperature.
13. The DNase was inactivated by the addition of 20 µl of 250 mM EDTA and extracted by the addition of 220 µl of phenol-chloroform (1:1). The tube was vortexed, spun and the top, aqueous layer was decanted.
14. Chloroform-isoamyl alcohol (24:1) was also used to complete the extraction of phenol which may contain contaminating RNases.

15. The RNA was precipitated with 1/10 volume of 3M NaAc and 3 volumes of ethanol and washed with 70% ethanol, as above.
16. The RNA pellet was resuspended in 40 µl Millipore-Q water and was stored at -70 °C. The concentration was determined spectrophotometrically using the relationship $A_{260} = 1$ for 25 µg/ml of single-stranded RNA.

A1.14.2 Denaturing RNA gel electrophorsis

RNA gels were run according to Fourney *et al.* (1988).

1. A 13 × 24 cm gel tray, comb and gel tank were soaked overnight in 5% hypochlorite and rinsed well with Millipore-Q water.
2. 1.5 g agarose and 10 ml 10×MOPS and 87 ml Millipore-Q water were mixed and microwaved to dissolve the agarose.
3. When the mixture was about 70 °C, 5.1 ml of 37% formaldehyde was added in a fume hood (the mixture thickened on addition).
4. The gel was poured and allowed to set for at least 1 h.
5. Prior to loading, the wells were flushed with 1×MOPS (running buffer).
6. Less than 10 µg per lane was run on a slide gel to check the relative concentrations of RNA, 30 µg was run per lane for the Northern blot.
7. A maximum RNA sample volume of 10 µl only, was loaded. The volume should be adjusted to at least 5 µl with RNase-free water
8. 20 µl of Electrophoresis sample buffer was added, the tubes heated to 65 °C for 15 min and then placed straight onto ice.
8. 1 µl of EtBr (1 mg/ml) was added per sample.
9. The gel was run at 30V overnight.

A1.14.3 Random primed labeling of DNA probe with [$^{32}\alpha$P]-dCTP

The DNA probe was prepared according to the instructions of the Random Primed DNA Labeling Kit (No. 1004 760), Boehringer Mannheim (SA).

1. 100 ng of DNA in a 9 µl volume was denatured by heating for 10 min at 100 °C and rapid cooling on ice.
2. The following were added to the microfuge tube on ice:

dATP (vial 2)	1 µl
dGTP (vial 4)	1 µl
dTTP (vial 5)	1 µl
reaction mix (vial 6)	2 µl
[$^{32}\alpha$P]-dCTP	5 µl
H_2O	to 19 µl
Klenow enzyme (2U)	1 µl

3. Labeling was allowed to proceed for 2 h at 37 °C in a heating block.
4. The reaction was stopped by the addition of 2 µl of 0.2M EDTA (pH 8.0).
5. 1 µl of the reaction mixture was diluted in 19 µl of distilled water and was carefully spotted onto two small pieces of filter paper in two 10 µl amounts.
6. The paper was allowed to air dry and one was then washed with ice-cold trichloroacetic acid (TCA) for 10 min to precipitate the DNA.
7. The paper was then given two ethanol washes of 10 min each and then air-dryed. The filter papers were placed in two scintillation vials and the amount of radioactivity present measured in a scintillation counter. The incorporation of radioactivity was the ratio of the amount of radioactivity on the treated filter to the amount on the untreated filter.

A1.14.4 Northern hybridization

Northern hybridization was according to the Hybond N+ protocol for nucleic acid blotting and hybridization (Amersham International, UK).

1. A plastic tupperware box and vacuum blotting equipment was soaked overnight in a 5% solution of hypochlorite and rinsed well.

2. The RNA gel was soaked for 20 min in 0.05M NaOH with gentle agitation and then vacuum-blotted for 45 min onto Hybond N^+ in the same solution.
3. The membrane was rinsed for 1 min in 2×SSPE.
4. Prehybridization and hybridization were performed in: 5×SSPE, 2×Denhardt's solution, 0.1% (w/v) SDS, 50% (w/v) formamide and 0.1 mg/ml denatured salmon sperm. (The pH of the formamide was reduced from approximately 8.5 to 5.5 by equilibration with a Duolite MB6113 mixed resin.)
5. The membrane was rinsed once in 1×SSPE/0.1% SDS (w/v) and then washed for 15 min in the same solution at 60 °C.
6. The membrane was wrapped in clingwrap and exposed to XAR-5 autoradiographic film.

A2. Buffers and solutions

Standard buffers and solutions are from Sambrook *et al.* (1989). Solutions were autoclaved at 121 °C for 15 min or filter sterilized through a 0.22 µm Millipore filter.

A2.1 Tris-EDTA (TE) buffer (pH 8.0)

Tris base	1.21 g
EDTA (0.5 M, pH 8.0)	2.0 ml
Distilled water to	1.0 l

The pH was adjusted to 8.0 with 0.1 M HCl.

A2.2 50 × Tris-Acetate EDTA (TAE) buffer (pH 8.0)

Tris base	242.0 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M, pH 8.0)	100.0 ml
Distilled water to	1.0 l

A2.3 10 × Tris-Borate EDTA (TBE) buffer (pH 8.0)

Tris base	108.0 g
Boric acid	55.0 g
EDTA (0.5 M, pH 8.0)	20.0 ml
Distilled water to	1.0 l

A2.4 Gel electrophoresis loading buffer

0.25% bromophenol blue	0.05 g
25% Ficoll (Type 400)	5.0 ml
EDTA (0.5 M, pH 8.0)	1.6 ml
Distilled water to	20.0 ml

A2.5 Universal restriction enzyme dilution buffer

Stock solution	Final conc	/10.0 ml
Tris-Cl (1 M, pH 7.5)	10 mM	0.1 ml
NaCl (5 M)	50 mM	0.1 ml
Distilled water		5.3 ml
Filter sterilize then add		
2-Mercapto-ethanol	10 mM	7.0 µl
Gelatin (10 mg/ml)	100 µg/ml	0.1 ml
Glycerol	44% (v/v)	4.4 ml

Store at -20 °C.

A2.6 Restriction enzyme buffers

The four buffers at 0, 50, 100 and 150 mM NaCl are stored at -20 °C.

Stock solution	0	50	100	150
Tris-Cl (1M, pH 7.9)	1 ml	1 ml	1 ml	1 ml
$MgCl_2$ (1 M)	1 ml	1 ml	1 ml	1 ml
DTT (0.5)	0.2 ml	0.2 ml	0.2 ml	0.2 ml
BSA (10 mg/ml)	1 ml	1 ml	1 ml	1 ml
NaCl (0.5 M)	0	1 ml	2 ml	3 ml
Sterile water	2.4 ml	1.4 ml	0.4 ml	2.4 ml
Glycerol	4.4 ml	4.4 ml	4.4 ml	4.4 ml

Add all components to a sterilin tube and make up to the 10 ml mark with glycerol.

A2.7 10 × Ligation buffer

Stock solution	Final concentration	/1 ml
Tris-Cl (1 M, pH 7.6)	66 mM	660 µl
$MgCl_2$ (1 M)	6 mM	66 µl
DTT (1 M)	100mM	100 µl
ATP (0.1 M, pH 7.0)	10 mM	100 µl
Distilled water		174 µl

Store at -70 °C.

A2.8 Exonuclease III shortening solutions

1. 10 × S1 buffer

KOAc (3 M)	1.1 ml	
NaCl (5 M)	5.0 ml	
Glycerol	5.0 ml	
$ZnSO_4$	30.0 mg	autoclave

2. Exo III buffer

Tris-Cl (1 M, pH 8.0)	660 µl	
$MgCl_2$ (100 mM)	66.4 µl	
Distilled water	9.27 mls	autoclave

3. S1 mix

10 × S1 buffer	41 µl
Sterile water	258 µl
S1 nuclease	60 U

4. S1 stop

Tris base	(0.30 M)
EDTA	(0.05 M)

Autoclave but do not pH since a high pH is needed to inactivate the S1 nuclease.

5. Klenow mix

Tris-Cl	(20 mM, pH 8.0)	
$MgCl_2$	7 mM	autoclave

6. Ligase mix

10 × ligation buffer	90 µl
T4 ligase	12 µl
Sterile water	618 µl

Enough for 12 tubes. Use 60 µl per tube.

A2.9 Transformation and storage buffer (TSB)

LB	150 ml
PEG (4000)	15 g
$MgSO_4$ (1 M)	1.5 ml
$MgCl_2$ (1 M)	1.5 ml

Dispense into 20 ml amounts and autoclave. Add 1 ml DMSO before use and glucose to a final concentration of 20 mM.

A2.10 DNA hybridization solutions

1. 0.25 M HCl	stock	1 l
HCl (9.1 g/l)	33%	23.8 ml
2. Alkali transfer solution		
NaOH (0.5 M)		20 g
NaCl (1.5 M)		87.7 g
3. Prehybridization and hybridization buffer		
	stock	200 ml
SSC (5 ×)	20 ×	50 ml
Oxoid skim milk powder (1.5%)		3 g
N-lauroylsarcosine, Na (0.1%)		0.2 g
SDS (0.02%)	10%	400 µl
Dissolve for 1-2 h at 50 °C.		
4. 2 × SSC / 0.1% SDS		1 l
SSC (2 ×)	20 ×	100 ml
SDS (0.1%)	10%	20 ml
5. 0.1 × SSC / 0.1% SDS		1 l
SSC (0.1 ×)	20 ×	5 ml
SDS (0.1%)	10%	20 ml
6. Buffer 1		2 l
Maleic acid (0.1 M)	23.2 g	
NaCl (0.15 M)		17.6 g
Adjust pH to 7.5 with NaOH pellets and autoclave.		
7. Wash buffer		1 l
Buffer 1		997 ml
Tween 20 (0.3%)		3 ml

8. Block buffer

1% Oxoid skim milk powder in Buffer 1. Microwave on high for 3 min.

9. Buffer 3	Stock	500 ml
Tris-Cl (0.1 M)	1 M	50 ml
NaCl (0.1 M)	5 M	10 ml
$MgCl_2$ (50 mM)	1 M	25 ml

A2.11 SDS PAGE solutions

All solution recipes are contained in the Hoefer Electrophoresis Catalog and Exercises (1990-1991) pg 123-126, Hoefer Scientific Instruments, San Francisco, California, USA.

After staining the gel for 20-30 min, the gel was destained 3 × in:

Methanol (30%)	300 ml
Acetic acid (10%)	100 ml
Distilled water	600 ml

Gels were stabilised in 3% glycerol for 2 h or overnight.

A2.12 DNA sequencing gel mix

6% acrylamide gel mix was used:

Urea		12.0 g
Bis acrylamide		0.21 g
Acrylamide		4.8 g
10 × TBE		8.0 ml
Distilled water (35 ml)	to	80.0 ml

55 ml of the mix was filtered through a 0.8 µm Millipore filter. 50 µl of TEMED and 50 µl of 50% ammonium persulphate was added and mixed before the gel was poured.

A2.13 Denaturing RNA gel solutions

1. 10 × MOPS/EDTA buffer

MOPS [3-(N-morpholino)propanesulfonic acid] (0.2 M)
Sodium acetate (50 mM)
EDTA (10 mM)
Adjust pH to 7.0 and autoclave

2. RNA electrophoresis sample buffer

Deionized formamide	0.75 ml
10 Ê MOPS	0.15 ml

Formaldehyde	0.24 ml
RNase-free water	0.1 ml
Glycerol	0.1 ml
Bromophenol blue (10%)	0.08 ml

Store at -20 °C in small aliquots.

A3. Media

Solid media contained 1.5% (w/v) agar. Media were autoclaved at 121 °C for 20 min.

A3.1 Luria-Bertani medium (LB)

Bacto typtone	10 g	per litre
Yeast extract	5 g	
NaCl	5 g	

A3.2 Yeast tryptone medium (YT)

Bacto-tryptone	16 g	per litre
Yeast extract	10 g	
NaCl	5 g	

A3.3 *E. coli* M9 minimal media

Na_2HPO_4	6 g	per litre
KH_2PO_4	3 g	
NaCl	0.5 g	
NH_4Cl	1 g	

The pH of the solution was adjusted to pH 7.4 before autoclaving. The following sterile solutions were added after autoclaving:

	Stock	/100 ml
$MgSO_4.7H_2O$	20%	0.1 ml
$CaCl_2.2H_2O$	0.1 M	0.1 ml
Glucose	20%	1 ml

For the T7 expression system 100 ml of medium was supplemented with:

Thiamine (vit B_1)	2 mg/ml	1 ml
18 amino acids (not cys and met)	each 1 mg/ml	100 µl

A3.4 Clostridium basal medium (CBM)

The recipe is according to O'Brien and Morris (1971).

	Stock	/l
Glucose		10 g
Sigma casein hydrolysate		4 g
Difco yeast extract	4 g	
$MgSO_4.7H_2O$	20%	1 ml
$MnSO_4.4H_2O$	1%	1 ml
$FeSO_4.7H_2O$	1%	1 ml
PAB	0.1%	1 ml
Biotin	0.002%	1 ml
Thiamine HCl	0.1%	1 ml
After autoclaving add:		
$NaHCO_3$	10%	10 ml
Cysteine HCl	5%	10 ml
Resazurin stock solution (not for plates)		2 ml

A4. Antibiotics and media additives

	Concentration	Stock
Ampicillin	100 µl/ml	100 mg/ml in water
Kanamycin	50 µl/ml	25 mg/ml in water
Rifampicin	200 µl/ml	50 mg/ml in DMSO
Metronidazole	0-50 µl/ml	5 mg/ml in water

Only kanamycin and metronidazole were filter sterilized. Ampicillin was divided into 1 ml amounts and stored at -20 °C.

IPTG (isopropyl-b-D-thio-galactopyranoside)

IPTG (100mM)	23.4 mg
Distilled water	1.0ml

The solution was aliquoted and stored at -70 °C.

X-Gal (5-bromo-4-chloro-3-indolyl-b-galactoside)

X-Gal (2% w/v)	0.2 g
Dimethylformamide	10.0 ml

The solution was stored at -70 °C.

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