

**Characterization of a transposon-induced
pleiotropic metronidazole resistant mutant of
Clostridium acetobutylicum P262**

Helen Collett

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the Faculty of Science, University of Cape Town

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J. REID

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Dr S.J. Reid
Department of Microbiology
University of Cape Town

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Abstract

Metronidazole is a pro-drug which must be reduced to elicit a bactericidal effect. In the clostridia, some of the electron transport proteins that provide the source of electrons for the reductive activation of metronidazole play a key role in electron distribution, which in turn regulates the direction of carbon flow in the cell. The aim of this research project was to isolate electron transport gene(s) from the solvent-producing *Clostridium acetobutylicum* strain P262, using transposon-induced metronidazole resistance as a selection system. In the process, the feasibility of transposon mutagenesis in this strain, which lacks conventional systems for DNA delivery, was assessed, and the nature of metronidazole susceptibility in the *C. acetobutylicum* wild type was investigated.

The metronidazole resistant transconjugant of interest, referred to as mutant 3R, was shown to harbour a single insertion of the Tn925::Tn917 transposon cointegrate within a structural gene, designated *sum* (susceptibility to metronidazole). The *sum* gene encoded a 334 amino-acid protein which shared 36% identity and 56% similarity with a putative pyruvate-formate lyase (PFL)-activating enzyme identified in the anaerobic archaeobacterium, *Thermococcus litoralis*. The PFL-activating enzyme is a reductase required for post-translational activation of PFL, which in turn catalyses the CoA-dependent cleavage of pyruvate to formate and acetyl-CoA. Northern hybridization analysis and *in vitro* protein synthesis indicated that the *sum* product was translated as a 38 kDa protein from a monocistronic operon. The putative PFL-activase gene was located between an unidentified ORF encoding a membrane-bound protein, and a convergent cluster of stationary phase genes encoding *inter alia* a spore-cortex-lytic enzyme (SCLE), a putative SCLE-processing protease, and a regulatory protein sharing homology with, most notably, the *Bacillus* Sin and bacteriophage ϕ 105 Cro-like proteins.

Physiological characterization of mutant 3R revealed a number of pleiotropic characteristics which included enhanced autolysin activity, increased motility, impaired clostridial cell formation, a moderate decrease in solvent production, loss of sporulation, and resistance to the toxic tripeptide analogue, bialaphos. Mutant 3R also displayed resistance to the DNA damaging agents, UV and bleomycin. The formate-producing pathway in *C. acetobutylicum* is uncharacterized, but as gauged from the pleiotropic phenotype of mutant 3R, is required for cellular differentiation in the organism. The increased capacity for DNA repair appeared to account for the metronidazole resistant phenotype of the mutant.

Abbreviations

A	adenosine
A_x	absorbance at x nm
AB	acetone-butanol
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
bp(s)	base pair(s)
C	cytidine
CBM	<i>Clostridium</i> Basal Medium
cfu	colony forming units
CoA	coenzyme A
CSC	Commercial Solvents Corporation
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	ethylenediaminetetra-acetic acid
Fd	ferredoxin
Fe	iron
g	gram
g	gravitational force
G	guanosine
ΔG	Gibbs free-energy change
h	hour(s)
kb	kilobase pairs
kCal	kilocalories
kDa	kilodaltons
kV	kiloVolts
l	litre(s)
LB	Luria-Bertani broth
log	logarithmic
m	metre(s)
MIC	minimal inhibitory concentration
min	minute(s)
mol	mole(s)
mRNA	messenger RNA

NAD ⁺	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NCP	National Chemical Products
NCIMB	National Collection of Industrial and Marine Bacteria
nm	nanometers
OD _x	optical density at x nm
ORF	open reading frame
ori	origin of replication
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PFL	pyruvate formate-lyase
PFOR	pyruvate:ferredoxin oxidoreductase
RNA	ribonucleic acid
rRNA	ribosomal RNA
s	second(s)
SD	Shine-Dalgarno
SDS	sodium dodecyl sulfate
σ	sigma
T	thymidine
TAE	Tris-acetate EDTA buffer
Tn	transposon
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UV	ultraviolet (light)
v/v	volume/volume
w/v	weight/volume
YT	yeast tryptone medium
::	novel joint (fusion)
()	plasmid carrier state
Δ	deletion

Chapter 1

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Chapter 1

General Introduction

1.1. Introduction to *Clostridium acetobutylicum*

1.1.1 *C. acetobutylicum* research in context

The world oil crisis of 1973 and the Gulf Crisis of 1990-1991 served to illustrate the dependence of the industrial world on inexpensive petroleum reserves. The motivation to dispense with oil-based economies, together with increasing environmental awareness, has revived interest in the *Clostridium acetobutylicum* acetone-butanol (AB) fermentation process. The bioconversion of industrial waste or surplus agricultural crops into refined chemicals is an attractive alternative to the exploitation of fossil fuel reserves.

So far, however, interest in the AB fermentation has been sustained only at the laboratory level (Verhasselt and Vanderleyden, 1993). Unless environmental considerations eventually outweigh economic considerations, the economic viability of the process depends on improved product yields and more importantly, on product selectivity. *C. acetobutylicum* produces five major products from acetyl-CoA via three branch points in the fermentation pathway. Although this metabolic variety is essential to the physiology and adaptability of the organism, *C. acetobutylicum* would only be effective commercially if the fermentation could be directed towards one or two specific products (Rogers and Gottschalk, 1993). The expanding field of clostridial genetics means that genetic manipulation of the clostridia for industrial purposes is now feasible.

1.1.2 Historical background of *C. acetobutylicum* and the acetone-butanol fermentation

The production of acetone and butanol by *C. acetobutylicum* via large scale industrial fermentation was pioneered by Chaim Weizmann at the request of the UK government, following the outbreak of the First World War in 1914 (Jones and Woods, 1986). Acetone, a strategic material required for the manufacture of munitions, was produced from maize mash by a starch-utilizing strain of *C. acetobutylicum*, initially designated BY. After the war, butanol became the main product of interest, and the Commercial Solvents

Corporation (CSC) in the United States acquired the patent rights for the Weizmann acetone-butanol (AB) fermentation process. Following the expiry of the CSC Weizmann patent in 1936, fermentation plants were established worldwide. This coincided with conversion of the starch-based fermentation to a molasses-based fermentation (blackstrap molasses became available as a plentiful and convenient substrate), and necessitated the isolation of effective sugar-fermenting *C. acetobutylicum* strains (Jones and Keis, 1995). The AB fermentation process served as the main supply of industrial solvents until it was replaced by cheaper synthesis from petrochemicals in the early 1960's. Escalation in the price of the molasses substrate due to its development as a cattle feed supplement also helped to cripple the economic viability of the process. The AB fermentation process continues to operate in the People's Republic of China, however.

1.1.3 Taxonomy of *C. acetobutylicum* strains

C. acetobutylicum has become the saccharolytic, solventogenic representative of the clostridia. At present, the genus *Clostridium* comprises a heterogenous group of Gram-positive, anaerobic, non-sulphate-reducing, endospore-forming bacilli (Cato and Stackebrandt, 1989). It is estimated that the clostridia alone represent 1.2-1.5 billion years of evolutionary distance (Ochman and Wilson, 1987), and therefore some of the 100 species currently recognised (Hippe *et al.*, 1992) are, technically speaking, as divergent from each other as yeast species are from human beings. Furthermore, recent phylogenetic studies based on comparative 16s RNA sequence analysis show that many non-sporeforming bacteria are relatives of the *Clostridium* species (Lawson *et al.*, 1993; Collins *et al.*, 1994), and confirms that classification of the clostridia requires major revision.

Although the majority of industrial solventogenic strains are classified as *C. acetobutylicum*, there has been increasing evidence for differences in their physiology and genetic properties (Wilkinson and Young, 1993; Johnson and Chen, 1995; Wilkinson *et al.*, 1995b). A recent systematic study combining biotyping procedures, DNA fingerprinting and 16S rRNA gene sequence analysis (Keis *et al.* 1995), has provided a scientific basis for reclassification, and indicates that commonly used laboratory strains of the solvent-producing clostridia can be divided into four taxonomic groups as shown in Fig.1.1. Group I is represented by

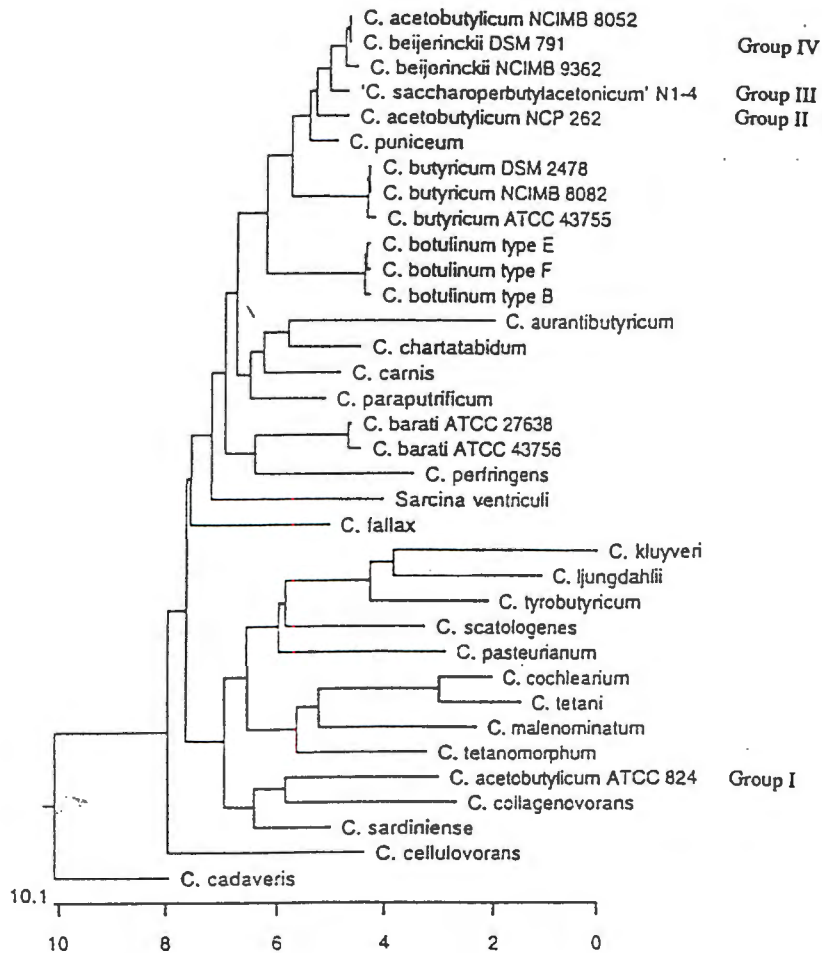


Fig. 1.1. Unrooted phylogenetic dendrogram showing the relationships of commonly used laboratory strains of *C. acetobutylicum* to other clostridial species (Keis *et al.*, 1995). The dendrogram is based on comparison of the complete 16S rRNA gene sequences (positions 100 to 1434 of the *E. coli* numbering system), and the scale bar indicates the evolutionary distances.

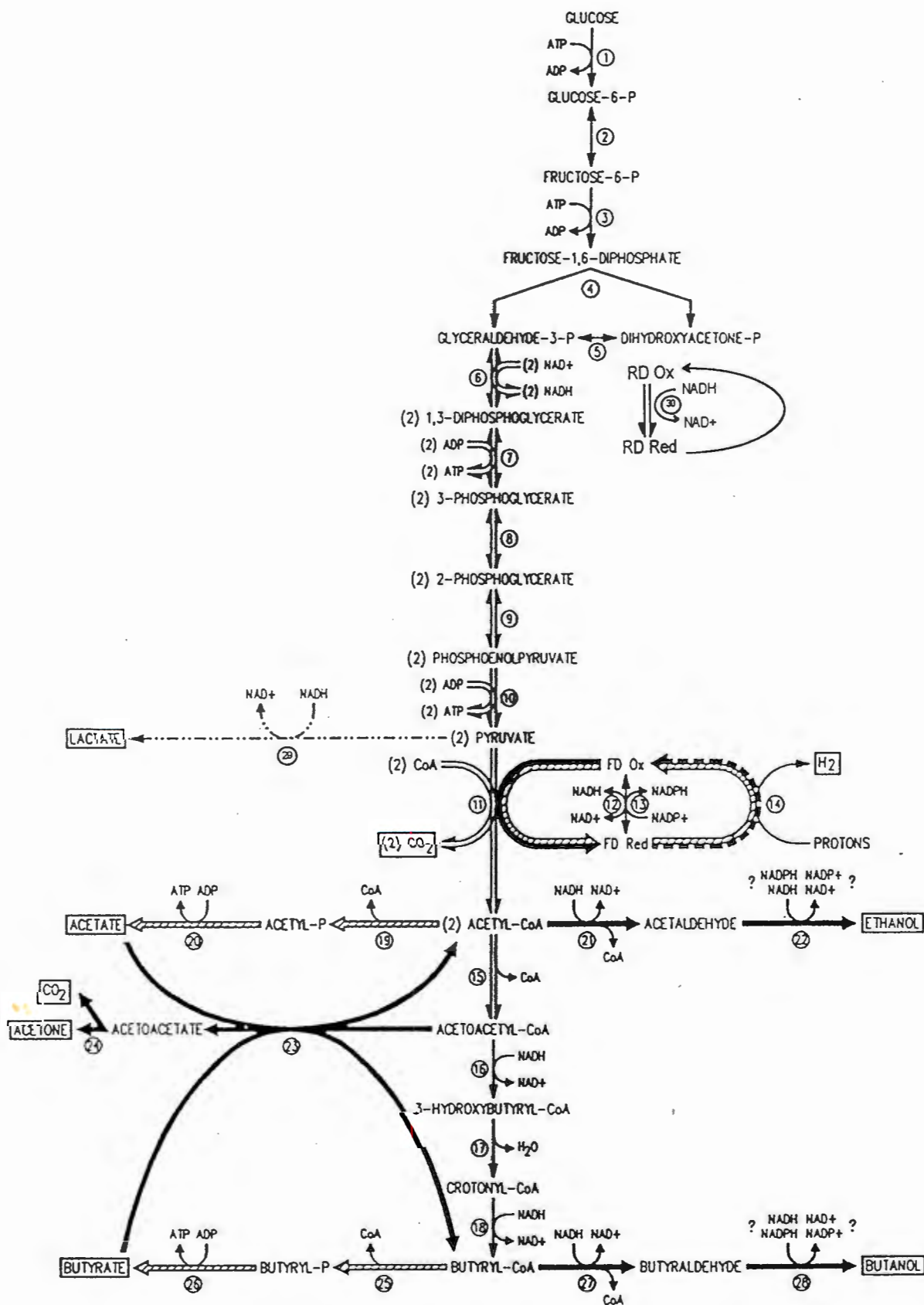
ATCC 824, the *C. acetobutylicum* prototype, and includes strain DSM 792 (the DSM culture collection equivalent) and strain DSM 1731, a descendent of the original patented Weizmann strain. Group II is represented by P262, an NCP production strain derived from *Clostridium saccharo-butyl-aceticum-liquifaciens* (patented by CSC). Group III consists of "*Clostridium saccharoperbutylacetonicum*" strains e.g. N1-4, and Group IV includes *C. acetobutylicum* NCIMB 8052 and *Clostridium beijerinckii*.

The historical background of the AB fermentation is significant with respect to the origins and taxonomic relationships of *C. acetobutylicum* strains (Jones and Keis, 1995; Johnson and Chen, 1995). There is a notable division between the starch-utilizing *C. acetobutylicum* strains (Group I) and the sugar-utilizing strains (Groups II-IV). This demonstrates that the transition from a starch-based fermentation process to a sugar-based fermentation process selected for organisms that were genetically distinct from *C. acetobutylicum*. Groups II - IV are closely related to *Clostridium butyricum* and *Clostridium botulinum*, and only distantly related to *C. acetobutylicum*.

Official reclassification of the solvent-producing clostridia is imminent, and in recent publications, *C. acetobutylicum* NCIMB 8052 is now referred to as *C. beijerinckii* NCIMB 8052. It is possible that the original patent name *C. saccharo-butyl-acetonicum-liquefaciens* may be retained to define *C. acetobutylicum* P262 (the laboratory strain used in this study), and other members of taxonomic group II as a new species.

1.2 Fermentative metabolism

A normal *C. acetobutylicum* batch fermentation is characterized by two distinct phases (Jones and Woods, 1986). During the exponential growth period, cells convert sugars to acids (predominantly acetate and butyrate) with the concomitant production of carbon dioxide and hydrogen. This is followed by a metabolic shift as the culture enters the stationary growth phase. Residual sugars and preformed acids are then converted to solvents (acetone and butanol) via reduction reactions. Small amounts of ethanol are produced throughout the fermentation. Solventogenesis is thought to be a response to acid toxicity (Kim and Zeikus, 1985) and can be triggered by a combination of low pH and high intracellular concentrations of undissociated acids (Terracciano and Kashket, 1986). The undissociated acids act as uncouplers, resulting in a collapse of the pH gradient. The biochemical pathways of acidogenesis and solventogenesis in *C. acetobutylicum* are well-formulated (Fig.1.2), and most of the relevant enzymes have been purified and/or the corresponding genes isolated from various species (Chen, 1993; Papoutsakis and Bennett 1993; Chen, 1995; Dürre *et al*, 1995).



1.2.1. Central pathways

Solvent production is linked to acid production through shared metabolic intermediates. These key intermediates in the branched fermentation pathways are acetyl-CoA, acetoacetyl-CoA and butyryl-CoA. Acetyl-CoA, generated by the phosphoroclastic cleavage of pyruvate, is considered to be the starting compound for the acidogenic and solventogenic pathways, and is converted by the thiolase (acetyl-CoA acetyltransferase) to acetoacetyl-CoA (Fig. 1.2, reaction 15). This enzyme is important in controlling the ratio of two-carbon products (acetate and ethanol) to four-carbon products (butyrate, acetone and butanol). The identification of two different thiolase genes in *C. acetobutylicum* DSM 792 (Winzer and Dürre, 1994) suggests that one enzyme is specific for acidogenesis and the other for solventogenesis (Dürre *et al.*, 1995). The sequential action of three enzymes, viz. 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase, reduces acetoacetyl-CoA to butyryl-CoA (Fig. 1.2, reactions 16, 17 and 18). The fate of the three precursors differs for acid and solvent formation, depending on the direction of electron and carbon flow.

1.2.2 Acidogenic pathways

During the acid-producing phase, acetate and butyrate are produced from acetyl-CoA and butyryl-CoA, respectively, via a parallel set of reactions, resulting in ATP formation. The CoA intermediates are first converted to a phosphorylated form by a phosphate acyltransferase (Fig. 1.2, reactions 19 and 25), followed by enzymatic hydrolysis by a kinase to generate the relevant acid and ATP (Fig. 1.2, reactions 20 and 26). A unique set of enzymes is responsible for each of the analogous pathways. The phosphotransbutyrylase and butyrate kinase genes of the butyrate-synthesis pathway have been cloned and sequenced (Oultram *et al.*, 1993; Walter *et al.*, 1993). The corresponding enzymes exhibit reversible activity under certain fermentation conditions e.g. carbon-monoxide sparging (Hartmanis and Gatenbeck, 1984; Hüsemann and Papoutsakis, 1989).

1.2.3 Solventogenic pathways

During the solvent-producing phase, ethanol and butanol are produced from acetyl-CoA and butyryl-CoA, respectively, also via an analogous set of reactions. The process requires the sequential action of two dehydrogenases, viz. an aldehyde dehydrogenase (Fig 1.2, reactions

21 and 27) and an alcohol dehydrogenase (Fig. 1.2, reactions 22 and 28), and excess NAD(P)H generated by glycolysis serves as the reducing cofactor. Although NAD(H) and NADP(H) have similar physicochemical properties, their physiological roles differ greatly, as the former is mainly involved in oxidative reactions for energy conservation, whereas the latter is required for reductive biosynthetic reactions (Chen, 1995). The identification of *C. acetobutylicum* alcohol dehydrogenases with different coenzyme specificities indicates a difference in the regulatory aspects of these enzymes. Elucidation of the role of the individual alcohol dehydrogenases is complicated by their overlapping substrate specificities.

Ethanol production is constitutive (Dürre *et al.*, 1987; Gerischer and Dürre, 1992) and is independent of butanol formation (Bertram and Dürre, 1989; Babb *et al.*, 1993). Ethanol formation in *C. acetobutylicum* DSM 792 requires the activity of a specific NADH-dependent acetaldehyde dehydrogenase and a NADPH-dependent alcohol/ethanol dehydrogenase (Bertram *et al.*, 1990). The structural gene (*adh-1*) for the latter enzyme has been cloned from *C. acetobutylicum* P262 (Youngleson *et al.*, 1988, 1989). The physiological function of the enzyme may be to regulate the pool of NADPH which is required for biosynthesis and is generated by the NADPH-ferredoxin oxidoreductase (Dürre *et al.*, 1995) (see Section 1.3).

The enzymology of butanol formation is complex. Three different butanol dehydrogenases have been identified in the *C. acetobutylicum* strains DSM 792 and ATCC 824. These are the two NADH-dependent butanol dehydrogenase iso-enzymes I and II encoded by the *bdhA* and *bdhB* gene cluster (Petersen *et al.*, 1991; Walter *et al.*, 1992), and the putative multifunctional aldehyde/alcohol dehydrogenase encoded by the *adhE* or *aad* gene of strains DSM 792 and ATCC 824, respectively (Fischer *et al.*, 1993; Nair and Papoutsakis, 1994). The latter enzyme is presumably responsible for the concomitant decrease or loss in NADH-dependent butyraldehyde and butanol dehydrogenase activities in butanol-defective mutants of *C. acetobutylicum* DSM 792 and ATCC 824 (Clark *et al.*, 1989; Bertram *et al.*, 1990), as the cloned *aad* gene restores butanol production and butyraldehyde activity (Nair and Papoutsakis, 1994). The different order of induction observed for the three butanol dehydrogenase genes implies different physiological roles for the enzymes e.g. high and low level butanol production (Sauer and Dürre, 1995).

The *adhE* gene, together with the genes encoding the two subunits of the CoA transferase (*ctfA* and *ctfB*) forms part of the *sol* operon. The gene encoding acetoacetate decarboxylase (*adc*) is divergently and independently transcribed. The CoA transferase and acetoacetate decarboxylase are responsible for acetone production from acetoacetyl-CoA, the second key intermediate in the fermentation pathway (Fig. 1.2, reactions 23 and 24). Acetone is in fact a by-product of a system for recycling accumulated acids to form alcohols (Rogers and Gottschalk, 1993), and so acetone production is directly coupled to the reassimilation of acetate and butyrate. Reassimilation of acids via the CoA transferase results in formation of predominantly butyryl-CoA and some acetyl-CoA. Small amounts of butyrate can also be converted back to butyryl-CoA by the reverse action of the butyrate kinase and the phosphotransbutyrylase (Hüsemann and Papoutsakis, 1989). Butyryl-CoA is then converted to butanol by the multifunctional butyraldehyde/butanol dehydrogenase, and acetyl-CoA is condensed to acetoacetyl-CoA, presumably by a solvent-specific thiolase. The sequential action of the CoA transferase and the aldehyde/alcohol dehydrogenase justifies their genetic organization in a common transcription unit, the *sol* operon (Dürre *et al.*, 1995). The acetoacetate decarboxylase serves only to channel the CoA transferase in the thermodynamically less favourable direction of butyryl-CoA formation, and this might explain the separation of the two structural genes for acetone formation into two different operons (Dürre *et al.*, 1995).

1.2.4 Subsidiary pathways

Side pathways that are responsible for the formation of acetoin and lactate can compete with the conversion of pyruvate to acetyl-CoA. Pyruvate is reduced to lactate by the action of the lactate dehydrogenase (Fig. 1.2, reaction 29). The *C. acetobutylicum* lactate dehydrogenase is a non-reversible enzyme and is activated by fructose 1,6-diphosphate (FDP) (Contag *et al.*, 1990). Lactate is a major fermentation product when the mechanism for the disposal of electrons and protons via hydrogen evolution is blocked e.g. by inhibition of hydrogenase activity via iron limitation or carbon monoxide sparging (Kim and Zeikus, 1985; Meyer *et al.*, 1986). Bahl *et al.* (1986) have shown that in a continuous culture subject to iron or sulfate limitation, acetone and butanol are produced at pH 4.5, whereas lactate production is favoured above pH 5.0. The lactate dehydrogenase gene has been cloned from *C. acetobutylicum* B643 by complementation of an *Escherichia coli*

fermentation-defective mutant and appears to compete with the pyruvate-formate lyase (PFL) of the *E. coli* host (Contag *et al.*, 1990).

Acetoin (3-hydroxy-2-butanone) is a structural analogue of acetoacetate and can therefore compete with acetone production. Although not an acid, it is produced mainly during the acidogenic phase, also when electron disposal is blocked (Doremus *et al.*, 1985). The acetoin biosynthetic pathway is uncharacterized, but is linked to the formation of lactate. In butanediol-producing organisms, it is derived from pyruvate by the successive action of acetolactate synthetase and acetolactate decarboxylase (Störmer, 1975). Doremus *et al.* (1985) propose that in *C. acetobutylicum*, acetoin formation might occur by the reaction of lactic acid with acetyl-CoA.

1.3 Carbon and electron flow

1.3.1 Energy and electron distribution

Growth of anaerobic organisms via fermentation is restricted by the limited availability of ATP (Thauer *et al.*, 1977). The complexity of the branched fermentation pathways in *C. acetobutylicum* reflects the ability of the organism to adapt to energy limitation. ATP is required for 1) biosynthesis and 2) membrane energization via the proton-translocating ATPase, to generate the proton motive force (electrochemical gradient across the cell membrane) which is essential for metabolic function (Herrero, 1983).

Another problem encountered by anaerobic bacteria which ferment substrates is the disposal of reducing power. An elegant route for the oxidation of reducing equivalents in the clostridia is the evolution of hydrogen via the phosphoroclastic reaction (refer to Fig.1.2). During the conversion of pyruvate to acetyl-CoA by the pyruvate:ferredoxin oxidoreductase (PFOR), electrons are transferred to ferredoxin (Fig. 1.2, reaction 11). These electrons are in turn donated to the hydrogenase which couples them with protons to form molecular hydrogen, the terminal electron acceptor (Fig. 1.2, reaction 14). The NADH-ferredoxin oxidoreductase acts in combination with this reaction and is responsible for the oxidation or reduction of NAD by equilibration of electrons between NAD and ferredoxin (Fig. 1.2, reaction 12). An analogous enzyme is the NADPH-ferredoxin oxidoreductase which

catalyses the production of NADPH, required for biosynthesis, from reduced ferredoxin (Fig. 1.2, reaction 13). Ferredoxin is therefore an important electron carrier, and acts as the fulcrum for electron distribution in the cell. Vasconcelos *et al.* (1994) propose that the so-called NADH-ferredoxin oxidoreductase in fact represents two distinct enzymes, viz. a NADH-ferredoxin reductase responsible for reduction of ferredoxin, and a ferredoxin-NAD⁺ reductase active for reduction of NAD (see Section 1.3.2). An alternative route for the disposal of reducing equivalents is in the form of electron sink products such as solvents or lactate.

The regulation of energy and electron flow during acidogenic and solventogenic metabolism has been reviewed by Jones and Woods (1986), and is outlined as follows. During glycolysis, *C. acetobutylicum* generates less ATP and more NAD(P)H than is required for biosynthesis and growth. Additional ATP is generated via the production of acids, and thus acid-producing pathways play a major role in energy metabolism. While the carbon flow is directed to acid and energy production, most of the electron flow is directed to hydrogen production, and during this time hydrogenase activity is at a maximum. Some of the excess NADH produced during glycolysis is removed via the oxidoreductase pathway to stimulate hydrogen production, or is consumed via butyrate synthesis. Hydrogen production serves as an efficient route for the disposal of electrons and protons until acids accumulate.

Towards the end of acidogenesis, ATP concentrations drop considerably as ATP is redirected from biosynthesis to be consumed by ATPase as protons are extruded across the cell membrane to maintain the pH gradient (ΔpH). The role of the ATPase in the maintenance of ΔpH in *C. acetobutylicum* has been demonstrated (Riebeling *et al.*, 1975; Bowles and Ellefson, 1985). There is also a large increase in NADH and NADPH concentrations prior to the induction of solventogenesis (Grupe and Gottschalk, 1992). Solventogenesis is accompanied by a decrease in hydrogen production and an increase in carbon dioxide production. To accommodate the decrease in hydrogen evolution, electron and carbon flow are redirected to the production of solvents. Excess NADH generated during glycolysis is now used in alcohol formation and the flow of electrons from reduced ferredoxin is shifted to the generation of NADH and NADPH via the appropriate ferredoxin oxidoreductase.

Apart from the contribution of ATP and coenzymes in directing metabolism, intermediate metabolites such as acetyl-CoA also play a significant role in enzyme regulation. For example, increased levels of butyryl-CoA and decreased levels of CoA and acetyl-CoA have been shown to be associated with the transition to solventogenesis (Boynton *et al.*, 1994).

1.3.2 Regulation of carbon and electron flow

As outlined above, ATP and NAD(P)H concentrations are important in product selectivity, and the ability of *C. acetobutylicum* to alter its fermentation strategy can be viewed as an adaptation of the organism to balance the levels of both these metabolites. Much effort has been directed at defining the metabolic shift-inducing conditions responsible for the onset of solventogenesis. Studies by Soucaille and co-workers on the metabolic flexibility of *C. acetobutylicum* in chemostat culture serve to highlight the complexity of electron and carbon flux, and indicate that the scenario presented in Section 1.3.1 is oversimplified. A review article by Girbal *et al.* (1995) provided the basis for much of the information presented here.

1.3.2.1 Electron, carbon and energy flux

For continuous cultures of *C. acetobutylicum*, two distinct sets of conditions under which alcohol formation may be initiated have been identified, resulting in either classical solventogenic metabolism (acetone, butanol and ethanol) or alcohologenic metabolism (butanol and ethanol only). As for a typical batch fermentation, initiation of classical solventogenesis is brought about by low pH or addition of organic acids (Bahl *et al.*, 1982, 1986; Roos *et al.*, 1985; Terracciano and Kashket, 1986; Meyer and Papoutsakis, 1989a, 1989b; Grupe and Gottschalk, 1992), and is associated with high ATP and butyric acid concentrations and normal to low levels of NAD(P)H. On the other hand, induction of an alcohologenic metabolism is related to high NAD(P)H levels and variable ATP concentrations (Girbal *et al.*, 1995). It is achieved by growth on glucose-glycerol or glucose-glycerol-pyruvate substrates at neutral pH (since glycerol, a more reduced substrate, generates extra NAD(P)H in its catabolism) (Vasconcelos *et al.*, 1994), or by inhibiting hydrogen production. *In vivo* activity of the hydrogenase enzyme can be artificially decreased by: 1) an increase in hydrogen partial pressure (Doremus *et al.*, 1985; Yerushalmi and Volesky, 1985), 2) iron limitation (Bahl *et al.*, 1986; Junelles *et al.*, 1988), 3) addition

of an artificial electron carrier e.g. methyl viologen or neutral red (Rao and Mutharasan, 1986, 1987; Kim and Kim, 1988; Peguin *et al.*, 1994, 1995; Girbal *et al.*, 1995), and 4) carbon monoxide sparging (Kim *et al.*, 1984; Datta and Zeikus, 1985; Meyer *et al.*, 1986; Meyer and Papoutsakis, 1989a). Carbon monoxide is a reversible inhibitor of hydrogenase which reacts with iron in the active site of the enzyme to form an FeCO complex (Thauer *et al.*, 1974). Under iron limitation, the hydrogenase is not synthesised or is non-functional (Junelles *et al.*, 1988), whereas methyl viologen replaces ferredoxin as the substrate for the ferredoxin-NAD(P)⁺ reductase, thereby increasing its activity 60-fold (Peguín and Soucaille, 1995). Peguin and Soucaille (1995) have demonstrated that the effects of iron limitation and methyl viologen are additive and that they have different, yet complementary sites of action. Alteration of electron flow by inhibition of hydrogenase activity correlates not only with an alcohologenic fermentation, but also with enhanced lactate production (Datta and Zeikus, 1985; Bahl *et al.*, 1986) (see Sections 1.2.4 and 1.7.5). Moreover, high NADH levels were shown to inhibit the activity of glyceraldehyde-3-phosphate dehydrogenase, a key enzyme in the glycolytic pathway (Girbal and Soucaille, 1994). It is appropriate that high NAD(P)H levels should stimulate the exclusive production of alcohols for NAD(P)H consumption, since acetone formation does not require NAD(P)H, and is thus a redox neutral pathway.

1.3.2.2 Regulation of enzyme expression

The solventogenic and alcohologenic metabolic shift systems are characterized by two different electron transport and solventogenic enzyme profiles and this provides evidence for the existence of two different mechanisms for butanol production represented in Fig.1.3 (Girbal *et al.*, 1995). Classical solventogenesis is associated with high-level expression of the NADPH-dependent alcohol and butyraldehyde dehydrogenases, as well as the enzymes responsible for acetone formation, viz. CoA transferase and acetoacetate decarboxylase (Fig. 1.3A). In addition, the *in vitro* activity of the hydrogenase is decreased, and surprisingly, there is no expression of the ferredoxin-NAD(P)⁺ reductases which provide an alternative route for regeneration of ferredoxin. By contrast, exclusive production of alcohols by glycerol-grown or neutral red-treated cultures is accompanied by high-level expression of the ferredoxin-NAD(P)⁺ reductases and the NADH-dependent alcohol and butyraldehyde dehydrogenases, and decreased activities of the NADH-ferredoxin reductase (Vasconcelos *et al.*, 1994; Girbal *et al.*, 1995) (Fig. 1.3B). Sauer and Dürre (1995) have

also observed, by transcription analysis, that the artificial induction of butanol formation with methyl viologen is mediated by a different group of enzymes. There appears to be no expression of the *bdhA* and *bdhB* genes (encoding NADH-dependent butanol dehydrogenase isozymes I and II), the *sol* operon (encoding the alcohol/aldehyde dehydrogenase and the CoA transferase) and the *adc* gene (encoding the acetoacetate decarboxylase). Instead, activity of a NAD(P)H-dependent aldehyde dehydrogenase and a NADH-dependent alcohol dehydrogenase is increased.

It is interesting that no ferredoxin-NAD(P)⁺ reductase activities were detected for solventogenic metabolism, yet NAD(P)H is produced. Girbal *et al.* (1995) propose that NAD(P)H synthesis from reduced ferredoxin occurs via an alternative membrane-bound electron and proton transport system. Another possibility which has not been addressed by these authors is that the non-sulfur protein, rubredoxin may substitute for ferredoxin in reduced pyrimidine biosynthesis (Fig. 1.2, reaction 30). Rubredoxin and NADH-rubredoxin oxidoreductase activity have been demonstrated in many solvent-producing clostridia, including *C. acetobutylicum* (Marczak *et al.*, 1983a; Petitdemange *et al.*, 1983). Unlike ferredoxin which is constitutively produced, high levels of rubredoxin correlate with the induction of solventogenesis (Marczak *et al.*, 1985). The rubredoxin oxidoreductase is positively regulated by low pH and high acetic acid concentrations (Petitdemange *et al.*, 1981) i.e. the same conditions which induce solventogenic metabolism.

For induced alcohologenic metabolism there is no change in hydrogenase activity and so the decrease in hydrogen production is ascribed to increased activities of the ferredoxin-NAD⁺ reductases and decreased activity of the NADH-ferredoxin reductase. This represents a shift in redox state in which reduced coenzymes are produced at the expense of hydrogen production. Girbal *et al.* (1995) propose that the subsequent increase in the NADH/NAD⁺ ratio may serve as the signal for decreased expression of the NADH-ferredoxin reductase gene and increased expression of the ferredoxin-NAD⁺ reductase and the NADH-dependent dehydrogenase genes (Fig. 1.3 B).

1.3.2.3 Regulation of transmembrane pH gradient

The solventogenic and alcohologenic cultures each employ a different mechanism for

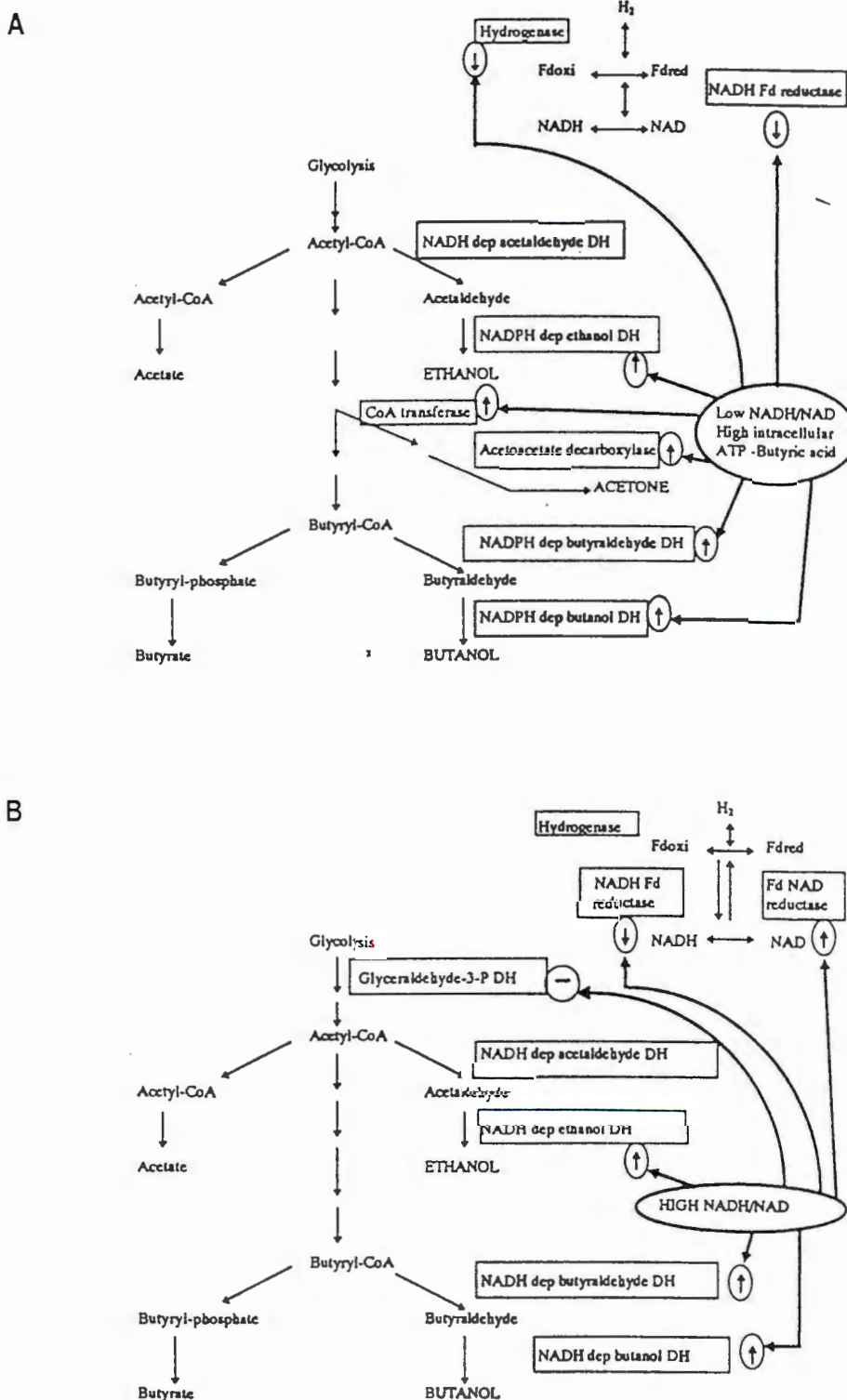


Fig. 1.3. Schemes of the regulatory mechanisms involved in carbon and electron flow orientation in continuous phosphate-limited steady-state cultures of *C. acetobutylicum* ATCC 824 during solventogenic metabolism at pH 4.4 (A) and alcohologenic metabolism (B) (Girbal *et al.*, 1995). [\uparrow] high-level enzyme expression; [\downarrow] low-level enzyme expression; [-] inhibition at the enzymatic level.

regulation of the intracellular pH (Girbal *et al.*, 1994, 1995). Maintenance of the transmembrane pH gradient in solventogenic cultures is not linked to ATPase or hydrogenase activity, since inhibition of these enzymes does not affect the ΔpH (Girbal *et al.*, 1995). This evidence for the existence of some other proton extrusion mechanism is substantiated by a previous report showing that inhibition of ATPase results in only partial dissipation of the ΔpH (Huang *et al.*, 1985). These authors suggested that rubredoxin participates in proton extrusion, since both rubredoxin and NADH-rubredoxin oxidoreductase are induced at low pH.

Induced alcohologenic metabolism is characterized by inversion of the transmembrane pH gradient. In other words, cells maintain their intracellular pH at a lower value than the extracellular pH, resulting in a negative ΔpH value. This is related to the decreased rate of hydrogen production, since the hydrogenase appears to be involved in the alkalization of the cytoplasm via proton consumption (Girbal *et al.*, 1994). In the absence of hydrogenase activity, protons accumulate and under these conditions, the cell appears to maintain a constant proton motive force by interconverting chemical and electrical potentials.

The features of the two different metabolic shift mechanisms characterized by Girbal *et al.* (1995) are summarised in Table 1.1 for easy reference. It is apparent that only the alcohologenic fermentation is associated with increased availability of reducing power and that ATP is not a key factor in determining product selectivity, as previously thought (Papoutsakis, 1983) (See Section 1.3.1). The results of this study reiterate the important role played by the ferredoxin-NAD(P)⁺ reductases and the hydrogenase enzyme in regulating electron and carbon flow, and the way in which their activity determines the fate of reduced ferredoxin. In addition, the continued production of NAD(P)H in the absence of ferredoxin-NAD(P)⁺ reductase activity during solventogenic metabolism suggests that a third system is operational for regeneration of reduced ferredoxin. Girbal *et al.* (1995) propose that this system is also responsible for ΔpH regulation in a solventogenic culture.

1.3.3 Cloning of *C. acetobutylicum* electron transport genes

The hydrogenase gene from *C. acetobutylicum* P262 has recently been cloned and sequenced (Santangelo *et al.*, 1995). It was isolated by heterologous hybridization, using

Table 1.1. Features of solventogenic and alcohologenic metabolism in *C. acetobutylicum*

Solventogenic metabolism	Alcohologenic metabolism
<ul style="list-style-type: none"> • Normal-low NAD(P)H • High ATP • High butyric acid • Acetone, butanol, ethanol • Low H₂; low hydrogenase activity • No ferredoxin-NAD⁺ reductase activity • High NADPH-dependent alcohol and butyraldehyde dehydrogenase activity • High CoA transferase and acetoacetate decarboxylase activity • No inversion of ΔpH 	<ul style="list-style-type: none"> • High NAD(P)H • Variable ATP • Addition of neutral red or growth on reduced substrates • Butanol, ethanol • Low H₂; hydrogenase activity constant • High ferredoxin-NAD⁺ reductase activity • High NADH-dependent alcohol and butyraldehyde dehydrogenase activity • No CoA transferase and acetoacetate decarboxylase activity • Inversion of ΔpH

the *Clostridium pasteurianum* hydrogenase-1 gene as a probe, and has been shown to be constitutively expressed during the course of a typical batch fermentation. This indicates, as do the results of Girbal *et al.* (1995), that the NADH-ferredoxin oxidoreductase and possibly an alternative NADH cycling system are the sites of solventogenesis-associated regulation in *C. acetobutylicum*. The flavodoxin gene has also been isolated from *C. acetobutylicum* P262 via a cloning system in which metronidazole was used as a selection tool and *E. coli* F19 served as the selection host (Santangelo *et al.*, 1991). The low molecular weight electron carrier, flavodoxin, can substitute for ferredoxin in the *C. acetobutylicum* phosphoroclastic reaction under conditions of iron limitation (Meinecke *et al.*, 1989). Metronidazole is a drug which is activated by chemical/enzymatic reduction and can accept electrons from components of the electron transfer network of anaerobic organisms.

Electron transfer occurs from those proteins with sufficiently low redox potential to metronidazole, a molecule with a more positive redox potential. The role of the clostridial hydrogenase enzyme and the ferredoxin and flavodoxin proteins in metronidazole activation has been demonstrated (Chen and Blanchard, 1979; Lockerby *et al.*, 1984; Church *et al.*, 1988). Metronidazole will be discussed in detail in Section 1.7.

1.4 Cell differentiation and sporulation

1.4.1 Link between solventogenesis, sporulation and heat shock response

Sporulation (the formation of highly resistant spores) is viewed as an adaptive response which ensures survival of the microorganism under unfavourable conditions. The onset of solventogenesis in *C. acetobutylicum* is strongly linked to endospore formation (Long *et al.*, 1984*a*, 1984*b*; see Section 1.4.3), but is not a sporulation-specific event. This is evidenced by the isolation of asporogenous solvent-producing mutants (Jones *et al.*, 1982; Meinecke *et al.*, 1984; Babb *et al.*, 1993) and sporulating asolventogenic mutants (Jones *et al.*, 1982; Babb *et al.*, 1993). It is possible that both processes are initiated simultaneously in response to acid stress, as there are indications that sporulation in *C. acetobutylicum* is triggered by the same factors which induce solvent formation e.g. a decrease in pH and an accumulation of acid end products (Long *et al.*, 1984*b*; Terracciano and Kashket, 1986; Babb, 1993). Solventogenesis may provide *C. acetobutylicum* with a temporary mechanism to overcome the toxic effects of accumulated acid end products, until the time-consuming process of sporulation is complete (Dürre *et al.*, 1995). The sporulation process must in turn be completed before the cell succumbs to the toxic effects of accumulated solvents. Butanol is the most toxic of the solvents (Hermann *et al.*, 1985) and the mechanism of toxicity is associated with the hydrophobic nature of the compound. It exerts a chaotropic effect on the cell membrane, causing leakage of ions and other solutes, which in turn leads to dissipation of the proton gradient across the cytoplasmic membrane (Bowles and Ellefson, 1985; Hutkins and Kashket, 1986). Butanol toxicity also appears to be linked to the autolytic activity of solvent-producing *C. acetobutylicum* cells, and it has been suggested that inhibitory concentrations of butanol are involved in triggering the release of autolysin during solventogenesis (Barber *et al.*, 1979; Van der Westhuizen *et al.*, 1982; Jones and Woods, 1986; Soucaille *et al.*, 1987). Sauer and Dürre (1995) have shown that expression

of the *lyc* autolysin gene (Croux and Garcia, 1991) is induced following artificial induction of solventogenesis.

The interpretation of the solventogenic switch as a metabolic response to environmental stress is reinforced by the observation that heat shock gene expression also increases during the initiation of the solventogenic phase (Pich *et al.*, 1990). Heat shock protein synthesis can be induced by the addition of solvents and exposure to oxygen (Terracciano *et al.*, 1988), changes in pH and growth rate (Pich *et al.*, 1990), and the addition of methyl viologen (Sauer and Dürre, 1995). The important role of some electron transport proteins in regulating solventogenesis has been outlined in Section 1.3. It is therefore significant that the N-terminus of the 21-kDa heat shock protein HSP21 (Pich *et al.*, 1990) reveals high amino acid homology to the 6-kDa rubredoxin protein of anaerobic bacteria (unpublished results cited in Bahl *et al.*, 1995); this implies some oxidative-reductive element in the regulation of the heat shock response. Bahl *et al.* (1995) propose that the heat shock response might be part of a global regulatory network that integrates the different stress responses e.g. heat shock, the solventogenic switch and sporulation.

1.4.2 Morphological changes during sporulation

The sporulation process is well characterized in *Bacillus subtilis* which is recognised as a model system for examining cellular differentiation in bacteria. The process involves seven defined morphological stages (reviewed by Errington (1993)) which are summarised briefly. In response to nutrient deprivation, the vegetative stationary-phase cell (stage 0) is asymmetrically divided into the mother cell (larger) and forespore (smaller) compartments by development of the forespore septum (stage II). The forespore is then engulfed by the mother cell (stage III), the cortex of cell wall-like material is synthesised (stage IV) and the proteinaceous spore coat is deposited (stage V). The characteristic properties of resistance, dormancy, and germinability develop during spore maturation (stage IV), and the process culminates in lysis of the mother cell to release the mature spore (stage VII).

Whereas sporulation in *Bacillus* is initiated by nutrient starvation, sporulation in *C. acetobutylicum* is triggered by cessation of growth in the presence of excess nutrients (carbon and nitrogen) or exposure to oxygen, yet it proceeds via a similar sequence of

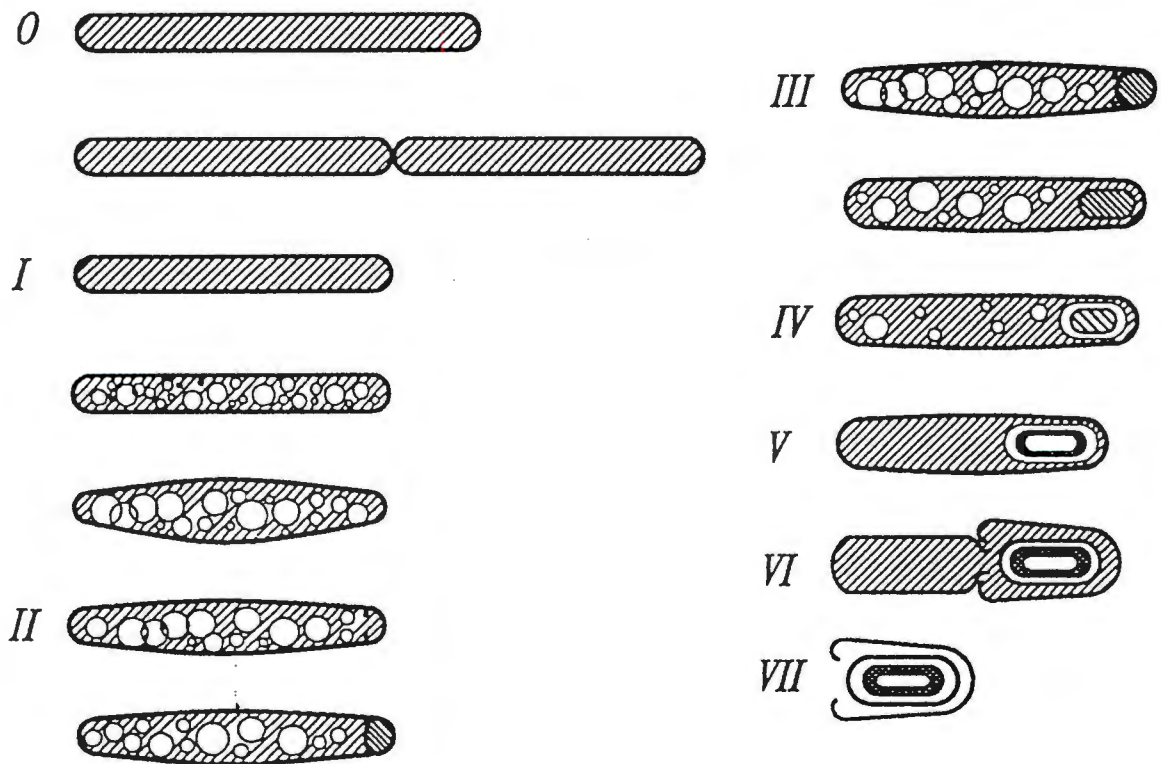


Fig. 1.4. Stages of developmental morphology as *C. acetobutylicum* P262 sporulates, adapted by Babb (1993) from electron micrographs (Long, 1984). Sporulation stages are indicated with Roman numerals and are described in the text.

morphological changes (Long *et al.*, 1983) (Fig.1.4). Analysis of the molecular basis of the differentiation and sporulation processes in *C. acetobutylicum* has only recently been undertaken. Emerging evidence reveals basic similarities between *Bacillus* and *Clostridium* in the regulation of sporulation (see Sections 1.4.3, 1.4.4 and 1.4.5), despite differences in their induction patterns.

1.4.3 Spo0A: initiation of sporulation and solventogenesis

The simultaneous loss of spore-forming potential and solvent-producing ability in several *C. acetobutylicum* mutants and degenerate strains (Jones *et al.*, 1982; Long *et al.*, 1984a, 1984b; Meinecke *et al.*, 1984; Woolley and Morris, 1990) is an indication that solventogenesis and sporulation share common regulatory features. The *spo0A* gene is a likely candidate for global regulation of these processes (see Wilkinson *et al.*, 1995a). The

Spo0A regulatory protein is the master controller of sporulation, but is also essential for other aspects of stationary phase metabolism such as the development of competence, motility, autolysins and degradative enzyme synthesis in *B. subtilis* (Hoch, 1993; Smith, 1993). It is a two component signal transduction protein, consisting of a N-terminal sensor domain and a C-terminal effector domain. Its function is controlled both by its concentration (dual σ^A and σ^H -dependent promoters regulate its transcription) and by its activation state (it is reversibly activated via a multicomponent phosphorelay system). In its phosphorylated form, Spo0A acts as an ambivalent transcription factor. It directly regulates the expression of the genes it controls by binding to sequence motifs (TGNCGAA) referred to as 0A boxes, usually located upstream of the target genes. It also indirectly controls gene expression via the sporulation cascade or by means of transition state regulators (see Section 1.4.4) which are themselves directly controlled by Spo0A e.g. AbrB (Hoch, 1993).

Presumptive *spo0A* homologues have been isolated by PCR from several other *Bacillus* and *Clostridium* species, including *C. acetobutylicum* NCIMB 8052 (Brown *et al.*, 1994a). This suggests that Spo0A is a key regulator in all endospore-forming bacteria. Amino acid comparison of the *spo0A* sequences served to identify a helix-turn-helix motif within the highly conserved effector domain which is thought to represent the 0A DNA-binding surface of the protein. The sequence of the *spo0A* gene of *C. acetobutylicum* NCIMB 8052 has recently been completed (Wilkinson *et al.*, 1995a) to reveal conserved regulatory elements and features of self-regulation i.e. a putative σ^H -dependent promoter overlapped by a 0A box (Ferrari *et al.*, 1985). There are three extra 0A motifs located in the intergenic region between *spo0A* and its upstream neighbour, *spoIVB*. Juxtaposition of the *spoIVB* and *spo0A* genes is preserved as in a number of *Bacillus* species (Van Hoy and Hoch, 1990; Brown *et al.*, 1994a).

The functionality of Spo0A in *C. acetobutylicum* was confirmed by insertional activation of the *spo0A* gene via targeted integration (Wilkinson and Young, 1994). The resultant Spo0A⁻ strain was defective in three related stationary phase responses, viz. sporulation, solventogenesis and granule production (Brown *et al.*, 1994a). Compelling evidence that Spo0A directly controls the initiation of solventogenesis, is the identification of 0A box motifs in the upstream regulatory regions of several *C. acetobutylicum* NCIMB 8052,

ATCC 824 and DSM 792 genes whose expression is activated (*adc*, *ctfAB*, *adhE*, *bdhA* and *bdhB*) or repressed (*ptb*, *butK*) at the onset of solventogenesis (Wilkinson *et al.*, 1995a) (Table 1.2). Except in the case of the *bdhB* gene, the 0A boxes are located upstream of the promoters.

Table 1.2. *C. acetobutylicum* genes which contain 0A boxes in their 5' regions

Genes	Encoded enzymes	No. 0A copies
• <i>adc</i>	Acetoacetate decarboxylase	3
• <i>adhE-ctfAB</i> (<i>sol</i> operon)	Alcohol/aldehyde dehydrogenase and 2 subunits of CoA transferase	1
• <i>bdhA</i>	Butanol dehydrogenase I	1
• <i>bhdB</i>	Butanol dehydrogenase II	1
• <i>ptb-butK</i> operon	Phosphotransbutyrylase and butyrate kinase	2

1.4.4 Transition state regulation

Spo0A appears to be an essential regulatory feature for the transition from exponential growth to stationary phase in all endospore-forming bacteria, including *C. acetobutylicum*. The transition state should not be viewed simply as a "gear-shifting period", but as a process as important as sporulation itself (Hoch, 1993). During this time the cell "has to decide" in the face of nutrient (*B. subtilis*) or acid (*C. acetobutylicum*) stress whether to sporulate or not. The role of the transition state regulators e.g. AbrB, Hpr and Sin is to delay sporulation until such time as it is appropriate. At the same time they coordinate complex regulatory pathways and direct the cell towards alternative developmental fates e.g. competence, motility, degradative enzyme synthesis and autolysin production (Smith, 1993). Redundancy is a characteristic feature of transition state regulator control. For example, AbrB positively

regulates Hpr, and together with Hpr, represses Sin, all of which are negative effectors of subtilisin expression. Presumably redundant control provides a "rheostat" through which the level of gene expression can be sensitively adjusted (Strauch and Hoch, 1993).

It is likely that transition state regulation is a feature of any microbial species that undergoes a differentiation process. The *B. subtilis* hpr gene homologue and multiple copies of Hpr consensus binding sites have been identified upstream of the sporulation-associated enterotoxin gene of *Clostridium perfringens* (Brynstad *et al.*, 1994). Additional evidence for transition state regulation in the clostridia includes tentative identification of alternative sigma (σ) factors e.g. σ^D and σ^H which are involved in transition state gene expression (Béguin *et al.*, 1986; Mishra *et al.*, 1991; Sauer *et al.*, 1994; Wilkinson *et al.*, 1995a). In *B. subtilis*, σ^D controls transcription of chemotaxis, motility and autolysin functions, and σ^H controls postexponential expression functions such as competence and early sporulation genes (Haldenwang, 1995). Transition state regulators complement the role of alternative σ factors and themselves participate in the regulation of alternative σ factor gene expression (Strauch and Hoch, 1993).

1.4.5 Sporulation-specific sigma factors

Alternative σ factors are responsible for the most significant changes in gene regulation during sporulation. They serve to alter the specificity of transcription by reprogramming the RNA polymerase for different promoter recognition. Four sporulation-specific σ factors in *B. subtilis* mediate the sequential pattern of gene expression which guides the cell through the series of developmental stages following forespore septation (reviewed by Errington, 1993 and Haldenwang, 1995). σ^E and σ^F establish early gene expression in the mother cell and prespore, respectively, and strains deficient in either of these two σ factors terminate in a stage II phenotype. σ^G and σ^K are responsible for late gene expression in the prespore and mother cell compartments, respectively. σ^K gene expression contributes to final spore maturation e.g. development of the cortex and coat protective layers which seal off the prespore from the mother cell, and σ^G gene expression is directed towards the development of UV resistance and germination functions. Because untimely or inappropriate gene expression might be lethal to the cell, σ factor activity is subject to multiple control at both

the transcriptional and translational levels. For example, production of σ^K depends on 1) an extraordinary chromosomal rearrangement that pieces together two truncated genes more than 40 kb apart to form the σ^K coding sequence; 2) compartment-specific transcription of its structural gene, *sigK* under the control of σ^E and σ^K , both acting in conjunction with SpoIIID; and 3) conversion of the inactive pro- σ^K to its active form by proteolytic processing. Irreversible activation by pro-sequence cleavage appears to be an appropriate method of posttranslational processing for σ^K , the terminal σ factor specific to the mother cell which, by this time, is committed to the sporulation process and destined to lyse (Haldenwang, 1995).

Homologous genes for three of the four *B. subtilis* sporulation-specific σ factors have been cloned and sequenced from *C. acetobutylicum* DSM 792 (Sauer *et al.*, 1994). The *spoIIGA-sigE-sigG* gene region of *C. acetobutylicum* DSM 792 and ATCC 824 (Sauer *et al.*, 1994; Wong *et al.*, 1995) displays the same genomic organization as found for the corresponding genes in *B. subtilis* and *Bacillus thuringiensis*. This is conservation of an unlikely grouping, considering that these two encoded σ factors (σ^E and σ^G) direct gene expression in different cell compartments and at different stages in sporulation (Sauer *et al.*, 1994). The putative *spoIIGA* gene is thought to encode the pro- σ^E -processing protease. The *sigK* gene of *C. acetobutylicum* is contiguous, as are the corresponding genes in *B. thuringiensis* and *Bacillus megaterium* (Sauer *et al.*, 1994, 1995). Thus the chromosomal rearrangement of the *sigK* gene in *B. subtilis* can be viewed as a species-specific idiosyncrasy, rather than an essential regulatory feature (Sauer *et al.*, 1995). The primary structure of the *C. acetobutylicum sigE* and *sigK* genes indicates that the encoded σ factors are subject to endoproteolytic processing as demonstrated in *B. subtilis*. Identification of a σ^F promoter-homologous sequence upstream of the clostridial *sigG* gene provides indirect evidence for the existence of σ^F (Sauer *et al.*, 1994).

None of the solventogenic genes characterized so far has sporulation-specific promoters (Wilkinson *et al.*, 1995a); instead they all appear to be transcribed by the vegetative form of the RNA polymerase ($E\sigma^A$). It is therefore unlikely that they are expressed as part of the sporulation cascade.

1.5 Genetics

Until recently, *C. acetobutylicum* has lacked well-developed genetic systems. Several plasmid vectors are now available, however, and methods for transferring these plasmids have been developed. In addition, conjugative transposons have been exploited for mutagenesis (see Section 1.6.5), a physical and genetic map of the *C. acetobutylicum* NCIMB 8052 genome has been constructed (Wilkinson and Young, 1995), and the establishment of a genetic exchange system (see Section 1.5.3) is a significant advance. The availability of these genetic techniques means that metabolic re-engineering of the solvent-producing clostridia is imminently feasible. This section, and the following section, focus on some of the latest developments in clostridial genetics and those aspects which found application in this study.

1.5.1 Gene transfer systems

Protoplasting and electroporation render any bacterial strain potentially transformable, even in the absence of natural competence, and both these techniques have been successfully applied for DNA transformation of several clostridial species (Reysset and Sebald, 1993). Good PEG-mediated protoplast transformation frequencies ($\sim 10^{-6}$ transformants/ μg phage or plasmid DNA) have been obtained for e.g. *C. acetobutylicum* N1-4081 (Reysset *et al.*, 1988; Truffaut *et al.*, 1989; Azeddoug *et al.*, 1992), but protoplast regeneration is a tricky and time-consuming technique, and for this reason electroporation seems to be the preferred method of DNA transfer. The first clostridial species to be transformed by electroporation was *C. acetobutylicum* NCIMB 8052 (Oultram *et al.*, 1988), with reported frequencies of $\sim 10^2$ - 10^3 transformants/ μg plasmid DNA. Because of these low frequencies, shotgun cloning of manipulated DNA directly into *C. acetobutylicum* is impractical, and this has obvious limitations for those genes which are not expressed in *E. coli* (Oultram *et al.*, 1988). Procedures have subsequently been developed for successful electroporation of *C. acetobutylicum* ATCC 824 (Mermelstein *et al.*, 1992) and derivatives of N1-4 (Reysset and Sebald, 1993) with frequencies of $\sim 10^5$ transformants/ μg plasmid DNA.

Conjugal mobilization of plasmids to *C. acetobutylicum* is a suitable alternative to transformation. The ability of *C. acetobutylicum* to act as a recipient in intergeneric plasmid transfer was first demonstrated by the transfer of the broad host range enterococcal plasmid

pAMB1 from Gram-positive donors e.g. *B. subtilis* and *Streptococcus lactis* to *C. acetobutylicum* strains 903, ATCC 824, NCIMB 8052 and P262 (Oultram and Young, 1985; Reysset and Sebald, 1985; Yu and Pearce, 1986). The conjugation mechanism of IncP plasmids has subsequently been exploited to mobilize non-conjugative vectors from *E. coli* to *C. acetobutylicum* 8052 (Williams *et al.*, 1990). These cloning vectors, complete with appropriate replicon, contain the origin of transfer (*oriT*) from IncP plasmid, RK2.

1.5.2 *C. acetobutylicum* cloning vector pFNK1

The lack of selectable markers in indigenous clostridial plasmids has necessitated the *in vitro* construction of vectors by combining replicons and antibiotic resistance genes that are functional in *C. acetobutylicum*. Shuttle vectors have been developed with replicons from various sources e.g. pAMB1 (*Enterococcus faecalis*), pIM13 (*B. subtilis*), pWV01 (*Streptococcus cremoris*), pCB101 (*C. butyricum*) and pCP1 (*Clostridium paraputrificum*) (Williams *et al.*, 1990;). The 2.4 kb *B. subtilis/C. acetobutylicum* shuttle vector pFNK1 has been designed to avoid the restriction system of *C. acetobutylicum* ATCC 824 (Mermelstein *et al.*, 1992). The restriction endonuclease *Cac824I* recognises the sequence 5'-GCNGC-3' which restricts ColE1 plasmids at high frequency, thus rendering *E. coli/C. acetobutylicum* shuttle vectors such as pMTL500E (Swinfield *et al.*, 1990) useless for transformation purposes. The pFNK1 shuttle vector contains the origin of replication, the macrolide, lincosamide and streptogramin B (MLS) resistance marker from the *B. subtilis* plasmid pIM13, and the multiple cloning site of pUC9. It harbours only one *Cac824I* recognition sequence, is stably maintained with a copy number of eight (Lee *et al.*, 1993), and has been successfully used for overexpression of primary metabolic genes in strain ATCC 824 (Mermelstein *et al.*, 1992).

Increased transformation efficiencies have been demonstrated with a pFNK1 derivative plasmid passaged through strain ATCC 824 (Mermelstein *et al.*, 1992). To eliminate the dependence on *B. subtilis/C. acetobutylicum* shuttle vectors with few *Cac824I* sites, a system for complete *in vivo* protective methylation of *E. coli/C. acetobutylicum* shuttle vectors with ColE1 origins of replication has been developed (Mermelstein and Papoutsakis, 1993). The ϕ 3T I methyltransferase gene from *B. subtilis* phage ϕ 3T I was cloned into plasmid pACYC184 which replicates via the p15A origin, to generate plasmid pAN1. The

pAN1-encoded methyltransferase can potentially be used to protect any coresident *E. coli* plasmid that is compatible with p15A-based plasmids. Ultimately, *in vitro* extracts may be used to protect those vectors that cannot replicate in *E. coli* from *Cac824I* restriction (Mermelstein and Papoutsakis, 1993).

1.5.3 Insertional mutagenesis with integrational plasmids

Gene transfer via homologous recombination is a recent and revolutionary development in the field of clostridial genetics. Wilkinson and Young (1994) have demonstrated integration of non-replicative plasmids in the *C. acetobutylicum* NCIMB 8052 chromosome at a frequency of 10^{-7} to 10^{-6} per recipient, which is two to three orders of magnitude less than that of the replication-proficient control. Integration appears to occur by Campbell-like recombination between the chromosomal insert of the plasmid and the corresponding region of the bacterial chromosome (Fig.1.5). In this way, internal fragments of the genes encoding the sorbitol dehydrogenase and Spo0A were used for gene disruption, resulting in the inability of the transconjugants to use sorbitol as a carbon source and form endospores, respectively (Wilkinson and Young, 1994). The replication-deficient pMTL30 and replication-proficient pCTC1 vectors are both equipped with the *oriT* region of plasmid RK2 (Williams *et al.*, 1990) and were therefore introduced into NCIMB 8052 by conjugative mobilization from *E. coli* donors. Any method which generates a sufficiently high frequency of transformation can be used, however. On the other hand, a gene inactivation system developed for *Lactococcus lactis* (Maguin *et al.*, 1992; Biswas *et al.*, 1993) employs a replication-thermosensitive plasmid with a pWV01 replicon which is functional in *C. acetobutylicum* (Williams *et al.*, 1990). This thermosensitive plasmid is capable of autonomous replication in the transformed host, until subjected to the non-permissive temperature which then triggers plasmid integration. Application of this system in *C. acetobutylicum* would eliminate the requirement for high transformation frequency which is necessary for detection of low-frequency events such as recombination.

Integrational plasmids will undoubtedly serve as invaluable tools for genetic analysis in *C. acetobutylicum*. Wilkinson and Young (1994) have demonstrated that in addition to their application for insertional mutagenesis, integrational plasmids may be used for gene

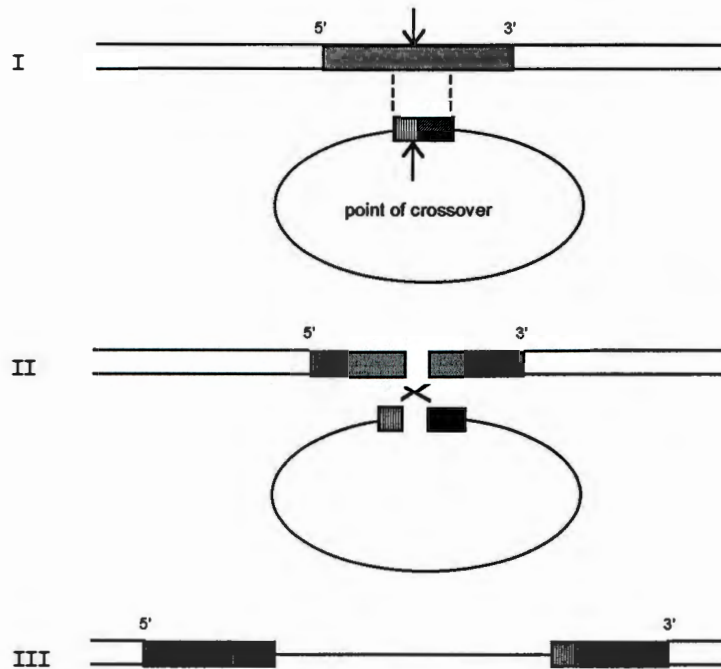


Fig. 1.5. Mechanism of directed plasmid integration by homologous recombination showing (I) the substrates of integration, viz. the gene of interest located on the chromosome and the integration vector containing a homologous DNA insert; (II) the recombination process; and (III) the integration product.

amplification (by integration of multiple plasmid copies) and determination of map location. Furthermore, gene replacement studies in which altered genes can be used to replace the resident wild type copy, are now a possibility.

An alternative method of chromosomal targeting is conjugative transposon mutagenesis.

1.6 Conjugative transposons

1.6.1 Introduction to conjugative transposons

Conjugative transposons are an unusual class of mobile genetic elements, distinctive in their ability to promote their own intercellular transfer by a DNase-resistant process requiring cell-to-cell contact (Shoemaker *et al.*, 1980; Franke and Clewell, 1981). They were first isolated from pathogenic streptococcal strains (Buu-Hoï and Horodniceanu, 1980; Franke and Clewell, 1981; Carlier and Courvalin, 1982; Courvalin and Carlier, 1986) and are found

predominantly in Gram-positive bacteria, where they are usually integrated in the host chromosome. They can conjugate naturally between Gram-positive and Gram-negative species (Sen and Oriel, 1990; Bertram *et al.*, 1991), and their host range does not appear to be affected by DNA restriction (Guild *et al.*, 1982). They represent a serious medical problem because of their remarkable promiscuity and the fact that they all carry drug-resistant determinants, yet it is precisely these features that make them useful tools for mutagenesis. The best-studied examples are Tn916 (*tetM*), an 18.3 kb element (Flannagan *et al.*, 1994) encoding resistance to tetracycline, and Tn1545 (*tetM*, *aphA-3*, *ermAM*), a 25.3 kb element encoding resistance to tetracycline, kanamycin and erythromycin. Other structurally and functionally related conjugative elements include Tn918 and Tn925 from *E. faecalis* (Franke and Clewell, 1981; Clewell *et al.*, 1985; Christie *et al.*, 1987) and Tn919 from *Streptococcus sanguis* (Fitzgerald and Clewell, 1985). Much of the information presented in this section is based on recent review articles by Scott (1992, 1993) and Scott and Churchward (1995).

1.6.2 Structure

The sequences of the ends (approximately 200 bp) of Tn916 and Tn1545 are almost identical for both transposons (Caillaud and Courvalin, 1987; Clewell *et al.*, 1988; Poyart-Salmeron *et al.*, 1989), although what is referred to as the "left" end of Tn916 corresponds to the "right" end of Tn1545. Here the Tn916 designations will be used. Imperfect inverted repeats have been identified at the immediate ends of the transposon and the position of three sets of direct repeats (DR-1, DR-2, and DR-3) relative to the ends of the transposon is shown in Fig. 1.6A. Three and two copies of the DR-2 repeats are located approximately 150 bp and 90 bp from the left and right ends, respectively, and their relevance is explained in Section 1.6.3.3.

A potential outward-reading promoter is located within one of the DR-1 direct repeats at the right terminus of Tn916 (Clewell *et al.*, 1988) (Fig. 1.6A). Transcriptional read-through from the putative promoter may account for the hyperhemolytic phenotype observed in a number of *E. faecalis* strains in which the transposon had inserted upstream of the hemolysin gene of resident plasmid pAD1. It therefore cannot be assumed that transposon-induced phenotypes will always be the result of disrupted gene expression.

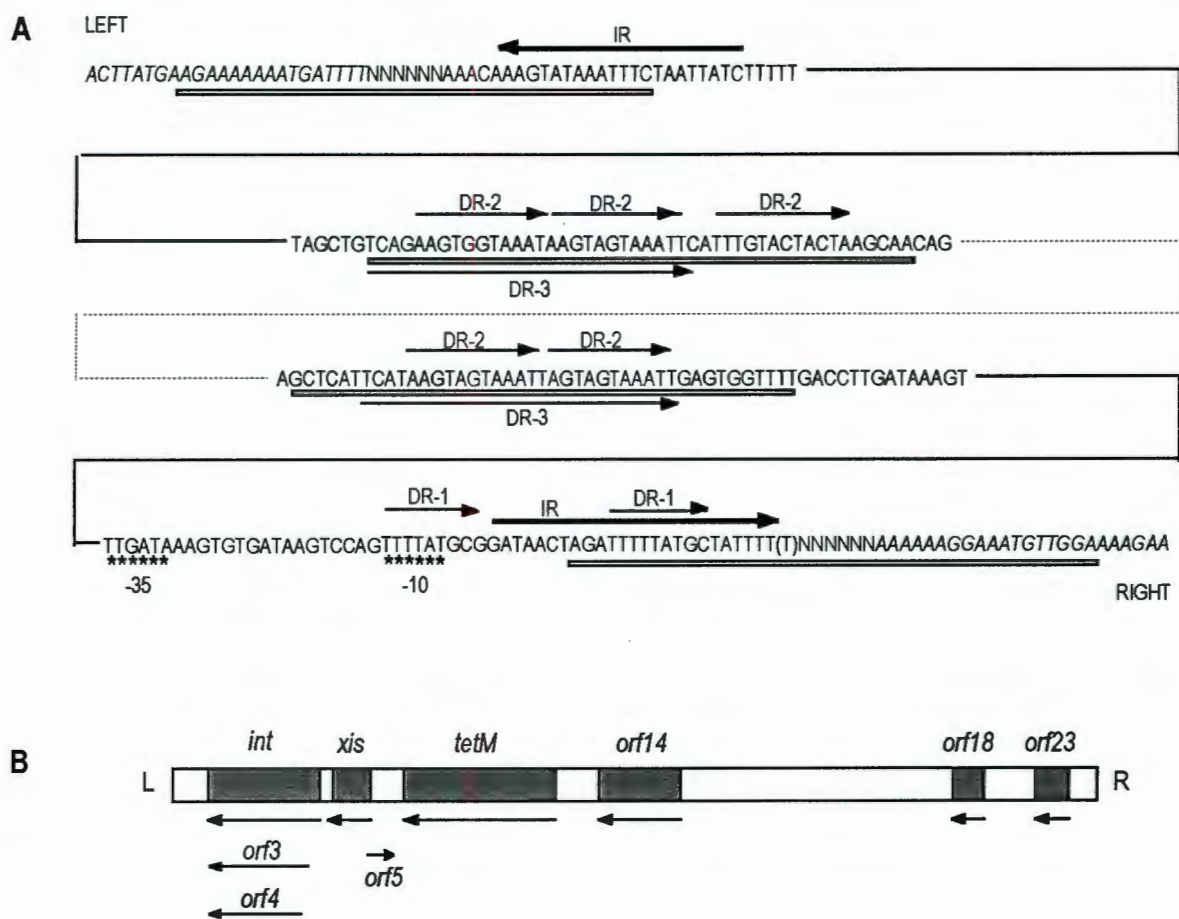


Fig. 1.6. A) Structure of the left and right ends of Tn916. Italicized characters represent host sequences and plain characters represent transposon DNA. The six Ns in each sequence represent the coupling sequences. The T in parentheses in the right transposon end is present when the transposon excises with five Ts, but absent when the transposon excises with four Ts. Arrows indicate inverted (IR) and direct repeats (DR-1, DR-2, DR-3). Potential promoter hexamers (-10 and -35 sequences) within the right end are indicated by asterisks. Open bars indicate the extent of DNA protected from DNase I cleavage by Int binding. B) Genetic organization of Tn916 (not drawn to scale) showing the relative position and orientation of relevant genes. The arrows indicate the direction of gene transcription. Adapted from Clewell *et al.* (1988), Su and Clewell (1993) and Scott and Churchward (1995).

The complete nucleotide sequence of Tn916 has recently been determined (Flanagan *et al.*, 1994) and the physical organization of relevant genes is depicted in Fig.1.6B. The *tetM* gene which specifies resistance to the broad spectrum antibiotic, tetracycline, has been separately cloned and sequenced (Burdett, 1990; Su *et al.*, 1992). Tet(M) is an elongation factor/translational initiation factor analogue (Sanchez-Pescador *et al.*, 1988; Su *et al.*,

1992) and renders protein synthesis insensitive to tetracycline via tRNA modification (Burdett, 1993). The *tetM* gene is located 10 kb from the right end of the transposon. Mutagenesis studies indicate that most of the DNA to the right of *tetM* is required for successful conjugation i.e. intercellular transfer (Yamamoto *et al.*, 1987; Senghas *et al.*, 1988), whereas the 4 kb region to the left of *tetM* contains the *int-Tn* and *xis-Tn* functions required for excision (Su and Clewell, 1993). The same functions are similarly located in Tn1545 (Poyart-Salmeron *et al.*, 1989).

The functions of three open reading frames (ORFs) in the right half of Tn916 have been tentatively identified by amino acid homology analysis (Flannagan *et al.*, 1994). ORF14 may encode an autolytic function which might enable the DNA to penetrate the cell wall of the donor and/or recipient during the conjugation process; ORF23 may specify a mobilization function responsible for nicking the circular transposon DNA at the hypothetical *oriT* (see Section 1.6.4); and ORF18 shares high homology with the restriction protection protein ArdA (alleviation of restriction of DNA). Presumably, the ORF18 product confers resistance to DNA restriction, a phenomenon which has been demonstrated in conjugative transposons (Guild *et al.*, 1982). Important aspects of conjugation are discussed in Section 1.6.4.

Besides the *xis* and *int* genes in the left 4 kb DNA region of Tn916, eight other ORFs have been identified, some of which are thought to specify Int/Xis regulatory elements (Su and Clewell, 1993). For example, two ORFs (ORF3 and ORF4) are encoded in-frame within the *int* gene. By analogy with a similar gene arrangement in Tn5, these ORFs may specify truncated forms of Int which regulate transposition at a post-translational level (Su and Clewell, 1993; Scott and Churchward, 1995). A divergent ORF (ORF5), adjacent to and overlapping *xis*, is required for conjugative transfer of Tn916 between *E. faecalis* strains, and also reduces the frequency of excision in *E. coli* (Su and Clewell, 1993). It is possible that this determinant generates a countertranscript which interferes with *xis* translation. The mechanism of transposition, as reflected in the transposon structure, is dealt with in the following section.

1.6.3 Mechanism of conjugative transposition

1.6.3.1 Excision

Conjugative transposition occurs by a lambda phage-related excision-insertion mechanism (Poyart-Salmeron *et al.*, 1990) in which the intermediate is a non-replicative circular molecule (Scott *et al.*, 1988). Excision is the first step of transposition and occurs by reciprocal site-specific recombination between non-homologous 6 bp coupling sequences (Caparon and Scott, 1989; Poyart-Salmeron *et al.*, 1990; Rudy and Scott, 1994). The process requires two transposon-encoded proteins, the excisionase (Xis) and the integrase (Int) (Poyart-Salmeron *et al.*, 1990; Storrs *et al.*, 1991; Su *et al.*, 1992), and possibly a host-encoded function (Bringel *et al.*, 1991). The transposon is excised by staggered endonucleolytic cleavage, generating 6 bp overhangs of DNA which flank the transposon on either side (Fig. 1.7A). The composition of these overlap regions or coupling sequences is non-specific, and since the sequence of the two strands is non-complementary, the joint of the circular transposon intermediate contains mismatches and is referred to as a heteroduplex (Caparon and Scott, 1989). The ends of the flanking (non-transposon) DNA are also joined to form a heteroduplex which is resolved by replication or repair, resulting in two possible excision products containing either of the junction sequences. In one excision product, the original DNA sequence is restored, in the other, a 6 bp substitution occurs. Imprecise excision e.g. excision of five bases instead of six, can result in a frameshift mutation (Caparon and Scott, 1989; Scott, 1992).

It is speculated that there is some connection between the expression of excision and resistance functions, since exposure of transposon donor cells to tetracycline during the conjugation event enhances the frequency of transfer (Torres *et al.*, 1991; Showsh and Andrews, 1992). It is interesting that excision of Tn916 from *Streptococcus pneumoniae* has also been observed to occur following exposure to streptomycin (Watson *et al.*, 1993). From a medical perspective, this provides the first evidence for selection pressure, due to the presence of one antibiotic, contributing directly to the spread of elements encoding resistance to unrelated compounds (e.g. tetracycline) (Watson *et al.*, 1993).

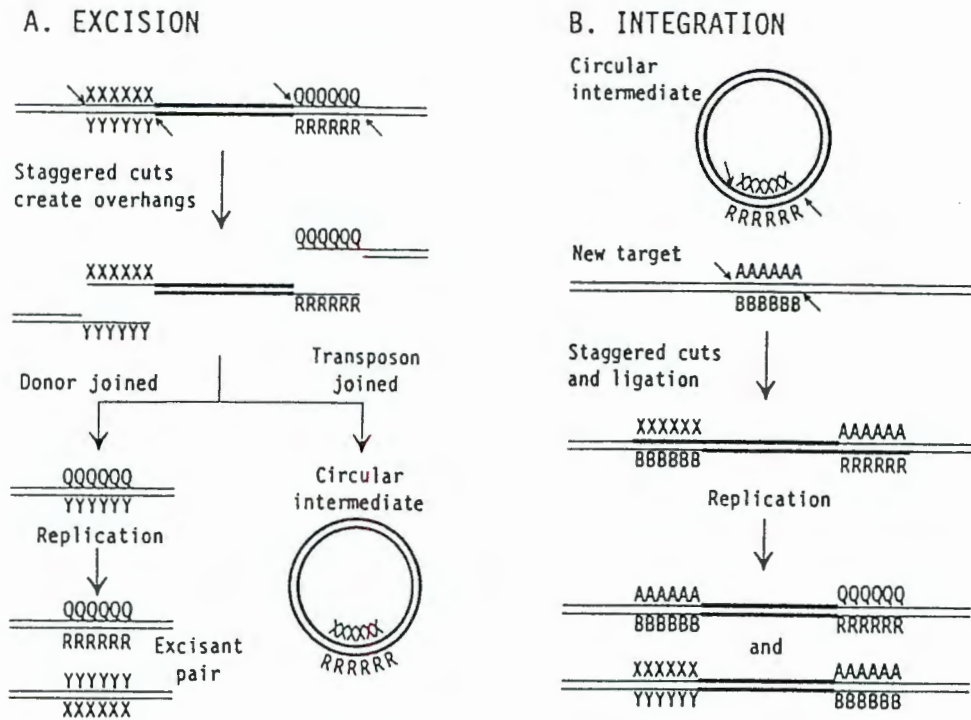


Fig. 1.7. A model for excision (A) and integration (B) of *Tn916* (Scott and Churchward, 1995). The thick lines represent *Tn916*, and the thin lines represent the DNA adjacent to the transposon. Coupling sequences are indicated by hypothetical complementary nucleotide pairs, X-Y, Q-R, and A-B. A) Staggered cleavages (arrows) of the phosphodiester backbone on the 5' side of the coupling sequence on both strands create molecules shown with 5' single ends. The ends are joined to generate an excisant molecule and a circular transposon intermediate, both of which contain heteroduplexes consisting of the base pairs originally present in the coupling sequences. Semiconservative replication resolves the heteroduplex in the excisant and generates a pair of molecules, one of which has the left coupling sequence at the site of excision, and the other the right. B) Staggered cleavages in the circular intermediate and the new target, followed by ligation, create a new insertion of *Tn916* with a heteroduplex at each end. Replication resolves the heteroduplexes and generates a pair of molecules in which each member is flanked by the target sequence at one end and a coupling sequence from the previous insertion at the other end.

1.6.3.2 Insertion

Insertion of the circular intermediate into its target occurs by reversal of the excision process. Staggered endonucleolytic cleavage of the target DNA and the joined transposon ends is followed by ligation (Fig.1.7B). The integrated transposon is flanked by mismatched coupling sequences which are resolved by DNA replication (Caparon and Scott, 1989). Whereas both the excision and integration functions are generally required for excision (see

Scott and Churchward, 1995), only the integrase function is needed for insertion (Storrs *et al.*, 1991; Bringel *et al.*, 1992). Once inserted in the chromosome of the new host, secondary transposition seems to be a rare event (Norgren and Scott, 1991).

The nucleotide composition of the 6 bp coupling sequence brought in by the transposon changes with successive excision-insertion and appears to dictate the frequency of conjugative transposition (Jaworski and Clewell, 1994). Independent Tn916 transposon insertions, all with the same orientation in identical target sites, have been shown to display significantly different transfer potentials. It is suggested that variations in the coupling sequences might affect the binding and/or activity of one or more of the proteins involved in the excision-insertion cycle (Jaworski and Clewell, 1994).

Defined in terms of the insertion site, the coupling sequence is the non-specific 6 bp spacer between two AT-rich target regions recognised by the transposon. Based on examination of a series of Tn1545 insertion sequences, Trieu-Cuot *et al.* (1993) proposed that target site specificity depends on the degree of homology between the immediate ends of the transposon and the segments flanking the spacer region in the target. When a synthetic target sequence identical to the joined ends of circular intermediate was inserted into a plasmid, a minitransposon Tn1545 construct integrated almost exclusively into this site in either orientation. However, this sequence failed to act as a target in the *E. faecalis* chromosome (Trieu-Cuot *et al.*, 1993), which suggests that another, or an additional factor determines target activity (Scott and Churchward, 1995). A recent study has shown that three functional Tn916 insertion sites consist of bent DNA (Lu and Churchward, 1995) and therefore the conformation of the target appears to be an important feature. Presumably target sites exhibit sequence-dependent bending as described for adenine-thymine tracts (Koo *et al.*, 1986) and the specific shape of the target determines the affinity for the transposon integrase (Lu and Churchward, 1995).

1.6.3.3 Recombination

Protein-DNA interactions are a fundamental aspect of the site-specific recombination event. The Tn916 integrase contains two independent DNA binding domains which may enable the Int protein to bridge the recombining sites, thereby ensuring that only correctly aligned

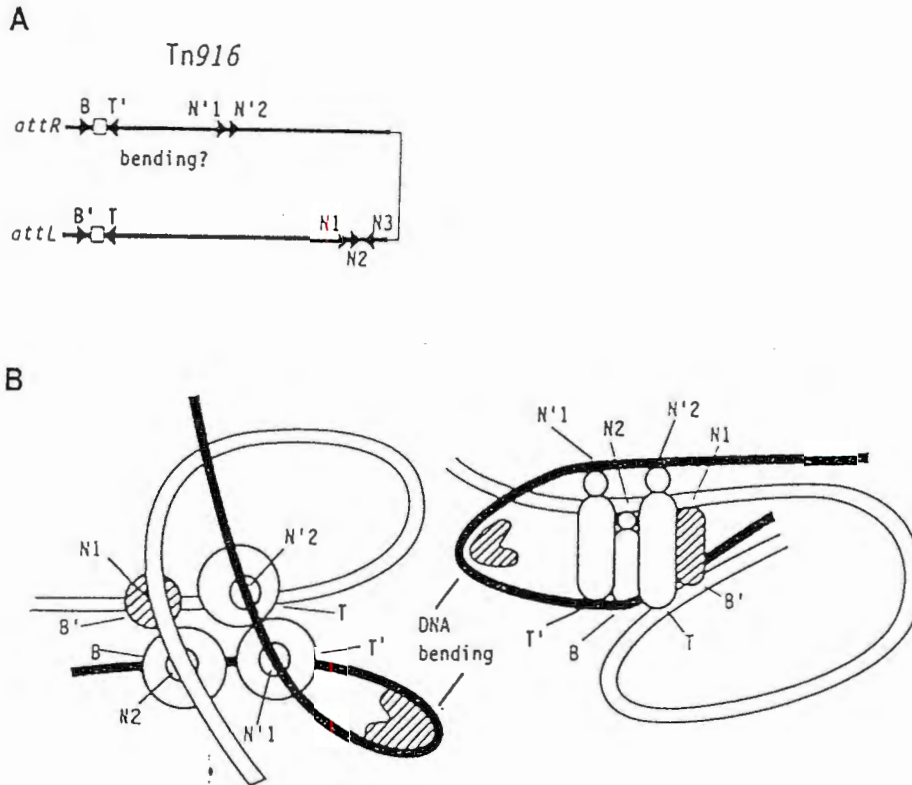


Fig. 1.8. A) *attL* and *attR* represent the left and right ends of the integrated transposon. Open boxes represent the coupling sequences at each end of the transposon. Arrows labelled B, T', B', and T represent binding sites for the C-terminal domain of Tn916 Int. Sites labelled N'1, N'2, N1, N2, and N3 represent the DR-2 repeats, shown in Fig. 1.6B, that bind the N-terminal domain of Tn916 Int. The region labelled "bending?" indicates the possible binding site for a host factor that bends the DNA between T' and N'1. B) Model for the alignment of the left and right ends of Tn916 during excision. The left and the right parts of the figure show two different views of the complex. The heavy black line represents the DNA of *attR*, and the open line represents the DNA of *attL*. The positions of the Int-binding sites B, T', B', T, N1, N2, N'1, and N'2 are indicated. In the left part of the figure, the large circles represent the C-terminal domain of Int molecules, and the small circles represent the N-terminal domains. On the right, the large cylinders represent the C-terminal domain of Int, and the small circles represent the N-terminal domain. The hatched circle on the left and the hatched cylinder on the right represent an Int molecule, which may have either two DNA-binding domains or only the C-terminal domain. The hatched object labelled "bending" represents a hypothetical host factor that binds and bends DNA between T' and N'1. Taken from Scott and Churchward, 1995.

molecules undergo recombination (Lu and Churchward, 1994). The N-terminal domain binds the DR-2 repeats located within the transposon, while the C-terminal domain has been shown to interact with the transposon-host DNA junction sequences (refer to Fig.1.6).

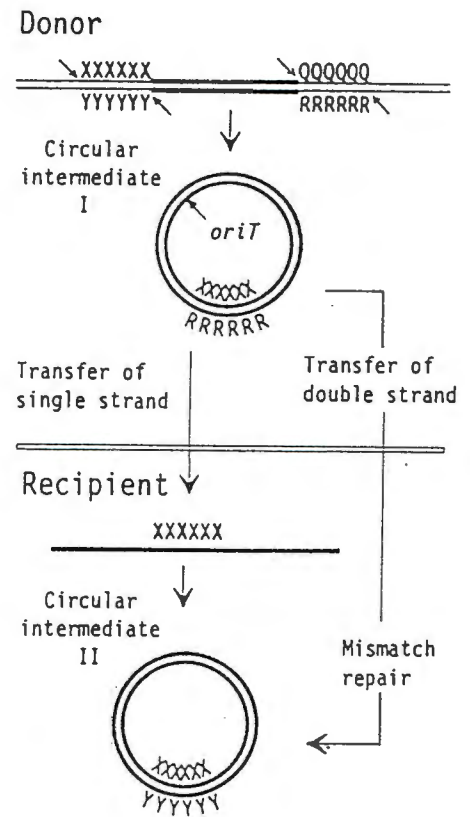
Scott and Churchward (1995) have proposed a model for excisive recombination in which formation of the nucleoprotein complex and alignment of the transposon ends requires four Int molecules to bridge the two recombining sites and arrange them in an antiparallel fashion via intrastrand looping (Fig.1.8). A host-encoded protein has been detected bound to ends of Tn916 (unpublished results cited in Scott and Churchward, 1995), and the inability of *L. lactis* to act as a transposon donor (Bringel *et al.*, 1991) provides indirect evidence for such a factor. It is presumably responsible for DNA bending, thereby playing a structural role in the formation of the nucleoprotein complex.

1.6.4 Conjugation

Not much is known about DNA transfer during conjugation between Gram-positive bacteria. Based on analysis of linkage of markers in crosses between Tn925 donor and recipient strains, Torres *et al.* (1991) have proposed that transposition may be accompanied by the transfer of chromosomal genes and that mating occurs by cell fusion which allows for extensive recombination between the donor and recipient genomes. Although not commented on, it surely raises the question as to whether transposon-containing transconjugants are in fact genetically defined as presupposed, but so far this cell fusion theory has not been substantiated. Excision is probably stimulated by mating-pair formation and appears to occur prior to transfer of the transposon (Scott, 1993). When Tn916 is present in a non-conjugative plasmid in the donor, there is no linkage of transposon and plasmid markers in the transconjugants; and when the transposon is present in a self-transmissible plasmid, transfer of the transposon is independent of plasmid transfer (Flannagan and Clewell, 1991; Scott and Churchward, 1995).

Since the Tn916 3' coupling sequence in the donor is never detected in the transconjugants, Scott *et al.* (1994) have interpreted this to mean that only one specific strand of the transposon is transferred to the recipient. Complementary strand synthesis then generates a double-stranded circle with no mismatches, which serves as the reaction intermediate (Fig. 1.9). Presumably both strands are excised from the donor target (to avoid the overload of strand replacement synthesis), and the strand selected for transfer is nicked at a hypothetical *oriT* site (see Section 1.6.2) (Scott *et al.*, 1994).

Fig. 1.9. A model for excision and transfer during conjugation (Scott and Churchward, 1995). Symbols as in Fig. 1.7. After excision and formation of a circular intermediate I in the donor strain, nicking at a putative *oriT* site allows transfer of a single transposon strand into the recipient. Complementary strand synthesis forms circular intermediate II with complementary coupling sequences. Alternatively, double-stranded circular intermediate I transfers to the recipient, and strand-specific mismatch repair corrects the heteroduplex in the intermediate to form the circular intermediate II.



1.6.5 Applications

Conjugative transposon mutagenesis is a simple and effective way of producing mutations, and can be exploited for those organisms which lack well-developed genetic systems e.g. *Bacillus pumilis* (Hendrick *et al.*, 1991), *Bacillus firmus* (Guffanti *et al.*, 1991), *C. botulinum* (Lin and Johnson, 1991) and *C. acetobutylicum* P262 (Babb *et al.*, 1993). The transposons are able to function in a wide variety of Gram-positive hosts and have been successfully introduced into *inter alia* a number of clostridial species e.g. *Clostridium tetani* (Volk *et al.*, 1988), *Clostridium difficile* (Mullany *et al.*, 1991), *C. perfringens* (reviewed by Rood and Cole, 1991) and *C. acetobutylicum* strains NCIMB 8052, DSM 792, DSM 1731, ATCC 824 and P262 (Davies *et al.*, 1988, Bertram and Dürre, 1989; Woolley *et al.*, 1989; Babb *et al.*, 1993; Kashket and Cao, 1993; Sass *et al.*, 1993) to generate an assortment of mutants. On account of the large size of these transposons, cloning the complete site of insertion is cumbersome, and so a minitransposon, Tn1545 Δ 3, consisting of the kanamycin resistance determinant cloned between left and right ends of transposon, has been developed to facilitate mutational cloning (Poyart-Salmeron *et al.*, 1989). In a similar

vein, the integrative vector, pAT112, exploits the transposition properties of Tn1545 and has been designed for easy recovery of insertionally inactivated genes in *E. coli* (Trieu-Cuot *et al.*, 1991).

Besides the use of transposons for mutagenesis, there are other useful applications. Plasmid pAT112 derivatives, pAT113 and pAT114 contain a multiple cloning site within the *lacZ'* region, and serve as convenient shuttle vectors for the introduction of foreign genes in Gram-positive bacteria (Trieu-Cuot *et al.*, 1991). Moreover, Tn916 has successfully been used to effect gene replacement (Norgren *et al.*, 1989). In the presence of a resident copy of Tn916, a second copy of the element (Tn916 Δ E) can either insert elsewhere in the chromosome at high frequency or integrate into the resident transposon by homologous recombination at low frequency (Norgren and Scott, 1991). Therefore, if the recipient chromosome shares a region of homology with the DNA cloned into a conjugative transposon, the transposon can insert into this DNA region via homologous recombination. The transposon is subsequently lost, and the resident allele is replaced with the new one. A successful system for homologous recombination would entail the use of a non-integrative mutant transposon to eliminate high frequency transposition events (Scott, 1992).

A novel application for these transposons is their use as genetic markers for examining microbial interactions in complex ecosystems. This has been neatly demonstrated using Tn916 to monitor fluctuating bacterial populations in the rumen of experimental sheep in response to diet (Brooker and Lum, 1993). A *Streptococcus bovis*: Tn916 population was shown to be stable in the presence of a grain-based ration, decline rapidly when the sheep were transferred to pasture, and reappear when the grain-based diet was resumed. Thus, conjugative transposons could provide a simple alternative to techniques such as species-specific gene probes and fluorescein-conjugated monoclonal antibodies for bacterial labelling (Brooker *et al.*, 1990). In addition, the conjugative transposon Tn1545 has been used as an important tool for genome mapping of the *C. acetobutylicum* NCIMB 8052 chromosome (Wilkinson and Young, 1995). Tn1545 is a suitably large element and lacks sites for the rare restriction endonucleases that were used for map construction (Wilkinson and Young, 1995). Tn1545 insertions in restriction fragments ranging from 50 to 600 kb therefore

served to identify overlapping fragments and resolve comigrating bands during pulsed-field gel electrophoresis.

Conjugative transposons were used in this study to generate metronidazole-resistant mutants of *C. acetobutylicum* P262. An overview of metronidazole is provided in the following section.

1.7 Metronidazole

1.7.1 Introduction to metronidazole

Metronidazole, 1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole, is a synthetic derivative of the *Streptomyces* antibiotic, azomycin (McFazdean, 1986). It was first introduced for prescriptive use in 1960 (under the tradename Flagyl) to treat a common feminine affliction: trichomonal vaginitis. Since then it has found wide application as a broad spectrum antibiotic which is selectively toxic to other anaerobic protozoa, obligately anaerobic Gram-positive and Gram-negative bacteria, microaerophilic bacteria, hypoxic tumours, and even a few helminths (Edwards, 1993a; Johnson, 1993). Despite the widespread clinical use of the drug for over 30 years, the incidence of resistance is still very low and much remains to be elucidated about its complex and selective mechanism of action against anaerobic microorganisms (Edwards, 1993a, 1993b; Johnson, 1993; Church and Laishley, 1995).

1.7.2 Mechanism of action

The mechanism of action of metronidazole is represented diagrammatically in Fig.1.10 and involves the following steps: 1) uptake of the drug into the target cell, 2) intracellular reductive activation of the drug, and 3) the toxic effects of the reduced intermediate(s) (Müller, 1983). Each of these steps will be discussed individually.

1.7.2.1 Uptake of metronidazole

Metronidazole is a low molecular weight compound, and indirect evidence suggests that the drug penetrates all cell membranes easily, irrespective of whether the organism is susceptible or not. Metronidazole uptake in anaerobic protozoa and Gram-negative anaerobic bacteria appears to occur by a simple passive diffusion process in which the rate of uptake is

proportional to the rate of intracellular reduction of the drug. Significant accumulation of ^{14}C -labelled metronidazole has been observed in susceptible organisms, whereas non-sensitive cells contained, but did not accumulate the ^{14}C label (Ings *et al.*, 1974; Müller and Lindmark, 1976; Tally *et al.*, 1978). It is proposed that modification of the drug in susceptible cells diminishes the intracellular concentration of metronidazole, which promotes a continuous gradient for uptake (Müller, 1983). In non-sensitive cells, metronidazole remains in its inactive form, and the intracellular and extracellular concentrations of the drug soon equilibrate (Müller, 1983). Church and Laishley (1995) refer to preliminary evidence for metronidazole uptake in *C. pasteurianum* via an energy-dependent mechanism. Drug uptake in this organism is blocked by glycolytic and transmembrane proton motive force inhibitors (Church, 1985). However, this phenomenon is possibly a secondary function of impaired drug reduction, as inhibitors of glycolysis have been shown to block drug activation (Marczak *et al.*, 1983b).

1.7.2.2 Reduction of metronidazole

In its unmodified form, metronidazole is an inert pro-drug. Its antimicrobial activity presumably arises from the reactivity of short-lived intermediates which are generated as the 5-nitro group is reduced by sequential four-electron reduction to a hydroxylamine (Müller, 1983) (Fig. 1.10). The proposed reduction sequence is as follows: activation of metronidazole (1) yields the nitro-free radical (2), and a protonated form (3) which is further reduced to the nitroso compound (4), the nitroso-free radical (5), and finally, the hydroxylamine (6). This last step is followed by fragmentation of the parent molecule to release the inactive end products, acetamide (7) and N-(2-hydroxyethyl)-oxamic acid (8) (Goldman, 1980; Knox *et al.*, 1983). The first step in the biological activation of metronidazole i.e. generation of the nitroradical anion, is reversible. Electron-spin resonance spectrometry has shown that in the presence of oxygen, the nitro-free radical can be converted back to its original form via formation of superoxide in a process referred to as "futile cycling" (Perez-Reyes *et al.*, 1980; Moreno *et al.*, 1983, 1984; Lloyd and Pedersen, 1985; Müller *et al.*, 1988; Edwards, 1993a).

The potential toxicity of the superoxide radical anion can be counteracted by the superoxide dismutase enzyme present in aerotolerant bacteria (Tally *et al.*, 1977). Oxygen presumably

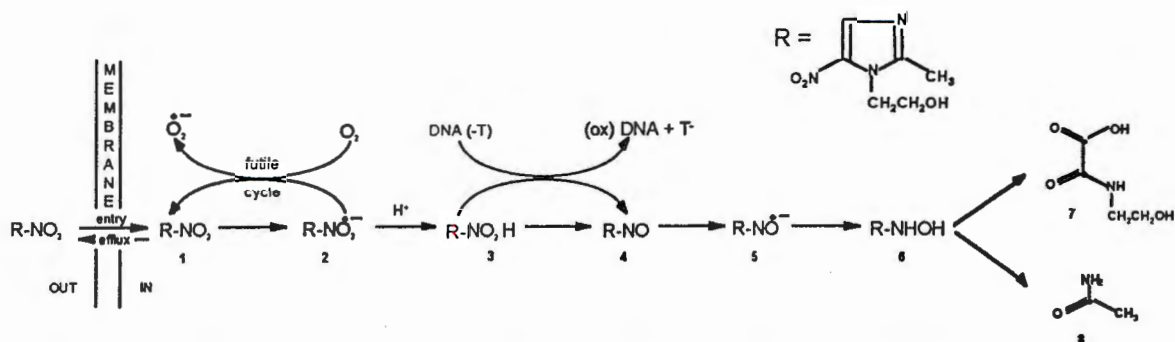


Fig. 1.10. Proposed reduction sequence of metronidazole and mechanism of action. Adapted from Edwards (1993a) and Johnson (1993).

protects cells from the drug either by "futile cycling" reoxidation of the nitro-free radical, or by direct competition for the electrons required to reduce metronidazole (Johnson, 1993). The selective ability of anaerobic organisms to activate metronidazole is a function of their reducing power since they are equipped with electron transport proteins and electron carriers of sufficiently low redox-potential to reduce the drug (Edwards, 1980; Ingham *et al.*, 1980).

1.7.2.3 Cellular cytotoxicity

Due to the instability of the proposed intermediates in the metronidazole reduction sequence, the transitory product(s) responsible for cytotoxicity has not been identified, although many studies point to the nitro-free radical as the toxic derivative (Ings *et al.*, 1974; LaRusso *et al.*, 1977; Moreno *et al.*, 1984; Edwards, 1986). Like other free radicals, it appears that the reactive species causes general damage to cellular components such as DNA, membranes and proteins.

DNA is believed to be the primary target of metronidazole action, and damage occurs mainly by single- and double-strand DNA breakage (Ings *et al.*, 1974; Goldstein *et al.*, 1977; Knight *et al.*, 1978; Rowley *et al.*, 1979; Dachs *et al.*, 1995). It is proposed that DNA is oxidised by a protonated form of the nitro-free radical (Fig 1.10, intermediate (3); Edwards, 1993a), and in the process of electron abstraction, the oxygen from the nitro group of the nitroimidazole radical is transferred to the DNA sugar-damaged product (Kappen *et al.*, 1989). As convincing *in vivo* evidence for metronidazole-induced DNA damage, Yeung

et al. (1984) and Jackson *et al.* (1984) have demonstrated that UV-sensitive *E. coli* strains with disrupted DNA repair systems are more susceptible to metronidazole treatment. AT-rich DNA is the preferred substrate for metronidazole and other related nitroimidazole drugs. Damage to DNA of a high A+T content is far more extensive than to DNA of a low A+T content (Edwards *et al.*, 1980; Knox *et al.*, 1981; Ludlum *et al.*, 1988; Tocher and Edwards, 1992) and predictably, microorganisms with a high A+T content show increased sensitivity to metronidazole (Edwards, 1993a). The major target site for nitroimidazole drugs appears to be the phosphodiester bonds of thymine residues (Rowley *et al.* 1980). Consistent with this is the apparent involvement of not only the SOS repair response, but also endonuclease III and exonuclease III, in the repair of metronidazole-induced DNA damage (Widdick and Edwards 1991; Edwards, 1993a). The latter two enzymes are responsible for the recognition and repair of oxidative damage to pyrimidines. The relationship between DNA damage and A+T content therefore serves as an additional factor in the selective toxicity of metronidazole.

Cytoplasmic membrane damage appears to be another mechanism of metronidazole cytotoxicity. A 99.99% loss of viability has been observed in *C. pasteurianum* cells shortly after treatment with a high concentration of the drug (10 µg/ml) (Church *et al.*, 1991). Cell death was accompanied by termination of end product formation and a significant increase in the protein content of the culture medium, both of which are indicative of rapid cell lysis. In this instance, rapid cell lysis would supersede the slower effects of metronidazole-induced DNA damage (Church *et al.*, 1991). This immediate bactericidal effect of metronidazole has not been described previously, but high concentrations of the drug have been shown to cause general disintegration of *Trichomonas vaginalis* cells (Buchner and Edwards, 1975). Morphological indications of metronidazole-induced damage in trichomonads and Gram-negative bacteria such as *E. coli* and *Bacteroides fragilis* include cell elongation, loss of cytoplasm, and alterations in cell surface charge and hydrophobicity (Skarin and Mardh, 1981; Jackson *et al.*, 1984; Silva-Filho and Souza, 1988; Cavalcanti *et al.*, 1991; Novaes *et al.*, 1991). These morphological changes may be indicative of damage to as yet, unidentified target sites, or of damage to the DNA or the cytoplasmic membrane. For example, induction of the SOS DNA repair system initiates a number of cellular responses such as the inhibition of cell division which results in cell filamentation. The metronidazole-

induced decrease in cell surface charges in pathogenic bacteria at clinically relevant concentrations raises the curious hypothesis that interaction between the host cell and the pathogen is favoured, thereby promoting engulfment of the pathogenic cell by phagocytosis (Silva-Filho and Souza, 1988; Calvacanti *et al.*, 1991).

1.7.3 Selective activation of metronidazole in the clostridia

It is recognised that reduced ferredoxin is required for the intracellular reduction of metronidazole, but the nature of its role, either as a direct chemical reducer (O'Brien and Morris, 1972; Lindmark and Müller, 1976; Chen and Blanchard, 1979; Marzak *et al.*, 1983*b*) or as a low potential electron carrier in enzymatic reduction (Edwards and Mathison, 1970; Tally *et al.*, 1978; Lockerby *et al.*, 1984), has been disputed. Church and co-workers have provided convincing evidence that in *C. pasteurianum*, metronidazole reduction occurs enzymatically via a ferredoxin-linked hydrogenase mechanism. Their results are summarised in a recent review article (Church and Laishley, 1995) and the conclusions of three important experiments are outlined below.

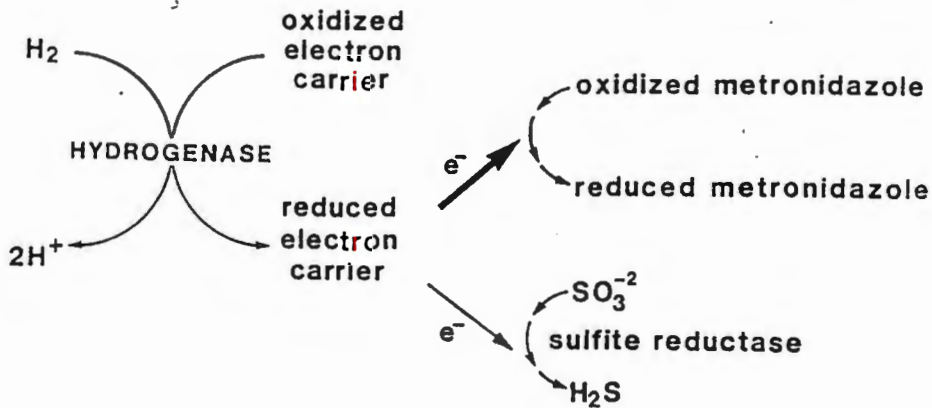
1.7.3.1 Reduction of metronidazole in *C. pasteurianum* is ferredoxin-linked

As measured by the uptake of hydrogen gas in the presence of metronidazole, ferredoxin-depleted cell-free extracts of *C. pasteurianum* were unable to reduce the drug, whereas untreated cell-free extracts could (Lockerby *et al.*, 1984). Low potential electron carriers such as methyl viologen, benzyl viologen and the flavin coenzymes, FAD and FMN, were able to substitute for ferredoxin in the ferredoxin-stripped cell-free extracts. This study demonstrated that ferredoxin is essential for the reduction of metronidazole by *C. pasteurianum*.

1.7.3.2 Ferredoxin-linked metronidazole reduction acts as a potent electrophile in *C. pasteurianum*

In vitro competition assays using *C. pasteurianum* cell-free extracts showed that 1) metronidazole was preferentially reduced before sulfite reductase activity was detected by sulfide evolution (Lockerby *et al.*, 1984), and 2) reduction of metronidazole accelerated the phosphoroclastic reaction and inhibited the production of hydrogen (Church *et al.*, 1985). In other words, the metronidazole-reducing reaction diverts electrons from these physiological ferredoxin-dependent reactions, temporarily affecting the overall metabolic

A



B

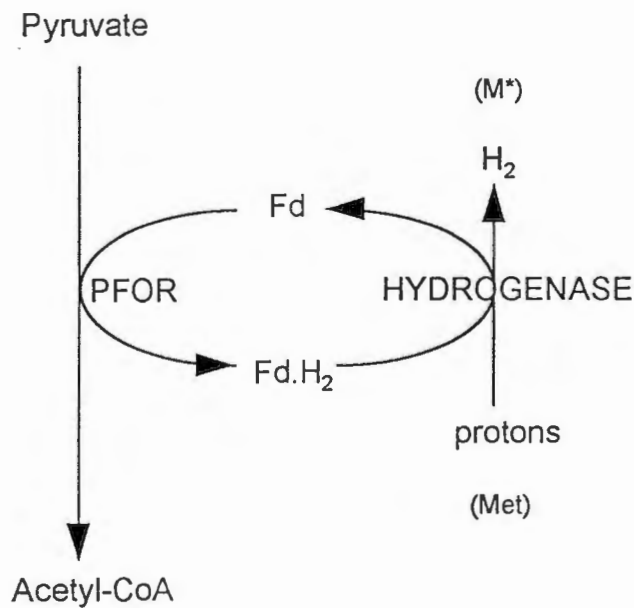


Fig. 1.11. Simplified scheme of A) the effect of metronidazole reduction on the inducible dissimilatory sulfite reductase pathway in *C. pasteurianum* (Lockerby *et al.*, 1984) and B) the role of the phosphoroclastic reaction of *C. pasteurianum* in metronidazole reduction. Metronidazole (Met) is reduced to its active derivative (M^*). Adapted from Santangelo (1991) and Church and Laishley (1995).

activity of the cell until the drug is completely reduced (Fig. 1.11). Since the reduction of ferredoxin in both the sulfite reductase and phosphoroclastic systems is coupled via the hydrogenase, to the oxidation of molecular hydrogen gas, the bidirectional hydrogenase was identified as the common denominator in these reactions.

1.7.3.3 Ferredoxin-linked metronidazole reduction in *C. pasteurianum* is enzymatically mediated by the hydrogenase

Metronidazole reduction activity was found to co-purify with *C. pasteurianum* hydrogenase activity during the course of a purification procedure (Church *et al.*, 1988). The reduction reaction required reduced ferredoxin, although other low potential electron carriers could be substituted with less efficiency. This led to the conclusion that metronidazole is enzymatically reduced by the hydrogenase via a ferredoxin-linked reaction, and a similar role in reducing other nitroimidazole drugs has been demonstrated for the *C. pasteurianum* hydrogenase (Church *et al.*, 1990). The rates of reduction of these nitroimidazole compounds correlated with their reduction potentials. These authors hypothesise that the reduction rates may also depend on the affinity of the nitroimidazole drug for the hydrogenase enzyme. They propose that metronidazole binds the hydrogenase in the vicinity of the electron carrier site, and that the nitro-group of the drug then preferentially scavenges electrons from reduced ferredoxin, thereby stimulating the activity of the enzyme.

1.7.4 Reduction of metronidazole in other anaerobic microorganisms

Does the ferredoxin-linked hydrogenase-mediated activation of metronidazole in *C. pasteurianum* represent a common drug reduction mechanism in most susceptible anaerobic organisms? The distribution of the hydrogenase and pyruvate:ferredoxin oxidoreductase (PFOR) enzymes correlates with metronidazole susceptibility in a wide range of pathogenic anaerobic bacteria (Narikawa, 1986; Church and Laishley, 1995) and these enzymes, together with ferredoxin, are all components of the phosphoroclastic reaction, an essential metabolic system for fermentative oxidation of pyruvate in anaerobic bacteria (see Section 1.3.1). A similar electron transport chain is present in the ancient aerotolerant eukaryotes, the trichomonads, which have evolved a specialised organelle, the hydrogenosome, for this purpose. Hydrogenosomal extracts from *T. vaginalis* have been shown to reduce metronidazole (Yarlett *et al.*, 1985), and as in *C. pasteurianum*, metronidazole reduction has been observed to inhibit hydrogen production *in vivo* in drug sensitive and resistant strains (Lloyd and Kristensen, 1985). On the other hand, highly susceptible anaerobic protozoa such as *Giardia* and *Entamoeba* lack the hydrogenase enzyme (Johnson, 1993). An enzyme tentatively identified as a pyruvate dehydrogenase in *Giardia duodenalis* has been shown to mediate metronidazole reduction *in vitro* when

coupled by ferredoxin purified from the organism (Townson *et al.*, 1994). It therefore seems that a variety of electron transport proteins that are involved in the oxidative fermentation of pyruvate provide the source of electrons for the reductive activation of metronidazole.

1.7.5 Mechanisms of metronidazole resistance

The incidence of clinical metronidazole resistance in anaerobic pathogens is relatively low, but nevertheless significant (Johnson, 1993). Plasmid-encoded metronidazole resistance has been identified in *Bacteroides* species (Rotimi *et al.*, 1981; Breuil *et al.*, 1989; Wehnert *et al.*, 1990, 1992; Haggoud *et al.*, 1992; Reysset *et al.*, 1992, 1993). The resistance phenotype is transferable by conjugation, and the specific gene products have recently been characterized, although their functions are unknown. The *metA* gene isolated from *B. fragilis* plasmid pBFC1 encodes a ~7 kDa polypeptide and has been shown to reduce metronidazole-induced DNA damage in *E. coli* (Wehnert *et al.*, 1990, 1992; Dachs *et al.*, 1995). The *nim* genes isolated from *Bacteroides* plasmids pIP417, pIP419 and pIP421 encode proteins of 18-20 kDa, all of which share 80-90% sequence similarity and are preceded by different insertion sequence elements (Haggoud *et al.*, 1994; Trinh *et al.*, 1995). It appears that transcription of these *nim* genes is directed by outward-oriented promoters located within the right ends of the insertion sequences (Trinh *et al.*, 1995). A *nim* gene of chromosomal origin has also been identified (Haggoud *et al.*, 1994).

Theoretically, metronidazole resistance can arise from 1) the inability to reduce the drug to its active intermediate(s), and 2) decreased susceptibility of the target sites. Although decreased permeability to drug uptake is sometimes cited as a mechanism of resistance, it appears to be a secondary function of impaired drug reduction in both protozoa and Gram-negative bacteria (Britz and Wilkinson, 1979; Tally *et al.*, 1979; Rowley and Edwards, 1982; Kulda *et al.*, 1993).

There have been no reports of decreased metronidazole susceptibility occurring via an enhanced DNA repair system, but it is a logical mechanism of resistance. Chrystal *et al.* (1980) have proposed a model in which survival requires that the rate of DNA repair must

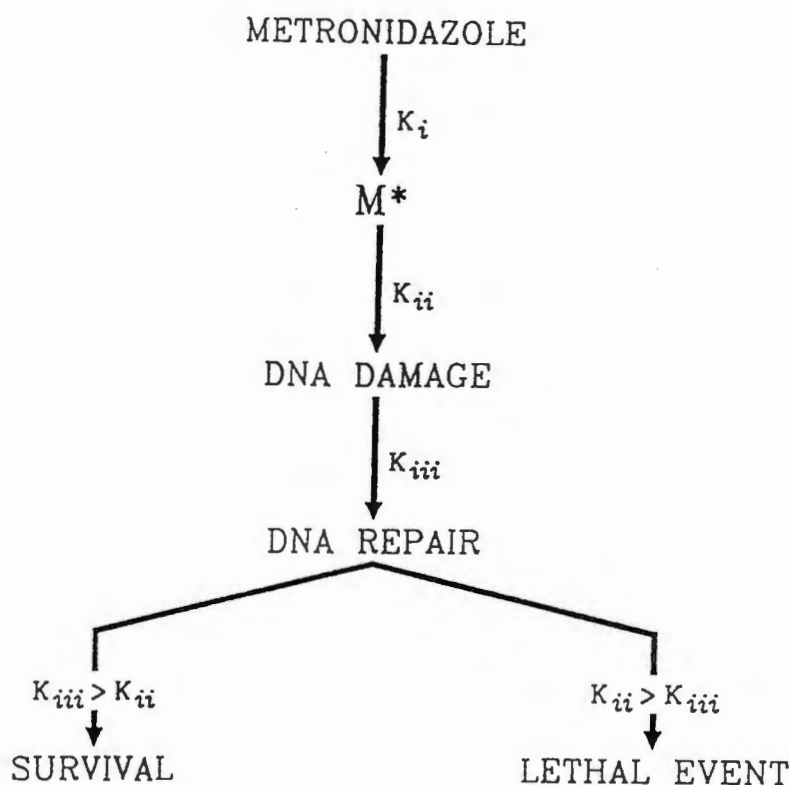


Fig. 1.12. A model relating metabolism of metronidazole to cell death. Metronidazole is reduced to its active derivative M^* by the cell. M^* reacts with DNA, causing damage which can be repaired by the DNA repair systems of the host. K_i is the rate of reduction of metronidazole, K_{ii} is the rate of DNA damage, and K_{iii} is the rate of DNA repair. If the rate of DNA damage (K_{ii}) is greater than the rate of DNA repair (K_{iii}), cell death occurs. Adapted by Santangelo *et al.* (1991) from Chrystal *et al.* (1980).

be greater than the rate of DNA damage (Fig.1.12). In this way, a cell with an efficient DNA repair mechanism will be able to tolerate higher levels of damage. The reverse scenario, in which mutants with impaired DNA repair systems are more sensitive to metronidazole than their wild type parents, has been demonstrated in *E. coli* (Jackson *et al.*, 1984; Yeung *et al.*, 1984). Furthermore, multiple DNA repair mutations have been shown to have a multiplicative effect on the sensitivity of the *E. coli* host to metronidazole. Loss of e.g. *uvrA* and *lexA* gene functionality decreased the metronidazole MIC by a factor of two and eight, respectively, and concomitant loss of both these gene functions decreased the metronidazole MIC by a factor of sixteen (Jackson *et al.*, 1984).

Examination of the mechanisms of induced or clinical resistance reveals that the predominant cause of decreased metronidazole susceptibility is inefficient drug activation via the

components of the phosphoroclastic reaction. Drug resistance is often associated with a decrease in PFOR activity and/or hydrogenase activity or ferredoxin levels, and is generally accompanied by alterations in carbohydrate metabolism. Decreased PFOR/pyruvate dehydrogenase activity has been observed in clinically resistant isolates of *Giardia intestinalis* (Johnson, 1993) and *B. fragilis* (Narikawa *et al.*, 1991), and in chemically-induced metronidazole resistant mutants of *B. fragilis* and *C. perfringens* (Britz and Wilkinson, 1979; Sindar *et al.* 1982). Church and Laishley (1995) point out that in some cases PFOR is probably mistakenly referred to as pyruvate dehydrogenase, the equivalent enzyme for pyruvate metabolism in aerobic systems. Pyruvate dehydrogenase is absent in anaerobic organisms and its activity is coupled by NAD, an electron carrier that cannot couple metronidazole reduction (Church *et al.*, 1988). Increased lactate production was associated with diminished PFOR activity in the *B. fragilis* and *C. perfringens* strains, and Narikawa *et al.* (1991) have shown that increased lactate dehydrogenase activity compensates for reduced PFOR activity. Decreased levels of both PFOR and hydrogenase activity also correlate with *in vitro* induced metronidazole resistance in trichomonads (Kulda *et al.*, 1987; Kulda *et al.*, 1993). Resistant strains of *Tritrichomonas foetus* were shown to substitute ethanol fermentation for hydrogenosomal pyruvate metabolism and displayed increased pyruvate decarboxylase activity, whereas resistant strains of *T. vaginalis* were characterized by enhanced levels of lactate glycolysis. In both cases the fermentation was shifted towards the production of electron sink products.

Reduced transcription of the ferredoxin gene is responsible for metronidazole resistance in a number of clinical isolates of *T. vaginalis* (Quon *et al.*, 1992). Immunoblot and Northern blot analysis revealed that levels of intracellular ferredoxin and ferredoxin mRNA were reduced by more than 50% in four metronidazole resistant strains. Two point mutations have been identified in the DNA region upstream of the ferredoxin structural gene, one of which results in a decreased binding affinity for a ~23 kDa regulatory protein.

The metronidazole resistant phenotype of the mutant described in this study is attributed to an increased capacity for DNA repair.

1.8 Aims of this thesis

In anaerobic microorganisms, the electron transport proteins that provide the source of electrons for the reductive activation of metronidazole are involved in pyruvate metabolism (Johnson, 1993). The motivation for this study was the potential to manipulate fermentation patterns in *C. acetobutylicum* via changes in electron distribution. The purpose of this project, therefore, was to use induced metronidazole resistance in *C. acetobutylicum* P262 as a selection tool for the isolation of genes involved in electron/carbon flow pathways. Previously in our laboratory, an *E. coli* F19 *recA*, nitrate reductase-deficient mutant has been used as a negative selection system to clone "metronidazole-activating" loci e.g. the flavodoxin gene, from *C. acetobutylicum* (Santangelo *et al.*, 1991). Such loci render the *E. coli* host strain sensitive to metronidazole. However, the disadvantages of this cloning system is that it is subject to heterologous host factor interference, and the molecular basis of the *TnphoA*-induced nitrate reductase mutation has not been characterized. Mutagenesis and mutational cloning in *C. acetobutylicum* offer a more direct approach. Conjugative transposons were used to generate metronidazole resistant mutants, and in the process, the potential for transposon mutagenesis in *C. acetobutylicum* P262 was assessed. (At present, the P262 strain has no other system for DNA delivery). A metronidazole resistant mutant was then used to clone and identify a gene implicated in pyruvate metabolism and metronidazole susceptibility. Although incidental to this study, mechanisms of metronidazole resistance in anaerobic bacteria are of medical interest.

Chapter 2

Investigation of transposon mutagenesis in *C. acetobutylicum* P262: isolation of two metronidazole resistant mutants

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Chapter 2

Investigation of transposon mutagenesis in *C. acetobutylicum* P262: isolation of two metronidazole resistant mutants

2.0 Summary

To obtain mutants that were resistant to metronidazole, *C. acetobutylicum* P262 was conjugated with *E. faecalis* OG1RF(pINY1275) containing the composite transposon Tn925::Tn917. The transfer frequencies of Tn925::Tn917 to *C. acetobutylicum* ranged from 10^{-4} to 10^{-3} transconjugants per recipient. Two metronidazole resistant mutants, designated 3R and 30R, were isolated from the 400 transconjugants screened. The metronidazole MIC value for the wild type was 0.05 $\mu\text{g/ml}$, whereas the MIC value for both the mutants was 0.10 $\mu\text{g/ml}$. Southern hybridization analysis established that mutants 3R and 30R were free of conjugative donor plasmid DNA and contained one and four copies of the Tn925::Tn917 cointegrate, respectively. The single left end transposon junction fragment of mutant 3R, and two left end transposon junction fragments of mutant 30R, were isolated by colony hybridization from size-selected *EcoRI-XhoI* genebanks constructed for each of the mutants. Nucleotide sequencing of these transposon-chromosome junctions revealed that a stretch of adenine residues was common to the target DNA, yet each of these DNA regions represented a different transposon insertion site in the *C. acetobutylicum* genome.

2.1 Introduction

C. acetobutylicum P262 is the most efficient natural producer of acetone and butanol of the solventogenic clostridia, yet unlike many other *C. acetobutylicum* strains, it is recalcitrant to most genetic manipulation procedures. Conjugative transposon mutagenesis has successfully been exploited for those microorganisms which lack conventional systems for DNA delivery. The distinguishing characteristics of conjugative transposons and their mechanism of transposition are described in Chapter 1, Section 1.6. The effectiveness of these transposons as insertional mutagens depends on the variety of target sites within a particular recipient and the number of transposon copies inherited, since multiple copies present an unwanted complication as regards physiological characterization and mutational cloning.

So far, conjugative transposons Tn916, Tn925 and Tn1545 have been introduced into a variety of clostridial species with reasonable success. Transposition is generally "random" e.g. Tn916 and Tn1545 have been shown to integrate at various sites in the genomes of *C. acetobutylicum* DSM 792 and *C. acetobutylicum* NCIMB 8052, respectively (Bertram and Dürre, 1989; Woolley *et al.*, 1989; Wilkinson *et al.*, 1995a). In some cases, however, transposition is restricted to a particular locus e.g. Tn916 integrates with one orientation in a single site in the *C. difficile* genome (Mullany *et al.*, 1991), and *C. acetobutylicum* NCIMB 8052 also appears to contain a hot spot for Tn916 integration (Woolley *et al.*, 1989). Preferential insertion in non-coding regions with increased A+T composition is presumably the reason that the majority of Tn1545 insertions in *C. acetobutylicum* NCIMB 8052 appear to be genetically silent (Woolley *et al.*, 1989; Young, 1993). Nevertheless, *C. acetobutylicum* transposon-induced mutants displaying various phenotypes have been isolated (Bertram *et al.*, 1990; Babb *et al.*, 1993; Kashket and Cao, 1993; Sass *et al.*, 1993; Young, 1993; Wilkinson *et al.*, 1995a). Multiple copies of the transposons have been detected in a number of *C. acetobutylicum* recipients (e.g. Bertram and Dürre, 1989; Young, 1993). Since Tn916 does not repress the transposition activity of an incoming copy of the element (Norgren and Scott, 1991), multiple insertions are most likely a consequence of multiple, consecutive conjugation events.

This chapter describes the construction of a *C. acetobutylicum* P262 transposon Tn925::Tn917 mutant bank, and the isolation and preliminary characterization of two

metronidazole resistant mutants, whereby the feasibility of transposon mutagenesis in *C. acetobutylicum* strain P262 is assessed. Metronidazole resistance was used as a selection system for the isolation of insertion mutants with altered electron transport pathways or carbon metabolism, as discussed in Chapter 3. The Tn925::Tn917 composite transposon (~23 kb) consists of the non-conjugative transposon Tn917 integrated in Tn925 (Christie *et al.*, 1987). The partially sequenced erythromycin resistant determinant of Tn1545 is almost identical to that of Tn917, although it is constitutive and non-inducible (Trieu-Cuot *et al.*, 1990). The transposons Tn916, Tn925, Tn925::Tn917 and Tn1545 are all identical in the features described, although by convention, the left end of Tn916 corresponds to the right end of Tn1545. Here, the Tn916 convention is used.

2.2 Materials and methods

2.2.1 Bacterial strains and plasmids

The wild type *C. acetobutylicum* strain P262 was originally obtained from National Chemical Products (NCP), Germiston, South Africa, and has been described by Jones *et al.* (1982). *E. faecalis* OG1RF(pINY1275) which contains the Tn925::Tn917 cointegrate and *E. faecalis* OG1SSp(pAD1) (Christie *et al.*, 1987) were used as the conjugal donor strain and the source of donor plasmid DNA, respectively. The donor strain was originally misidentified as *E. faecalis* OG1SSp(pCF10) which contains Tn925 (see Appendix A). *E. coli* JM105 (Yanisch-Perron *et al.*, 1985) served as the cloning host for DNA constructs in the pUC19 (Yanisch-Perron *et al.*, 1985) and the Bluescript SK (Stratagene) vectors. Restriction maps of the vectors are given in Appendix D.

2.2.2 Media and growth conditions

The wild type *C. acetobutylicum* was maintained aerobically as a spore suspension in sterile water at 4°C. Spores were activated by heat shock treatment at 70°C for 3 min, cooled on ice for 1 min, and used as the primary inoculum. The asporogenous *C. acetobutylicum* transconjugant 3R was maintained anaerobically as a glycerol stock at -70°C without antibiotic selection. The oligosporogenous *C. acetobutylicum* transconjugant 30R was stored both as a spore suspension and as a frozen glycerol stock. *C. acetobutylicum* strains were grown in *Clostridium* Basal Medium (CBM) as described by Allcock *et al.* (1982). CBM was supplemented with tetracycline (1 µg/ml) for mutant selection. Cultures were incubated at 34°C in an anaerobic glove cabinet (Forma Scientific Inc.) containing an

atmosphere of oxygen-free N₂, CO₂ and H₂ (85:10:5 by volume). Conjugation mixtures were plated on BASOL medium (Reysset and Sebald, 1985) supplemented with HY-casein (1%) and xylose (1%) as the carbon source for counterselection of the *E. faecalis* donor. *E. faecalis* OG1RF(pINY1275) was grown aerobically in Elliker Broth (Difco) supplemented with tetracycline (10 µg/ml). *E. faecalis* OGSp(pAD1) was grown at 37°C in Oxoid broth supplemented with 0.1M TrisCl (pH 7.5), glucose (0.2%) and streptomycin (1 mg/ml). *E. coli* clones were grown in 2xYT medium (Messing, 1983) containing ampicillin (100 µg/ml). Tetracycline (10 µg/ml) was used for selection of the *tetM* construct (pTet) in *E. coli*.

2.2.3 Conjugation procedure

The conjugation procedure for *C. acetobutylicum* strain P262 (Babb *et al.*, 1993) was adapted from the method described by Bertram and Dürre (1989). The *C. acetobutylicum* and *E. faecalis* cultures were grown to an OD₆₀₀ of approximately 1.0. Samples (100 µl) of the donor and recipient were plated onto BASOL medium which was selective for the recipient. After incubation (24 h), cells from each plate were resuspended in 2 ml sterile water. Dilutions of the cell suspension were plated onto BASOL medium supplemented with tetracycline (1 µg/ml). The frequency of transfer was calculated as the number of transconjugants per recipient in the conjugation mixture at the end of the conjugation period. The transconjugants were tested for resistance to erythromycin (1 µg/ml) to confirm the presence of Tn917.

2.2.4 Screening for metronidazole resistance and determination of MICs

C. acetobutylicum metronidazole resistant transconjugants were isolated by selection on CBM agar containing metronidazole (Sigma) (0.2 µg/ml). The minimal inhibitory concentration (MIC) of metronidazole for the *C. acetobutylicum* wild type and metronidazole resistant mutants was determined by plating cultures in the mid-exponential phase of growth onto CBM agar containing various concentrations of metronidazole. The MIC was defined as the lowest concentration of the drug that inhibited growth after overnight incubation.

2.2.5 Transposon stability assay

C. acetobutylicum mutants 3R and 30R were cultured in CBM without tetracycline selection

for 30 successive transfers. Serial dilutions of the subcultured samples were plated onto solid medium with and without tetracycline (1 µg/ml). The number of colony forming units (cfu) was compared after overnight incubation, and 100 colonies derived from the non-selective plate were subsequently duplicated onto solid medium with and without tetracycline (1 µg/ml) and metronidazole (0.2 µg/ml).

2.2.6 DNA extraction and manipulation

Plasmid DNA was isolated from *E. faecalis* according to Anderson and McKay (1983). Plasmid DNA was prepared from *E. coli* by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981). *C. acetobutylicum* genomic DNA was prepared according to the method of Marmur (1961) as modified by Zappe *et al.* (1986). Restriction endonuclease digestion of DNA was performed as recommended by the manufacturers, and routine cloning procedures were as described by Sambrook *et al.* (1989).

2.2.7 Construction of size-selected genebanks

Size-selected genebanks of the *C. acetobutylicum* mutants 3R and 30R were generated as follows: chromosomal DNA (50 µg) was digested to completion with the appropriate restriction enzymes and the resulting fragments fractionated by electrophoresis in 0.8% agarose gels in Tris/acetate buffer. DNA fragments of relevant size were recovered by the GeneClean (Bio 101, Inc.) procedure, ligated to the pUC19 or pBluescript SK vectors which had been restricted with the appropriate endonucleases, and transformed into *E. coli* JM109.

2.2.8 Colony hybridization

Colonies harbouring recombinant plasmids with *C. acetobutylicum* insert DNA were duplicated onto Hybond N⁺ membranes placed on 2xYT solid medium. The colonies were lysed after overnight incubation and the membrane filters were processed according to Sambrook *et al.* (1989). This was followed by hybridization and detection as described for Southern hybridization.

2.2.9 Southern hybridization

Digested plasmid DNA and *C. acetobutylicum* chromosomal DNA were fractionated by electrophoresis in 0.8% agarose gels in Tris/acetate buffer. The DNA was transferred by

capillary action to Hybond N⁺ nylon membranes and fixed according to the manufacturer's instructions. DNA restriction fragments selected as probes were gel-purified and labelled with Digoxigenin-11-dUTP (DIG) by the random primed method using a DIG DNA labelling kit (Boehringer Mannheim). Hybridization with a DIG-labelled probe (approximately 10-25 ng/ml) was performed at 68°C overnight. This was followed by chemiluminescent detection with Lumigen™ PPD (Boehringer Mannheim) according to the manufacturer's instructions.

2.2.10 Nucleotide sequencing

The transposon junction sites of plasmids p30R1, p30R2 and p3R were sequenced in one direction using the primer 5'-CGAAAGCATCTAGAATAAGGC-3' which is specific for the left end of Tn916. The primer was synthesised by D. Botes (Department of Biochemistry, University of Cape Town). DNA sequencing was performed by the dideoxy-chain termination method of Sanger *et al.* (1977) using [³⁵S] dATP and the T7 DNA sequencing kit (Pharmacia Biotech.).

2.3 Results

2.3.1 Conjugal transfer of Tn925::Tn917 to *C. acetobutylicum* P262

Approximately 1400 tetracycline resistant transconjugants were obtained from two small-scale conjugation experiments performed in conjunction with B. Babb (Department of Microbiology, University of Cape Town). The Tn925::Tn917 transposon cointegrate was transferred from *E. faecalis* to *C. acetobutylicum* at a frequency of 10⁻⁴ to 10⁻³ transconjugants per recipient. The erythromycin resistance (*erm*) determinant of Tn917 was not used as a selective marker in the conjugation experiments, but resistance of the transconjugants to erythromycin was confirmed. The *erm* determinant is unable to function as a selective marker for Tn925::Tn917-containing transconjugants of *C. acetobutylicum* DSM 792, as this strain displays a high level of resistance against the antibiotic (Bertram and Dürre, 1989).

2.3.2 Isolation of metronidazole resistant mutants

Two metronidazole resistant mutants, referred to as 3R and 30R, were isolated from the 400 *C. acetobutylicum* transconjugants screened. The MIC of metronidazole for the

C. acetobutylicum wild type was 0.05 µg/ml, whereas the MIC value for mutants 3R and 30R was 0.10 µg/ml, i.e. double that of the parent strain.

2.3.3 Transposon stability

Mutants 3R and 30R were examined for reversion to tetracycline sensitivity after 30 successive transfers (approximately 750 generations) in the absence of tetracycline selection. The results (Table 1.1) show that approximately the same number of colonies were obtained on plates with or without the antibiotic. The metronidazole resistant phenotype was also present in all 100 colonies tested from each of the samples, indicating stable inheritance of the Tn925::Tn917 cointegrate within the limits of detection. In contrast, Bertram *et al.* (1990) reported a 50% loss of Tn916 integration in a *C. acetobutylicum* DSM 792 solvent-deficient transconjugant after 15 transfers without tetracycline.

Table 1.1. Stability of *C. acetobutylicum* Tn925::Tn917 mutants in the absence of tetracycline

Cultures passaged for ~750 generations in the absence of Tet	Cell count*	
	Plate selection -Tet	Plate selection +Tet
3R	123	145
30R	400	385

*Samples were diluted x 10⁵

2.3.4 Check for presence of donor plasmid DNA

The *E. faecalis* donor plasmid pINY1275 that was used in the conjugation experiments consists of the Tn925::Tn917 composite integrated into the *Eco*RI D fragment of the 57 kb conjugative plasmid pAD1 (Christie *et al.*, 1987; see Appendix D for map). Transfer of a conjugative transposon present in a self-transmissible plasmid is independent of plasmid transfer, as indicated by the lack of linkage of plasmid and transposon markers in the transconjugants (Flannagan and Clewell, 1991). However, because pAD1 encodes a hemolysin-bacteriocin and resistance to UV light (Clewell *et al.*, 1986), it was important to

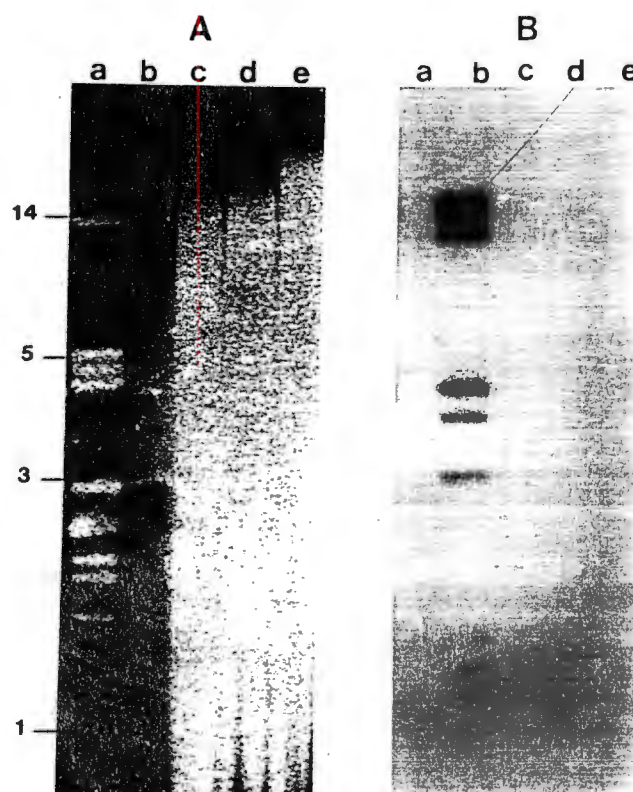


Fig. 2.1. Analysis of donor plasmid DNA by Southern hybridization. Agarose gel (A) and corresponding autoradiograph (B) of *EcoRI*-digested pAD1 plasmid DNA (lane b), and *EcoRI*-digested *C. acetobutylicum* chromosomal DNA from the wild type (lane c), mutant 3R (lane d) and mutant 30R (lane e). DNA was probed with DIG-labelled *EcoRI*-digested pAD1 plasmid DNA. Lane (a) contains *PstI*-digested lambda DNA as size markers.

ensure that these determinants did not contribute to the metronidazole resistant phenotype of mutants 3R and 30R. The mutants were examined for the presence of donor plasmid elements by Southern hybridization, using DIG-labelled *EcoRI*-digested pAD1 plasmid DNA as the probe (Fig. 2.1). The probe hybridized only to the eight restriction fragments generated by *EcoRI* digestion of plasmid pAD1 (lane b). No hybridization signal was detected for *EcoRI*-digested chromosomal DNA samples of the *C. acetobutylicum* wild type (lane c) and the *C. acetobutylicum* mutants 3R and 30R (lanes d and e, respectively). This indicated that the transconjugants were free of integrated pAD1 plasmid elements. Since the chromosomal DNA preparations were essentially total DNA preparations, it was concluded that independently-existing pAD1 plasmid DNA was also absent in the transconjugants.

2.3.5 Preliminary cloning of transposon DNA

A physical map of the Tn925::Tn917 cointegrate and the origin of the transposon DNA constructs described in this section are shown in Fig. 2.2. The Tn925 tetracycline resistance gene, *tetM*, was cloned into pUC19 as a 4.8 kb *HincII* restriction fragment. The clone, designated pTet, was isolated by selection for tetracycline resistance. The DIG-labelled DNA insert of plasmid pTet provided a convenient probe for transposon copy number analysis and isolation of the 5.3 kb *HindIII* fragment containing Tn925::Tn917 DNA. The resultant plasmid is referred to as p30R0 and was isolated via colony hybridization from a pSK-based *HindIII* size-selected genebank of mutant 30R. The 1.3 kb *HindIII*-*XhoI* restriction fragment exclusive to Tn917 was subcloned from plasmid p30R0 into pSK to generate pTn917'. This recombinant plasmid served as a probe for transposon copy number analysis and for cloning the left end transposon-chromosome junction fragments.

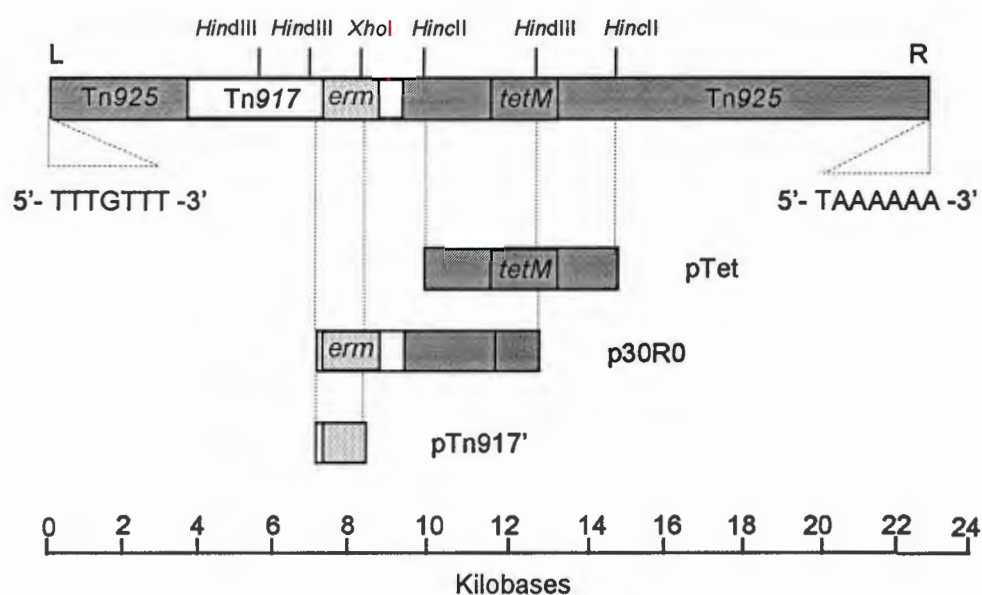


Fig. 2.2. Physical organization of the Tn925::Tn917 transposon cointegrate showing the relevant restriction sites and the origin of the DNA inserts of plasmids pTet, p30R0 and pTn917'. The *tetM* and *erm* determinants are carried by Tn925 and Tn917, respectively. The DNA sequence at the immediate left (L) and right (R) ends of Tn925 is assumed to be the same as reported for Tn916 (Clewell *et al.*, 1988).

2.3.6 Transposon copy number

The transposon copy number of mutants 3R and 30R was determined by Southern hybridization analysis (Fig. 2.3). The experiment was designed to detect the left end of the

transposon cointegrate and the flanking chromosomal DNA, such that each hybridization band represented a distinct transposon insertion. Chromosomal DNA from the mutants was restricted with *EcoRI* and *XhoI* endonucleases and probed with a DIG-labelled 1.3 kb *HindIII-XhoI* DNA fragment derived from plasmid pTn917'. Plasmid pTn917' DNA restricted with *HindIII-XhoI* (lane b), and *C. acetobutylicum* wild type chromosomal DNA digested with *EcoRI-XhoI* (lane c), served as positive and negative controls, respectively. The Tn917-exclusive probe hybridized to a single 7.4 kb *EcoRI-XhoI* fragment in the digested DNA of mutant 3R (lane d) and to four *EcoRI-XhoI* fragments, ranging from 7.3 to 12 kb in size, in the digested DNA of mutant 30R (lane e). This indicated that mutants 3R and 30R harboured one and four copies of the transposon, respectively, and also served to identify suitably-sized transposon-chromosome junction fragments for cloning. The number of transposon insertions was confirmed in a separate Southern hybridization experiment designed to detect the 3' transposon-chromosome junction fragments, using the 5.3 kb *HindIII Tn925::Tn917* DNA fragment as a probe (Appendix A). Since the same hybridization results were obtained using Tn917- and Tn925::Tn917-specific probes, the possibility of independent transposition of Tn917 can be excluded.

2.3.7 Cloning and DNA sequencing of left end transposon-chromosome junctions

To clone the left end transposon junction fragments identified by Southern hybridization, partial genebanks were prepared from *EcoRI-XhoI*-digested chromosomal DNA from the *C. acetobutylicum* mutants. Restriction fragments ranging from 6-8 kb and 6-12 kb in size were ligated into *EcoRI-XhoI*-digested Bluescript SK vector for construction of the mutant 3R and mutant 30R genebanks, respectively. A single clone, p3R, was identified from the *C. acetobutylicum* 3R genebank and two different clones, p30R1 and p30R2, were identified from the *C. acetobutylicum* 30R genebank by colony hybridization, using the 1.3 kb *HindIII-XhoI* DNA insert of pTn917' as a probe. Restriction analysis of the recombinant plasmids p3R, p30R1 and p30R2 revealed DNA inserts of 7.4 kb, 7.3kb and 5.2kb in size, respectively. Since the transposon-containing region of the DNA inserts was approximately 7 kb in size, it was deduced that plasmids p3R and p30R1 each contained 300-400 bp of *C. acetobutylicum* DNA. Plasmid p30R2 had presumably undergone a deletion, since the DNA insert (5.2 kb) was smaller than the size of the transposon-containing region (7 kb).

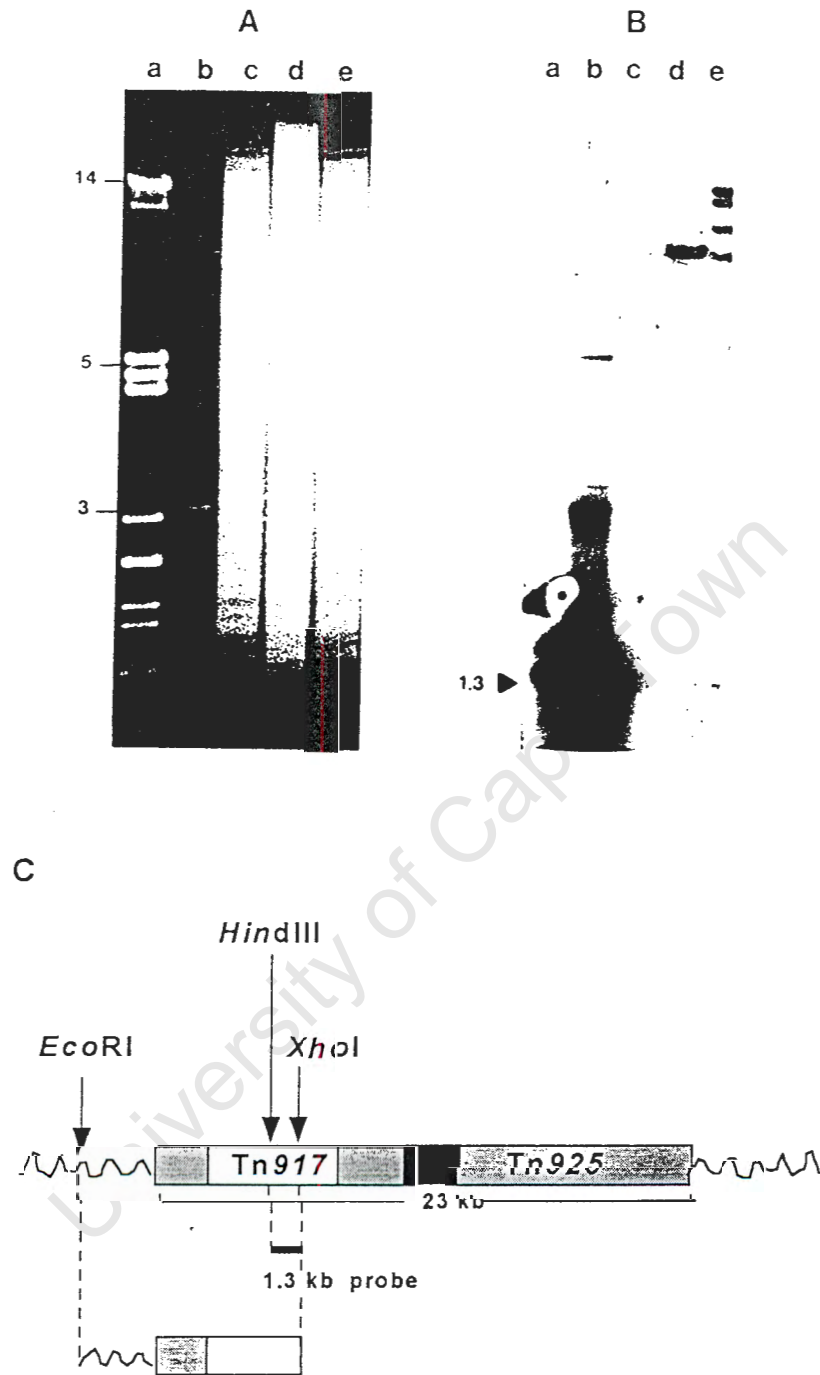


Fig. 2.3. Determination of transposon copy number by Southern hybridization. Agarose gel (A) and corresponding autoradiograph (B) of *Hind*III-*Xho*I-digested pTn917 plasmid DNA (lane b) and *Eco*RI-*Xho*I-digested *C. acetobutylicum* chromosomal DNA from the wild type (lane c), mutant 3R (lane d) and mutant 30R (lane e). DNA was probed with the 1.3 kb *Hind*III-*Xho*I DIG-labelled fragment of plasmid pTn917. Each hybridization band represents a unique transposon insertion, and the rationale is outlined in (C). Lane a contains *Pst*I-digested lambda DNA as size markers.

The left end transposon-chromosome junction sites were sequenced in one direction using a Tn916-specific primer, 5'-CGAAAGCATCTAGAATAAGGC-3', directed outward from the left end of the transposon. According to restriction mapping analysis, Tn916 and Tn925 are homologous (Christie and Dunny, 1986). The Tn925 DNA sequence flanking the left end junction was the same for plasmids p3R, p30R1 and p30R2, and identical to the sequence for the left terminus of Tn916 which has been sequenced by Clewell *et al.* (1988). The sequence of the neighbouring chromosomal regions was different for all three clones, indicating different sites of insertion in the *C. acetobutylicum* genome (Fig. 2.4). However, a stretch of adenine residues, located 5-6 bp from the transposon DNA sequence, was common to all three clones. Since the chromosomal DNA sequence for the similarly-sized

	Left end		Right end
<i>att</i> Tn916	TTATACTTTGTTT ---- -- - -->	NNNNNN	AAAAATAGCATAA <-- - -- -
p3R	TTATACTTTGTTT	GATTCA	***** * ** AAAAAAATTGTAG
p30R1	TTATACTTTGTTT	AACTTT	***** * ** AAAAAAAATGTAT
p30R2	TTATACTTTGTTT	ATTATA	***** ** AAAAATTAAATTT
pM5.1	TTATACTTTGTTT	ATATAC	***** ** AAAAAATTTCAAA
pM5.2	TTATACTTTGTTT	ATATAC	***** ** AAAAATAGTTAAA
pM5.3	TTATACTTTGTTT	AGAATC	***** ** AAAAAGAGATAGC
			+++++

Fig. 2.4. DNA sequence of the left junction sites of Tn925::Tn917 insertions in *C. acetobutylicum* P262 analysed for plasmids p3R, p30R1 and p30R2. The junction sequences for plasmids pM5.1, pM5.2 and pM5.3 (Babb *et al.*, 1993) are included for comparison. The first sequence is of the Tn916 transposon circle joint, designated *att* Tn916, containing the left and right ends of the transposon, separated by the 6 bp non-specific coupling sequence. Horizontal arrows denote terminal imperfect inverted repeats. The 6 bp coupling sequences or spacer regions of each junction site are boxed. Identical nucleotides, in pairwise comparison between right end of the transposon and every *C. acetobutylicum* target sequence are indicated by asterisks. Plus signs denote positions with 100% identity in all six target DNAs.

constructs, p3R and p30R1, was not identical, the metronidazole resistant phenotype of mutants 3R and 30R cannot be ascribed to a common transposon insertion. As evident from the nucleotide sequence, the left end transposon-chromosome junction of plasmid p30R2 was intact, despite *in vivo* deletion of the DNA insert as indicated by restriction analysis.

2.4 Discussion

Analysis of metronidazole resistant mutants 3R and 30R demonstrated that conjugative transposons provide a feasible approach for mutagenesis in *C. acetobutylicum* P262. Transposon Tn925::Tn917 integration appeared to be stable, suitably "random" and was not associated with integration or persistence of donor plasmid DNA. The major disadvantage of the transposon mutagenesis system is the incidence of multiple insertions (see Babb *et al.*, 1993). While mutant 3R contained a single copy of the transposon cointegrate, mutant 30R harboured four copies of the element. This makes it impractical to attempt to define the relationship between the genotype and the metronidazole resistant phenotype of mutant 30R. It was, however, established that the metronidazole resistant phenotypes of the two mutants were not the result of a common transposon insertion. Multiple insertions may sometimes lead to genomic rearrangements as a result of recombination between two or more transposon copies (Norgren and Scott, 1991; Wilkinson *et al.*, 1995a). Another potential disadvantage is instability of the cloned transposon-chromosome junction fragments (Babb, 1993), as observed for plasmid p30R2. One possible solution, however, is to target the transposon-chromosome junctions of interest by single specific primer PCR (see Sauer and Dürre, 1992). In this way, the cloning of large pieces of transposon DNA is avoided.

The ability of Tn925::Tn917 to insert at various loci on the *C. acetobutylicum* P262 chromosome was revealed by the different nucleotide sequences for each of the three left end transposon-chromosome junction fragments analysed. However, a stretch of at least five adenine residues was common to all three sequences, demonstrating that integration of the conjugative transposon is not a completely random process. The other *C. acetobutylicum* left end junction target sequences available to date (Sauer and Dürre, 1992; Babb *et al.*, 1993) also display a stretch of adenine residues adjacent to the left end of the transposon (Fig. 2.4 and Fig. 2.5).

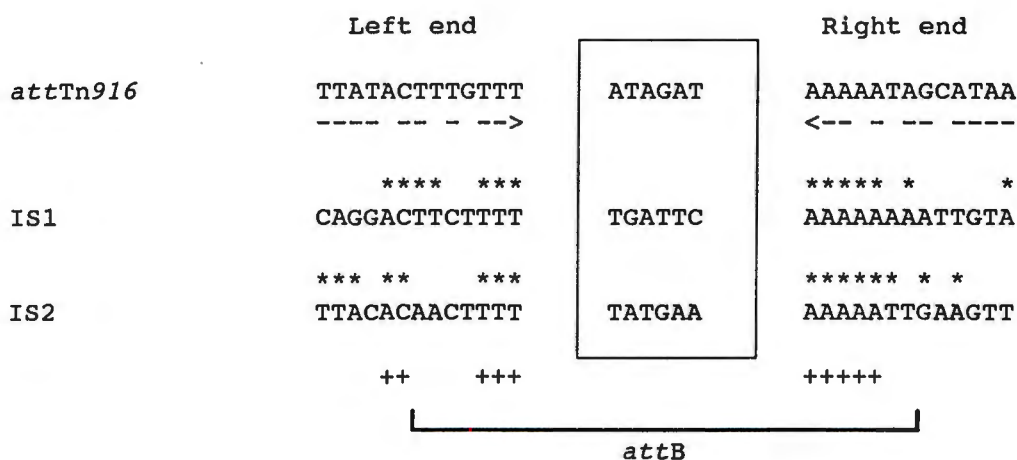


Fig. 2.5. Target DNA sequences at the integration sites of *Tn916* (IS1) and *Tn925::Tn917* (IS2) in *C. acetobutylicum* strains DSM 792 and P262, respectively. IS1 is the transposon target site inferred from sequence data presented by Sauer and Dürre (1992), and IS2 is the transposon insertion site in mutant 3R (elucidation of the complete target site is described in Chapter 4). The 20 bp segment containing the sequences required for target activity is designated *attB* (Trieu-Cuot *et al.*, 1993). Identical nucleotides, in pairwise comparison between right end of the transposon and every *C. acetobutylicum* target sequence are indicated by asterisks. Plus signs denote positions with 100% identity in both target DNAs.

Examination of target sequences for *Tn916* and *Tn1545* has revealed that these regions are structurally related and resemble the 7 bp A- and T-rich sequences at the right and left ends of the transposon, respectively (Caillaud and Courvalin, 1987; Clewell *et al.*, 1988; Poyart-Salmeron *et al.*, 1990; Trieu-Cuot *et al.*, 1993; Scott *et al.*, 1994). In the excised circular intermediate of the donor conjugative transposon, the left and the right ends of the transposon are separated by a non-specific 6 bp overlap region which is generated by staggered endonucleolytic cleavage at the time of excision. The 20 bp target site, designated *attB* (Trieu-Cuot *et al.*, 1993), can essentially be defined as a T-rich region separated by a 6 bp spacer from an A-rich region (Scott 1993). Presumably the motif, based on the degenerated palindromes of the transposon termini, determines a conformation specific for the transposon integrase (Poyart-Salmeron *et al.*, 1990; Lu and Churchward, 1995). The transposon inserts with the T-rich terminus adjacent to a stretch of A's and the poly(A) terminus next to a stretch of T's. The complete target sequence for *Tn925::Tn917* integration in mutant 3R can be inferred from sequence analysis of the corresponding intact DNA region as described in Chapter 4. Because it is relevant to this chapter, it is included in

Fig. 2.5. The Tn916 target sequence deduced from the sequence information provided in the analysis of a *C. acetobutylicum* DSM 792 asolventogenic mutant (Sauer and Dürre, 1992) is included as a comparison.

The metronidazole MIC values for mutants 3R and 30R were double that of the parent strain. Moderate (four-fold) levels of metronidazole resistance have been shown to be significant (Quon *et al.*, 1992). An investigation of the mechanism of metronidazole resistance in mutant 3R, which was suitable for further study, on account of the single transposon insertion, is described in the following chapter.

University of Cape Town

Chapter 3

Metronidazole susceptibility of *C. acetobutylicum* P262 and investigation of the mechanism of metronidazole resistance in mutant 3R

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Chapter 3

Metronidazole susceptibility of *C. acetobutylicum* P262 and investigation of the mechanism of metronidazole resistance in mutant 3R

3.0 Summary

Metronidazole is a pro-drug which is activated by chemical/enzymatic reduction and causes cell death, mainly via DNA damage. Susceptibility of the *C. acetobutylicum* wild type and mutant 3R was compared during various stages in growth following treatment with a high concentration (10 µg/ml) of metronidazole. Wild type cells displayed maximum susceptibility to the drug in the early vegetative growth stage, demonstrating a loss of almost four log units of viability. Analysis of supernatant protein concentrations of metronidazole-treated wild type cultures gave no evidence of death by extensive cell lysis, as has been observed in *C. pasteurianum*. Stationary phase wild type clostridial cells acquired a level of resistance corresponding to that of mutant 3R i.e. a loss of less than one log unit of viability. The level of metronidazole resistance in mutant 3R was constant, irrespective of the growth stage of the cells. Three possible mechanisms of metronidazole resistance were examined, namely multidrug efflux, impaired ability to activate the drug, and increased tolerance to DNA damage. The mutant was not resistant to chloramphenicol and ethidium bromide, two toxic compounds associated with multiple drug resistance. No difference was detected in the metronidazole reduction rates of wild type and mutant 3R cell-free extracts. However, mutant 3R was resistant to UV-irradiation and bleomycin, physical and chemical DNA damaging agents, respectively.

3.1 Introduction

Metronidazole is selectively toxic to anaerobic organisms since it requires electron transport proteins with sufficiently negative redox potential for drug activation (Edwards, 1993a). The electron transport proteins that are known to provide the source of electrons for the reductive activation of metronidazole are involved in the oxidative fermentation of pyruvate via the phosphoroclastic system - an important pathway for carbohydrate metabolism in anaerobic organisms (Johnson, 1993). The reduction of metronidazole is discussed in Chapter 1, Section 1.7, with particular reference to the ferredoxin-linked hydrogenase mechanism identified in *C. pasteurianum* (Church *et al.*, 1988, 1990). Reduction of the 5-nitro group of the drug generates the toxic intermediate(s), the primary target of which is DNA (Ings *et al.*, 1974; Edwards, 1993a). Damage occurs by means of single- and double-strand breaks, and a preference for AT-rich DNA has been demonstrated (Rowley *et al.*, 1980). Church *et al.* (1991) propose that an additional mechanism of drug cytotoxicity is the induction of rapid cellular lysis which has been observed to occur in *C. pasteurianum* within 5 min of high-concentration metronidazole treatment.

The aim of this project was to isolate electron transport genes implicated in solventogenesis, using metronidazole resistance as a selection system. Resistance to metronidazole in anaerobic protozoa and bacteria correlates with the inability to reduce the drug to its active form. This arises from disrupted or diminished activity of the components of the phosphoroclastic reaction i.e. PFOR and/or hydrogenase or ferredoxin (Britz and Wilkinson, 1979; Sindar *et al.*, 1982; Cerkasovova *et al.*, 1988; Narikawa *et al.*, 1991; Quon *et al.*, 1992; Kulda *et al.*, 1993), and is often associated with altered carbohydrate metabolism. Increased tolerance to metronidazole-induced damage, i.e. an improved DNA repair system, is a potential mechanism for resistance (Chrystal *et al.*, 1980), but as yet, there have been no reports to this effect. Decreased permeability to metronidazole uptake is sometimes cited as a mechanism of resistance, but appears to be a secondary function of inefficient drug activation (Tally *et al.*, 1979; Kulda *et al.*, 1993). It is generally assumed that metronidazole enters the cell by a simple passive or facilitative diffusion process in which the rate of uptake is controlled by the rate intracellular drug reduction via a concentration gradient (Müller, 1983). On the other hand, metronidazole is a likely substrate for multidrug transporters.

These are non-specific membrane proteins which recognise and efflux a range of structurally unrelated toxic compounds from the cell (Ahmed *et al.*, 1994).

This chapter describes an investigation of the susceptibility of the *C. acetobutylicum* wild type strain to metronidazole treatment at different stages in the course of a typical batch fermentation, and examination of the mechanism of metronidazole resistance in *C. acetobutylicum* mutant 3R. *C. acetobutylicum* metronidazole resistant mutant 30R was included in most experiments as a transposon-containing control.

3.2 Materials and methods

3.2.1 Bacterial strains and growth media

C. acetobutylicum P262 wild type (Jones *et al.*, 1982) and *C. acetobutylicum* mutants 3R and 30R (the isolation of which is described in Chapter 2) were routinely grown in CBM (Allcock *et al.*, 1982). For the cell lysis assay, wild type *C. acetobutylicum* was grown in BASOL medium (Reysset and Sebald, 1985), supplemented with 1% glucose.

3.2.2 Determination of MICs

Determination of nitroimidazole, chloramphenicol and ethidium bromide MICs were as described in Section 2.2.4. Tinidazole (Sigma) and 4-nitroimidazole (Aldrich) were first solubilized in dimethyl-formamide (final concentration 1.0% w/v) and the volume then made up with sterile distilled water.

3.2.3 Metronidazole susceptibility assay

The progress of wild type and mutant batch cultures was monitored by pH, OD at 600 nm and microscopic examination. Samples (1 ml) from wild type and mutant cultures were removed at various time intervals during the course of the batch fermentation and treated with metronidazole (final concentration 10 µg/ml) for 5 min at room temperature. This treatment was reported to kill 99.99% of the cells in an exponentially growing *C. pasteurianum* culture (Church *et al.*, 1991). Untreated samples were included as a measure of normal cell viability. Treated and untreated samples were diluted appropriately,

plated and incubated overnight. The surviving cell fraction was calculated from viability counts (cfu/ml) for treated and untreated cells.

3.2.4 Cell lysis assay

Metronidazole-induced lysis of *C. acetobutylicum* wild type cells was assayed according to the method described by Church *et al.* (1991) for *C. pasteurianum*. The protein concentration of the culture supernatant after metronidazole treatment was used as a measure of cell leakage, and cells were therefore cultured in a minimal medium to avoid protein contamination from the components of a complete medium. The *C. acetobutylicum* wild type strain was grown in BASOL medium supplemented with glucose (1%). Samples (20 ml) were removed at an OD₆₀₀ of 0.2 and 0.6, and treated with metronidazole (final concentration 10 µg/ml) for 5 min. Samples were then filtered through a 0.45 µm Millipore filter to remove cells, and the supernatants were concentrated to a volume of 1.5 ml with an Amicon PM10 filter apparatus. The amount of protein in the supernatants was determined by the method of Bradford (1976). Metronidazole-treated and -untreated samples were examined by phase contrast microscopy prior to filtration. A Zeiss photomicroscope fitted with phase- and interference contrast optics was used for microscopic examination.

3.2.5 Metronidazole reduction assay

Cell-free extracts for the metronidazole reduction assays were prepared under stringent anaerobic conditions in an anaerobic glove cabinet. Cells were harvested from early exponential cultures (OD₆₀₀ = 0.3) (200 ml) and protoplasted according to Zappe *et al.* (1986) as for chromosomal DNA extraction. Protoplasts were harvested and washed with 10 ml CBM containing 10% sucrose to remove residual lysozyme. Protoplasts were then resuspended in 4 ml of 50 mM Tris-Cl (pH 8.0) for 5 min to induce lysis. Samples were centrifuged at 10 000 x g for 10 min and the supernatant retained. Protein concentrations were determined by the method of Bradford (1976).

Metronidazole reduction assays were based on the hydrogenase-linked assay for ferredoxin and flavodoxin as described by Chen and Blanchard (1979). Assays were conducted at room temperature in an anaerobic glove cabinet. The reaction mixture consisted of 50 mM Tris-Cl (pH 8.0), 0.5 mM metronidazole and cell-free extracts containing approximately

0.5-1.0 mg/ml protein. The reactions were initiated by adding the cell-free extract and were terminated at various time intervals by exposure to oxygen. The rate of metronidazole reduction was monitored spectrophotometrically at OD₃₆₀ nm as specified for metronidazole concentrations of 0.5 mM.

3.2.6 UV survival assay

Early exponential phase cultures (10 ml) were harvested, washed and resuspended in sterile water (10 ml). Samples (5 ml) were irradiated in an open petri dish. The source of UV radiation was a Cole-Parmer 9815-series lamp with emission at 254 nm. The fluence rate was 1.0 J/m²/s. Samples were diluted appropriately, plated and incubated overnight. The surviving fraction of cells was calculated from viability counts (cfu/ml) for irradiated and non-irradiated cells.

3.2.7 Bleomycin susceptibility assay

Serial dilutions of *C. acetobutylicum* cultures in the early exponential phase of growth (OD₆₀₀ = 0.2) were spread onto CBM agar plates containing bleomycin (0.01-10 mg/ml). Plates were incubated overnight. Bleomycin was obtained from Groote Schuur Dispensary.

3.3 Results

3.3.1 Resistance to nitroimidazoles

The metronidazole resistant mutants 3R and 30R were examined for resistance to two other nitroimidazole drugs, viz. tinidazole and 4-nitroimidazole. The MICs of tinidazole for the *C. acetobutylicum* wild type, mutant 3R and mutant 30R were 0.015, 0.045 and 0.030 µg/ml, respectively. The MIC of 4-nitroimidazole was the same for the *C. acetobutylicum* wild type and both the mutants, i.e. 2.8 µg/ml. These MIC values are listed in Table 3.1.

3.3.2 Metronidazole susceptibility profiles

Because *C. acetobutylicum* is a differentiating organism, metronidazole susceptibilities of the wild type and mutants 3R and 30R were examined at different growth stages (Fig. 3.1). Samples were removed from the batch fermentation at various time intervals and treated with a high concentration of metronidazole (10 µg/ml). The wild type displayed maximum sensitivity to metronidazole in the early vegetative growth stage, demonstrating a loss of

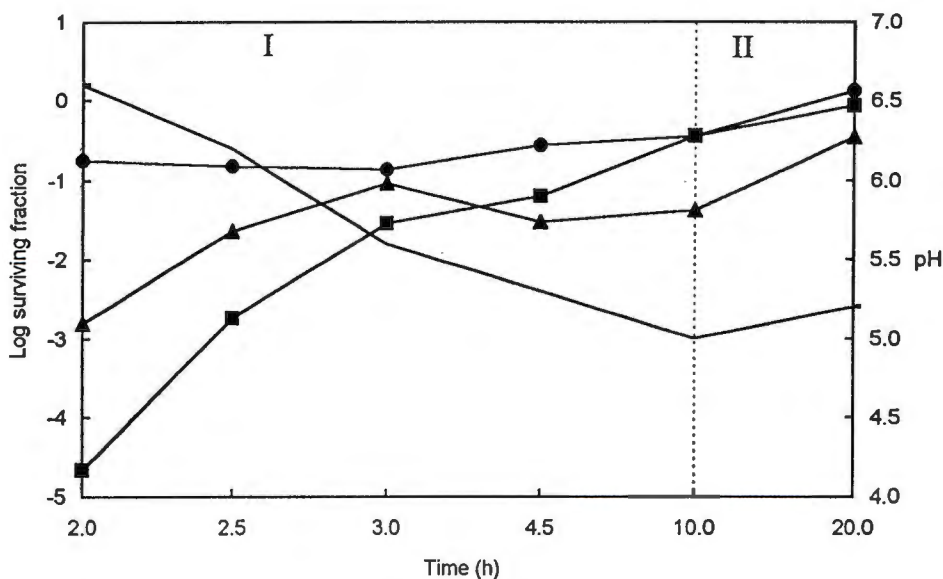


Fig. 3.1. Metronidazole susceptibility of wild type (■), mutant 3R (●) and mutant 30R (▲) *C. acetobutylicum* cells determined at various stages in growth. Unbroken line represents pH. Dotted line marks pH breakpoint and separates vegetative (I) and clostridial (II) phases. Results are the average of three experiments.

almost four log units of viability (99.99% cell kill). Resistance increased exponentially as the pH of the culture medium approached the breakpoint. The pH breakpoint marks the shift to solventogenesis and was accompanied by differentiation of the vegetative rods into stationary phase clostridial cells which acquired a level of resistance corresponding to that of mutant 3R, i.e. a loss of less than one log unit of viability. By contrast, the level of metronidazole resistance in mutant 3R was constant, irrespective of the growth stage of the cells. Mutant 30R displayed an intermediate level of metronidazole resistance during exponential growth. Surprisingly, stationary phase cells of mutant 30R appeared slightly more susceptible to metronidazole treatment than stationary phase wild type and mutant 3R cells.

3.3.3 Cell lysis

Church *et al.* (1991) proposed that an important mechanism of metronidazole cytotoxicity is the induction of rapid cell lysis. Wild type *C. acetobutylicum* cells were assayed for metronidazole-induced lysis as measured by supernatant protein concentration. Metronidazole treatment was the same as for the metronidazole susceptibility assays which

resulted in a four log unit loss of viability i.e. 99.99% cell kill. No protein was detected in the concentrated supernatants derived from wild type cultures taken from a batch fermentation at OD_{600} 0.2 and 0.6, and treated with metronidazole. Furthermore, the only visible difference observed in treated cells relative to untreated cells, was loss of motility which was indicative of cell death. On the basis of these results it appeared that cell death of exponentially-growing cells of *C. acetobutylicum*, following treatment with a lethal dose of metronidazole, did not arise from lysis as observed for *C. pasteurianum* (Church *et al.*, 1991).

3.3.4 Multiple drug resistance

Chloramphenicol and ethidium bromide are two of the many unrelated toxic compounds recognised by multidrug efflux transporters (Neyfakh *et al.*, 1993). The MIC of chloramphenicol for both the wild type and mutant 3R was 0.50 $\mu\text{g/ml}$. The MIC of ethidium bromide for both the wild type and mutant 3R was 0.05 $\mu\text{g/ml}$. It was therefore concluded that mutant 3R did not display multiple drug resistance.

3.3.5 Metronidazole reduction profiles

The reducing activity of *C. acetobutylicum* wild type and mutant cell free extracts was investigated by means of an assay designed to detect the hydrogenase-linked activity of electron carriers with low redox potential in a cell-free preparation (Chen and Blanchard, 1979). Metronidazole irreversibly loses its absorption peak at 320 nm upon reduction, and so the rate of reduction can be measured spectrophotometrically. Because of the strong absorption of metronidazole at 320 nm, the assays were monitored at 360 nm as specified by Chen and Blanchard (1979) for metronidazole concentrations of 0.5 mM. The preparation of anaerobic cell free extracts was essential: the slightest exposure of these preparations to oxygen resulted in a loss of metronidazole-reducing activity. Furthermore, the reactions were immediately and irreversibly terminated on exposure to oxygen. No overall difference in the metronidazole reduction rates of the wild type and mutant cell-free extracts was detected under these assay conditions (Fig. 3.2). The metronidazole-reducing power of mutant 3R appeared to be equivalent to that of the wild type.

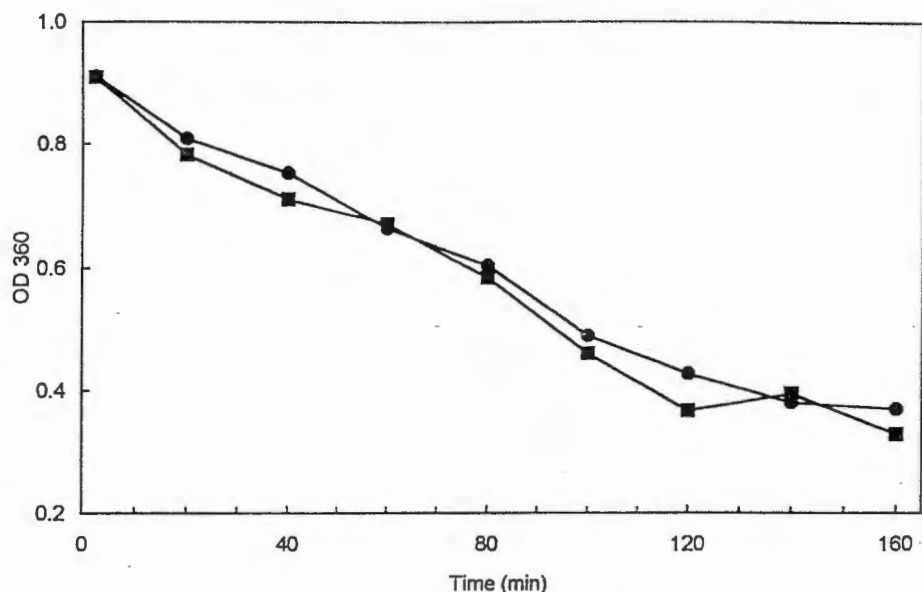


Fig. 3.2. Reduction of metronidazole by *C. acetobutylicum* wild type (■) and mutant 3R (●) cell extracts containing equivalent amounts of protein. Reduction of metronidazole corresponds with a loss in optical density at 360 nm. Results are representative of three experiments performed with samples in triplicate.

3.3.6 UV survival

Mutant 3R was examined for resistance to metronidazole via acquired tolerance to DNA damage. UV survival studies revealed that mutant 3R was more resistant to UV radiation than the wild type and mutant 30R (Fig. 3.3). The largest difference (more than two log units) in cell viability between the wild type and mutant 3R was observed after 30 seconds of UV irradiation. Since UV-induced DNA damage occurs by introduction of intra-strand cyclobutane-pyrimidine dimers which prevent base pairing, it was important to also test mutant 3R for resistance to a DNA-damaging agent with a mode of action similar to that of metronidazole.

3.3.7 Bleomycin resistance

Bleomycin is a glycopeptide antibiotic which introduces single- and double-stranded DNA breaks. The damaging agent, a hydroxyl radical, is generated in a reaction mediated oxygen (Pratviel *et al.*, 1986). The assays were performed anaerobically, and this presumably explains why only high concentrations of the antibiotic (10 mg/ml) were toxic to the *C. acetobutylicum* wild type. Mutant 3R, however, was not susceptible to the antibiotic.

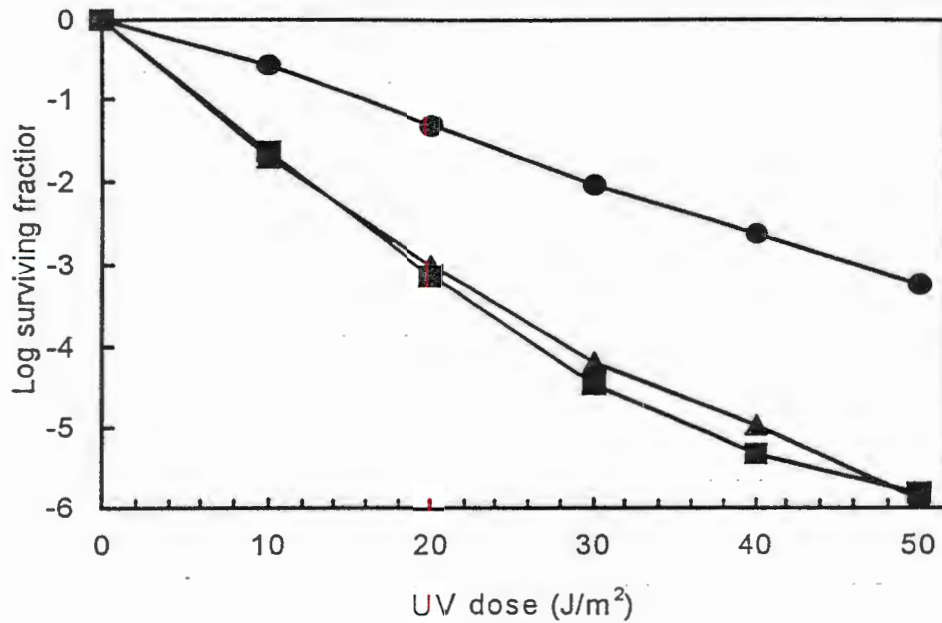


Fig. 3.3. UV survival of wild type (■), mutant 3R (●) and mutant 30R (▲) *C. acetobutylicum* cells exposed to various doses of UV radiation. Results are representative of a trend observed in three experiments.



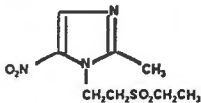
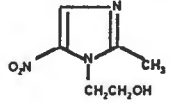
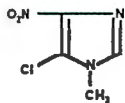
Fig. 3.4. Comparison of bleomycin susceptibility of the *C. acetobutylicum* wild type (WT) and mutant 3R (3R).

For wild type and mutant 3R cultures diluted $\times 10^4$, only mutant 3R colonies were obtained on CBM agar containing 10 mg/ml bleomycin (Fig. 3.4). The bleomycin resistant phenotype of mutant 3R was also demonstrated in a filter disc assay. Bleomycin had no inhibitory effect on mutant 3R (no zone of inhibition), whereas the wild type was sensitive to the antibiotic (zone of inhibition measured 2mm).

3.4 Discussion

The *C. acetobutylicum* wild type, mutant 3R and mutant 30R MIC values for metronidazole (reported in Chapter 2), tinidazole and 4-nitroimidazole, together with the electron affinities of these compounds, are listed in Table 3.1. Included in this table, are the corresponding MIC values for *B. fragilis* (Reynolds, 1981) and *C. pasteurianum* (Church *et al.*, 1990). These results demonstrate, in this instance, the correlation between the antibacterial activities and the electron affinities of the compounds (Reynolds, 1981). Compounds with electron affinities between -460 and -500 mV have been shown to have the strongest activity against *B. fragilis* (Reynolds, 1981), and therefore 4-nitroimidazole, with a reduction potential of -517 mV, is relatively biologically inert. Reduction of metronidazole, which according to Reynolds (1981) has an electron affinity of -482 mV, requires the activity of electron transport proteins with sufficiently negative redox potential.

Table 3.1. Nitroimidazole MICs

Bacterial strain	Minimal inhibitory concentration (mg/ml)		
	Tinidazole	Metronidazole	4-nitroimidazole
			
	- 0.464 mV	- 0.482 mV	- 0.517 mV
<i>C. acetobutylicum</i> WT	0.015	0.050	2.800
<i>C. acetobutylicum</i> 3R	0.045	0.100	2.800
<i>C. acetobutylicum</i> 30R	0.030	0.100	2.800
<i>C. pasteurianum</i>	ND	0.063	2.000
<i>B. fragilis</i>	0.037	0.170	37.200

ND, not done

Reduced ferredoxin has a redox potential of -470 to -527 mV (Edwards *et al.*, 1973; Chien 1976), and has been shown by Church and co-workers to serve as the low potential electron carrier in the hydrogenase-mediated reduction of metronidazole and other nitroimidazole drugs in *C. pasteurianum* (Lockerby, *et al.*, 1984; Church *et al.*, 1985; Church *et al.*, 1988; Church *et al.*, 1990). These reports, demonstrating that the reduction of metronidazole in *C. pasteurianum* occurs via the ferredoxin-dependent hydrogenase-mediated mechanism, is interesting in the analysis of the *C. acetobutylicum* metronidazole-susceptibility profiles.

Wild type *C. acetobutylicum* cells were most sensitive to metronidazole treatment in the early exponential phase of growth. Drug sensitivity decreased as the pH of the culture medium approached the breakpoint, and differentiated clostridial stage cells were resistant to metronidazole treatment. This pattern of metronidazole susceptibility in the *C. acetobutylicum* wild type is the inverse of the hydrogenase activity profile of the organism. Hydrogenase activity in *C. acetobutylicum* has been shown to be at a maximum in the exponential growth phase and to decrease significantly in the shift from acid production to solvent production (Kim and Zeikus, 1985). Although the *C. acetobutylicum* hydrogenase appears to be constitutively expressed (Santangelo *et al.*, 1995), the observed reduction in hydrogenase activity may be due to a decreased availability of reduced ferredoxin. Alternative explanations or contributing factors for inherent metronidazole resistance in the clostridial stage may be: 1) the reduced metabolic rate of the stationary phase clostridial cells; 2) impermeability of the clostridial cell or extracellular capsule to metronidazole; or 3) induction of the repair genes of the SOS system in the stationary growth stage as in the case of *B. subtilis* (Dubnau, 1993).

Actively growing vegetative cells of the *C. acetobutylicum* 3R mutant were approximately four-log units more resistant to metronidazole than the wild type cells. For this reason, assays which addressed the possible mechanisms of resistance (i.e. multiple drug resistance, decreased drug activation and increased tolerance to the drug's cytotoxic effects) were performed on early exponential phase wild type and mutant 3R cells in which the difference in metronidazole susceptibility was most pronounced.

Mutant 3R was no more resistant than the wild type to two unrelated toxic compounds, viz. chloramphenicol and ethidium bromide. These compounds have been identified as substrates for multidrug transporters e.g. Bmr of *B. subtilis*, which recognise and efflux structurally diverse compounds, thereby conferring multiple drug resistance (Neyfakh, 1992). The metronidazole resistant phenotype of mutant 3R was therefore not associated with the multiple drug resistance phenomenon, nor with general decreased permeability to drug uptake.

The metronidazole resistant phenotype also did not appear to be associated with a decrease in metronidazole-reducing activity as measured by the hydrogenase-linked assay for ferredoxin (Chen and Blanchard, 1979) using crude cell-free extracts. No difference was detected in the metronidazole reduction rates between cell extracts from wild type and mutant cells.

The metronidazole susceptibility assays involved treatment with a high concentration of metronidazole for 5 min and resulted in a 99.99% loss of cell viability for vegetative *C. acetobutylicum* wild type cells as observed for *C. pasteurianum* (Church *et al.*, 1991). However, there was no evidence of rapid cell lysis which these authors propose is an additional mechanism of drug cytotoxicity, superseding the slower effects of metronidazole-induced DNA damage.

Relative to the wild type and mutant 30R, mutant 3R showed decreased susceptibility to UV radiation and bleomycin. Although UV is a physical agent which introduces intra-strand pyrimidine dimers, and metronidazole and bleomycin are both chemical agents which cause DNA strand breaks, the same enzymes, viz. RecBCD and RecF are involved in the repair of metronidazole, bleomycin and UV-induced DNA damage in *E. coli* (Takeshita *et al.*, 1978; Jackson *et al.*, 1984; Yeung *et al.*, 1984; Simic *et al.*, 1991). These enzymes are part of the postreplication or recombination repair pathway and are RecA-dependent. Increased metronidazole susceptibility has been observed in DNA repair deficient strains of *E. coli* (Jackson *et al.*, 1984; Yeung *et al.*, 1984), but there have been no previous reports of metronidazole resistance associated with increased tolerance to DNA damage, as proposed by Chrystal *et al.* (1980).

These results indicated that the metronidazole resistant phenotype of mutant 3R is associated with an increased capacity to repair DNA damage (indirect evidence), rather than disruption of a gene involved in the reductive activation of the drug. Molecular characterization of the DNA locus implicated in metronidazole susceptibility is reported in the following chapter.

Chapter 4

Molecular characterization of *C. acetobutylicum* P262 metronidazole resistant mutant 3R

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Chapter 4

Molecular characterization of *C. acetobutylicum* P262 metronidazole resistant mutant 3R

4.0 Summary

A 2.4 kb *Xba*I-*Hinc*II DNA fragment containing the intact *C. acetobutylicum* wild type locus, corresponding to the disrupted locus in mutant 3R, was identified by Southern hybridization. This DNA fragment was isolated from a size-selected wild type genebank via colony hybridization, using a probe derived from the cloned transposon-chromosome junction of mutant 3R. The majority of this DNA fragment (1944 bp) was sequenced to reveal a truncated open reading frame, ORFB (which is described in Chapter 5), upstream of a complete open reading frame, ORFA (1005 bp), encoding a 334 amino-acid protein. Putative Shine-Dalgarno and σ^A -dependent promoter consensus sequences were detected upstream of ORFA, and the precise site of the transposon insertion within ORFA was extrapolated for mutant 3R. Initial amino acid homology searches failed to identify ORFA which was therefore designated *sum* (for *susceptibility to metronidazole*). A recent search of the databases showed that the deduced amino acid sequence of the *sum* product displays homology (36% identity and 58% similarity) with a new database entry for a pyruvate formate-lyase-activating enzyme homologue identified in *Thermococcus litoralis*. Northern blot analysis indicated that *sum* is produced as an independent 1.2 kb transcript in the wild type, and as a marginally truncated 1.1 kb transcript in the mutant. The predicted *sum* translation product (37.8 kDa) was detected in an *in vitro* transcription-translation assay.

4.1 Introduction

There has been only one report of the molecular characterization of a *C. acetobutylicum* transposon-induced mutant, in this case, an acetone-butanol deficient transconjugant of *C. acetobutylicum* strain DSM 792 (Sauer and Dürre, 1992). The Tn916 transposon insertion was located upstream of the structural *thrA* gene, encoding the tRNA^{Thr}_{ACG}. Initially it was thought that rare usage of the ACG codon in *C. acetobutylicum* might be implicated in translational regulation of stationary phase responses, but the function of this gene remains unclear (see Sauer *et al.*, 1995). There has also been only one report of the molecular characterization of mutations responsible for metronidazole resistance. Quon *et al.* (1992) have shown that drug resistance in four clinical isolates of *T. vaginalis* is associated with decreased expression of the ferredoxin gene. A single point mutation appeared to decrease the affinity for a DNA-binding protein implicated in the regulation of ferredoxin gene transcription.

The molecular characterization of the intact *C. acetobutylicum* wild type locus corresponding to the transposon-disrupted locus in mutant 3R, is reported in this chapter. These studies attempt to elucidate the molecular basis of the metronidazole resistant phenotype of mutant 3R.

4.2 Materials and methods

4.2.1 Bacterial strains and plasmids

C. acetobutylicum P262 wild type (Jones *et al.*, 1982) and *C. acetobutylicum* P262 mutants 3R and 30R, were used as the source of chromosomal DNA and total RNA. *E. coli* JM105 (Yanisch-Perron *et al.*, 1985) served as the cloning host for DNA constructs in the Bluescript SK (Stratagene) vector. Plasmid p3R is described in Chapter 2. The origins of the plasmid constructs generated in this study are described in Sections 4.3.2 and 4.3.6.

4.2.2 Media and growth conditions

C. acetobutylicum P262 strains were grown anaerobically at 34°C in buffered CBM (Allcock *et al.*, 1982) supplemented with tetracycline (10 µg/ml) for mutant selection. *E. coli* clones were cultured at 37°C in 2xYT medium (Messing, 1983) containing ampicillin (100 µg/ml).

4.2.3 Nucleic acid isolation and manipulation

Small and large-scale plasmid DNA extractions from *E. coli* were prepared by the alkali-hydrolysis method of Ish-Horowicz and Burke (1981). *C. acetobutylicum* genomic DNA was prepared according to the method of Marmur (1961) as modified by Zappe *et al.* (1986), and total *C. acetobutylicum* RNA was isolated from exponential-phase cultures ($OD_{600} = 0.2$) using the hot phenol extraction protocol described by Aiba *et al.* (1981). Restriction endonuclease digestion of DNA was performed as recommended by the manufacturers and general cloning procedures were as described by Sambrook *et al.* (1989).

4.2.4 Construction of a size-selected genebank

C. acetobutylicum wild type chromosomal DNA (50 μ g) was digested to completion with *Xba*I and *Hinc*II endonucleases and the resulting fragments fractionated by electrophoresis in a 0.8% agarose gel in Tris/acetate buffer. DNA fragments of approximately 2.4 kb in size were recovered by the GeneClean procedure (Bio 101, Inc.), ligated to *Xba*I-*Hinc*II-digested Bluescript SK vector and transformed into *E. coli* JM105.

4.2.5 Colony hybridization

Colonies harbouring recombinant plasmids with *C. acetobutylicum* insert DNA were processed for hybridization as described in Chapter 2, Section 2.2.8.

4.2.6 Southern hybridization

Southern hybridization was performed as described in Chapter 2, Section 2.2.9.

4.2.7 Nucleotide sequencing

The 0.3 kb *Hind*III-*Eco*RI DNA insert of plasmid pWT4, derived from plasmid p3R, was sequenced in both directions. The 1.8 kb *Xba*I-*Hind*III DNA insert of plasmid pWT2, containing most of the *C. acetobutylicum* *sum* gene, was subjected to Henikoff shortening (Henikoff, 1984). Nested deletions were generated in both directions by exonuclease III digestion of *Kpn*I-*Hind*III- and *Sac*I-*Xba*I-restricted DNA fragments. A range of suitably deleted cloned fragments was selected for sequencing. Double-stranded plasmid DNA templates were sequenced by the dideoxy-chain termination method of Sanger *et al.* (1977) using [³⁵S] dATP and the T7 DNA sequencing kit (Pharmacia Biotech.). The T3 and T7

primers were used for the sequencing reactions. The sequence data were analysed on a VAX 6000-330 computer using the Genetics Computer Group Inc. sequence analysis software package (Devereux *et al.*, 1984). The nucleotide sequence presented in this chapter was submitted to the GenBank data base and assigned the accession number L35099.

4.2.8 Northern hybridization

RNA markers (0.24-9.5 kb) were obtained from Gibco BRL. RNA was separated by electrophoresis in 1.5% denaturing formaldehyde agarose gels according to Fourney *et al.* (1988). The RNA was transferred by capillary action to Hybond N⁺ nylon membranes and fixed according to the manufacturer's instructions. The RNA probe was synthesised by *in vitro* transcription of *SlyI*-linearised "shortened" clone L11 (see Section 4.3.2), using a DIG RNA labelling kit (Boehringer Mannheim) and T7 polymerase to produce a run-off transcript antisense to the mRNA. Hybridization was performed at 50°C, using the probe at a concentration of approximately 50 ng/ml in a high SDS (7%) hybridization buffer.

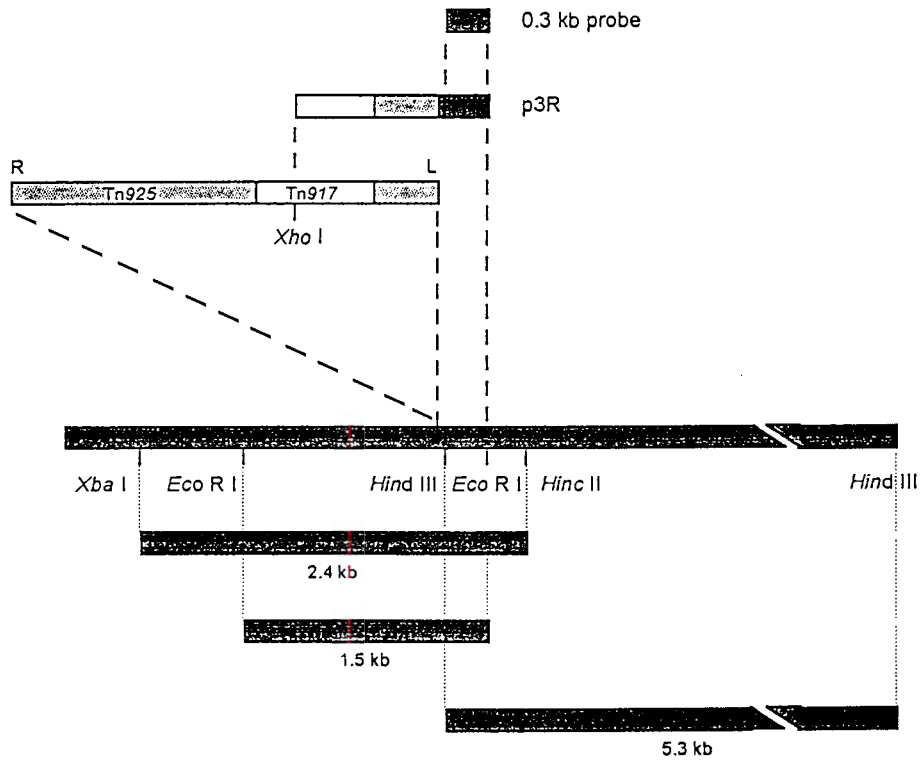
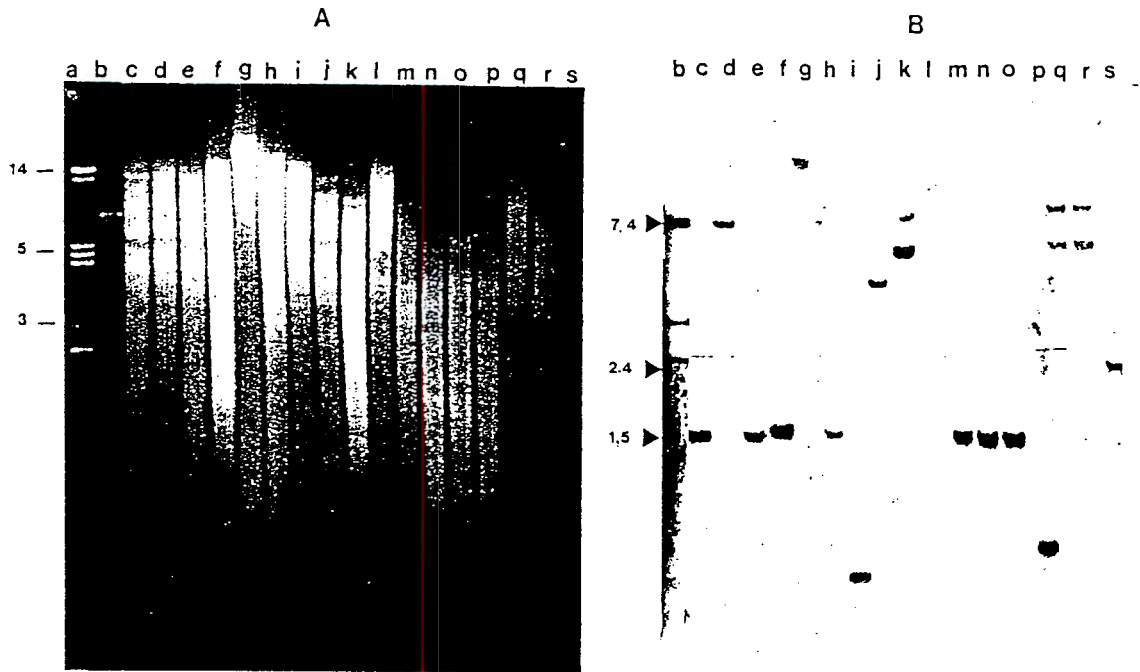
4.2.9 *In vitro* transcription-translation

[³⁵S] methionine-labelled proteins were produced by *in vitro* cell-free transcription-translation using the prokaryotic DNA-directed translation kit (Promega). These translation products were separated on a SDS polyacrylamide gel (4% stacking and 15% resolving phases) according to Laemmli (1970) and visualised by autoradiography. Low range molecular weight markers obtained from Pharmacia were stained with PAGE 83 blue (BDH) prior to autoradiography.

4.3 Results

4.3.1 Identification and cloning of the *C. acetobutylicum* wild type gene region corresponding to the 3R mutation

Southern hybridization confirmed that the chromosomal DNA region from plasmid p3R did originate from *C. acetobutylicum* mutant 3R, and that it was present in the *C. acetobutylicum* wild type genome (Fig. 4.1). Plasmid DNA from clone p3R and chromosomal DNA from the *C. acetobutylicum* wild type and *C. acetobutylicum* mutants 3R and 30R were digested with *EcoRI*-*XhoI* endonucleases and probed with a DIG-labelled 0.3 kb *HindIII*-*EcoRI* restriction fragment derived from plasmid p3R. Hybridization



signals at 7.4 kb were obtained for the digested DNA samples of plasmid p3R (lane b) and mutant 3R (lane d), thereby confirming the origin of the cloned DNA. A positive hybridization signal for a smaller sized fragment (1.5 kb) was detected for the digested wild type DNA (lane e). This represented the corresponding undisrupted locus in the wild type genome and was identical to the hybridization signal produced for the digested DNA sample of mutant 30R (lane c).

In the same Southern hybridization study, *C. acetobutylicum* wild type chromosomal DNA was cut with a variety of endonucleases (single and double digests, lanes f-s) to identify a suitable restriction fragment containing the relevant wild type DNA region for cloning. It was observed that wild type DNA restricted with *EcoRI* (single digest, lane f) and *EcoRI-XhoI* (double digest, lane e) produced equivalent 1.5 kb hybridization bands, indicating an internal 1.5 kb *EcoRI* restriction fragment. This, and additional information obtained from the hybridization analysis, facilitated preliminary mapping of the chromosomal DNA region of interest. A 2.4 kb *XbaI-HincII* restriction fragment (see lane s) was selected for cloning into plasmid Bluescript SK.

A mini gene library was constructed by cloning size-selected fragments (2-3 kb) of *XbaI-HincII*-digested wild type chromosomal DNA into *XbaI-HincII*-digested Bluescript SK vector. The relevant 2.4 kb *XbaI-HincII* DNA fragment was isolated from the mini-genebank by colony hybridization, using the 0.3 kb DIG-labelled probe derived from p3R. Five recombinant plasmids containing the desired insert were obtained from the 230 recombinants screened. These plasmids were characterized by restriction enzyme analysis and one of them, referred to as plasmid pWT1, was selected for sequence analysis.

4.3.2 Construction of templates for sequencing and sequencing strategy

The sequencing strategy is outlined in Fig. 4.2. Initially, the 0.3 kb *HindIII-EcoRI* DNA fragment derived from plasmid p3R was subcloned into the Bluescript vector SK to generate plasmid pWT4, and was sequenced in both directions. The DNA sequence of this region revealed the 3' end of an open reading frame which was truncated at the 5' end by the *HindIII* site. For this reason, it was considered necessary to determine the sequence of only the 1.8 kb *XbaI-HincII* fragment of plasmid pWT1 in order to complete the open reading

frame. This DNA fragment was subcloned into Bluescript SK to provide the template for exonuclease III shortening. The resultant clone is referred to as plasmid pWT2. A series of nested deletions was constructed across the 1.8 kb fragment in both directions as described in Section 4.2.7. The shortened constructs selected for sequencing are represented in Fig. 4.2, indicating the length of sequence provided by each clone for the coding and non-coding strands. A 0.26 kb *Dra*I fragment was subcloned into pSK in order to complete the sequence.

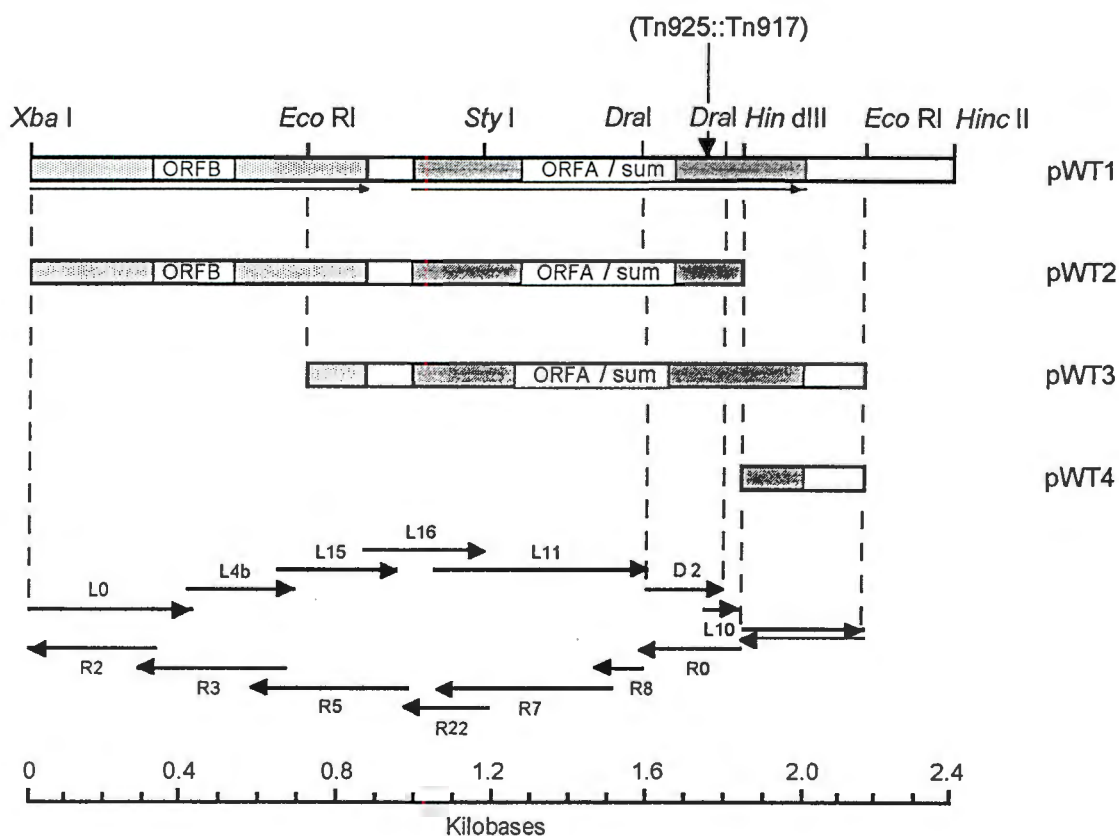


Fig. 4.2. Physical map of the 2.4 kb *C. acetobutylicum* DNA insert of plasmid pWT1 and derivative constructs (all pSK-based). The direction of transcription of ORFB (truncated) and the ORFA/*sum* gene is indicated by arrows. The site of transposon insertion in mutant 3R is shown. The sequencing strategy is outlined below the map. The numbered arrows show the extent and direction of sequence obtained from individual clones generated by exonuclease digestion of plasmid pWT2. D2 is the subcloned 0.26 kb *Dra*I fragment. The DNA insert of plasmid pWT4 was sequenced in both directions. The *sum*-specific RNA probe was synthesised from clone L11 linearised with *Sty*I.

4.3.3 Nucleotide sequence analysis

The combined nucleotide sequences of the *C. acetobutylicum* DNA inserts of plasmids pWT2 and pWT4 spanned a 1944 bp region (Fig. 4.2). Six-phase translation of this region revealed a complete ORF (ORFA) in frame with a partial ORF (ORFB). ORFA corresponded to the disrupted ORF identified in plasmid pWT4 (and plasmid p3R, by inference). ORFB (297 amino acid residues) was located upstream of ORFA and was truncated at the *Xba*I site. Additional cloning and nucleotide sequencing was required to complete this ORF which is described in Chapter 5.

ORFA is 1005 bp in size as represented by bp 124-1128 in Fig. 4.3, has a coding capacity of 334 aa and is preceded by an intergenic region of 123 nucleotides. A putative Shine-Dalgarno (SD; Shine and Dalgarno, 1974) sequence (5'-AAGGAG-3') was identified 10 bp upstream of the ATG start codon. A putative σ^A -dependent promoter sequence (5'-TTCAAA[19 bp]TATAAT-3') was located between positions 30 and 60. Although the proposed -35 region of the putative promoter is poorly conserved, there is a TG pair 3 bp upstream of the well-conserved -10 region which is sufficient to compensate for the absence of a specific -35 region (Ponnambalam *et al.*, 1986; Keilty and Rosenberg, 1987). Cloning of the 1.5 kb *Eco*RI fragment in the *Bacillus* promoter probe vector, pPL703, indicated promoter activity (data not shown).

Overlapping the TAG stop codon at position 1126 is an imperfect inverted repeat sequence which may play a role in transcription termination. This 17 bp stem/6 bp loop structure ($\Delta G = -12.1$ kcal/mol) lacks the features of typical prokaryotic rho-independent terminators (Brendel and Trifonov, 1984) which have been identified downstream of the coding sequence for several *C. acetobutylicum* genes (e.g. Gerischer and Dürre, 1990; Fischer *et al.*, 1993; Nair *et al.*, 1994; Santangelo *et al.*, 1995). However, alternative factor-dependent or factor-independent termination systems may also exist in Gram-positive bacteria. For example, the imperfect inverted repeats preceding the TAA stop codon of the *C. acetobutylicum* P262 xylanase gene may be implicated in transcription termination (Zappe *et al.*, 1990). The site of the transposon insertion in mutant 3R was located between position 873 and 874. This target site 5'-TCTTTTTGATTCAAAAAAAAAA-3' corresponds to the 20bp sequence required for target activity (Trieu-Cuot *et al.*, 1993). The 6 bp spacer region is underlined.

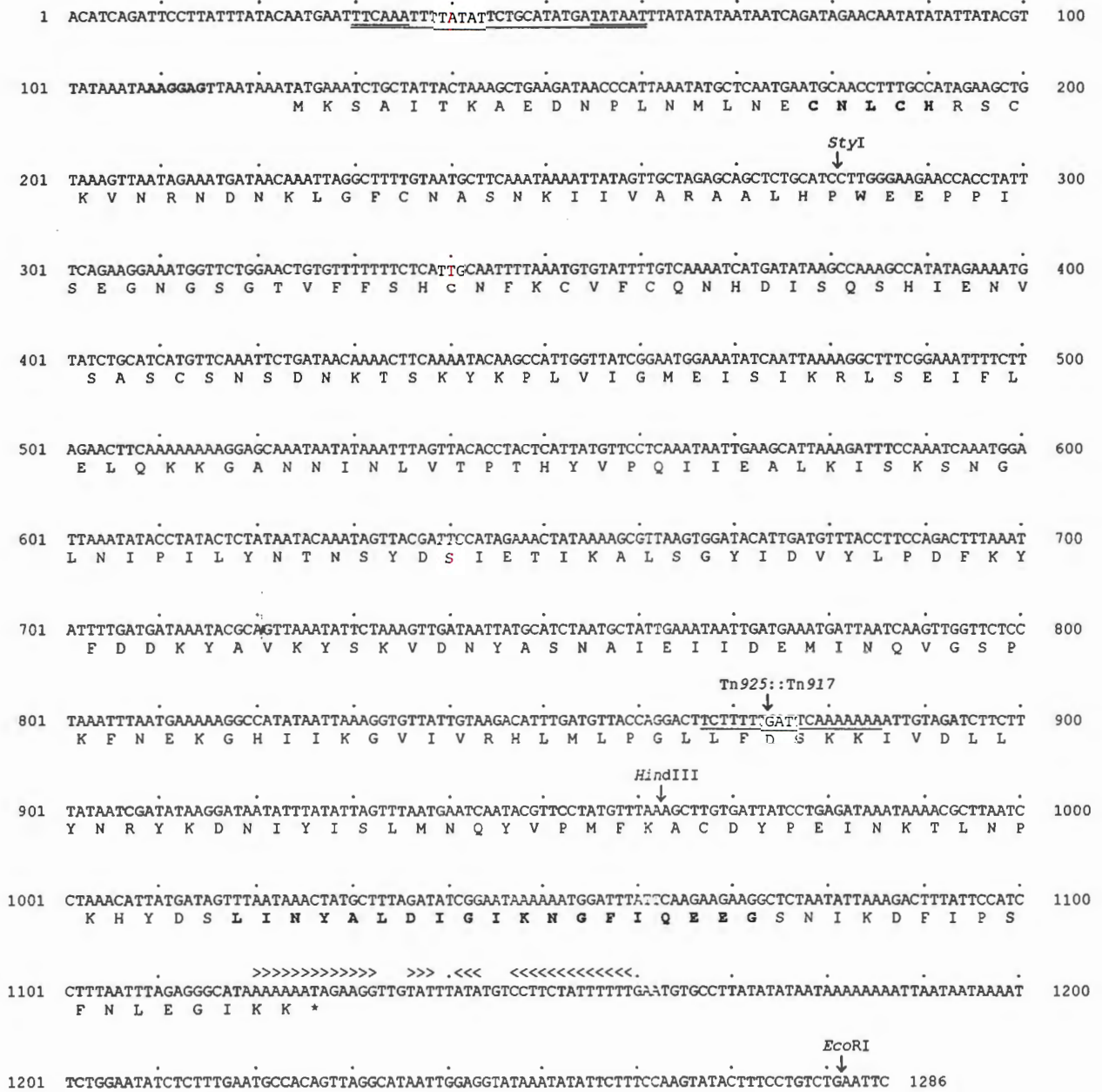


Fig. 4.3. Nucleotide and deduced amino acid sequence of the *C. acetobutylicum* P262 ORFA/sum structural gene and flanking regions. The -10 and -35 regions of the putative promoter (underlined) are double underlined and the putative Shine-Dalgarno sequence is shown in bold. The terminal inverted repeat sequence is marked with arrowheads. Relevant restriction sites and the site of the transposon insertion in mutant 3R are indicated. Consensus sequences for a cytochrome *c* heme-binding motif and a helix-turn-helix motif are identified in the amino acid sequence in boldface type.

4.3.4 Deduced amino acid sequence analysis

The BLAST subroutine (Altschul *et al.*, 1990) was used to screen the protein databases for sequences with homology to the hydrophilic amino acid sequence predicted for ORFA. Initial searches failed to identify the gene, and so it was designated *sum*, for *susceptibility to metronidazole*. Consensus sequences for a cytochrome *c*-type heme binding motif (CNLCH; Fig. 4.3, residues 19 to 23) (Mathews, 1985) and a helix-turn-helix motif (LINYALDIGIKNGEIQEEGS; Fig. 4.3, residues 299 to 317) (Brennan and Matthews, 1989) were identified at the N- and C-terminals of the sequence, respectively. The latter motif compared favourably with DNA binding domains identified in a number of regulatory proteins, but the secondary structure of this sequence, as predicted by Chou and Fasman (1978), does not exhibit a typical helix-turn-helix structure.

However, a recent database search indicated that the *sum* product shares homology (36% identity, 58% similarity according to BESTFIT alignment) with a pyruvate formate-lyase (PFL) activase homologue identified in *T. litoralis* (Kletzin *et al.*, 1995, sequence released September 1995). The PFL-activating enzyme is a Fe²⁺-dependent reductase required for post-translational activation of PFL, a key enzyme in the anaerobic glucose fermentation route in *E. coli* (Knappe and Sawers, 1990). Homology between the *sum* product and the two PFL-activating enzymes of *E. coli* is low (21% identity, 45% similarity according BESTFIT alignment), but amino acid alignment of these proteins (Fig. 4.5) shows conservation of three cysteine residues implicated in iron-binding. (Rödel *et al.*, 1988; Blattner *et al.*, 1993; Reizer *et al.*, 1995). Presumably the consensus sequences for the cytochrome *c*-type heme binding motif and the helix-turn-helix motif identified in the *sum* amino acid sequence are non-functional, as neither of these motifs are present in the other PFL-activase sequences. However, it is worth noting that a *c*-type heme has recently been identified in the formate dehydrogenase of *Desulfovibrio vulgaris*, and is associated with the peculiarly low redox potential of the anaerobic metabolism of the organism (Sebban *et al.*, 1995).

Ca Sum	:	:	-
Tl PFLA	:	MIKTQLMIMWFEIKRAKDALPHYFSILSGEEKPNFFYSKQVEVNFGEDEDLESLEWETHEEGMEKL	:	65
Ec PFLA1	:	:	-
Ec PFLA2	:	:	-
Ca Sum	:MCSAITKAEDNPLNMECECHRSKVENDNKLEFNASNKIIVARALPWE	:	56
Tl PFLA	:	RENDLKENPKKSLDLKALIAYKMESECEFEKCHANFFEEIQYRUKESLVASDFIIGTE	:	128
Ec PFLA1	:MSVIGRIISFES	:	12
Ec PFLA2	:MTSSAGQRISIVVETRRDDVARIFNIQR	:	29
Ca Sum	:* * * PPISEINISGIVFFSHUNFKVFCQNHISQSHENY.....SASCSNSDNKTSKYKPLV	:	112
Tl PFLA	:	EEIV...P.SYIVFFSCNFFCARCONWISQYR.....V	:	159
Ec PFLA1	:	CGIVDIPPIRFITFFQCLMRCLYCHNRRT.....WDTHG	:	47
Ec PFLA2	:	YSENDEEIRTVFFKCPHLCPWGANPESISGKIQTVRREAKCLHCCKLROADECPSGAFER	:	94
Ca Sum	:GMEIIKRSEIFL...ELQKKGANNINLVT..PTHYVFOIEA...ISNSLNIPKLYTNSYD	:	172
Tl PFLA	:	CLRYSPKEMAVKIA...VAYAEGAKNFV...PTPNLPILETIKYV...VPIFVWVSNMGM	:	218
Ec PFLA1	:	GKRVTVEDMKEVVIYRHFMNASGGVTSAGAILQAEVRDWFRAKKEIHTCLDTGFVRR	:	112
Ec PFLA2	:	GRDILDAEREVMKDDIFFRTGGGILSGVLMQAEATRFRQLRLWVSCAETAS....	:	155
Ca Sum	:SIETIKALSGYIV...PDEIVYFELKYVYVYVYDNVYASNAIEIIDEMINOVGSPFNEKCHI	:	234
Tl PFLA	:	SEKSMKLLDGIVV...GDFVWGNEDLKYSLAPRY.....WETVTRNFIKGNHY	:	269
Ec PFLA1	:	YDPVDELEVTIG...VMLDIPQNHIEIQNLVGVSH.....PTIEFARYANKNV	:	162
Ec PFLA2	:	..DAPASKLPLAKCDEVVFDLIMDATQARDVEMNLP.....EVLNLRIVSEGV	:	207
Ca Sum	:	IKGIVRHEMLPGLL.FDSKKVVDLYNRYKDYISLINCVMFKACDPEINIKTENPKH...	:	295
Tl PFLA	:	KAFLIRHLMPCHEECCTRPLEWISENLGRDVRVNVVFCRPEYRAHEFLIDREINDE...	:	331
Ec PFLA1	:	...WIRYVVPGWSDDDSAHRLGFTTDMGVVEKIELLP...HELGKHWVAMGEYKLDGVKP	:	224
Ec PFLA2	:	N...IPRLPLIPGFTLSRENMQAEDVLIPL...IROTILLEF...HOYGEPIRLLGKWSMKEVPA	:	268
Ca Sum	:YDSLINYALDI...GFIQEEGSGNIKDFIPSFNLEGIRK*	:	335
Tl PFLA	:MLKAAFLVKEFRIRLVG*	:	351
Ec PFLA1	:	...KKTETMERVKGILEOYH...ME*	:	247
Ec PFLA2	:	...SSADVATMRMAERAG...QSTVGG*	:	293

Fig. 4.4. Comparison of the amino acid sequence of the *C. acetobutylicum* Sum protein (Ca Sum) with two PFL-activase amino acid sequences from *E. coli* (Ec PFLA1 and Ec PFLA2; Rödel *et al.*, 1988; Blattner *et al.*, 1993; Reizer *et al.*, 1995; accession no.s S01789 and P32675) and the amino acid sequence for the PFL-activase homologue from *T. litoralis* (Tl PFLA; Kletzin *et al.*, 1995; accession no. X83963). Identical amino acids for all four sequences are indicated by a black background. Identical amino acids for two and three sequences are indicated by light and dark shading, respectively. The three conserved cysteine residues, thought to constitute the iron-binding site, are indicated with asterisks.

4.3.5 Determination of transcript size

Since *C. acetobutylicum* cells displayed maximum sensitivity to metronidazole in the early vegetative growth stage (see Chapter 2), total RNA was isolated from *C. acetobutylicum* wild type and mutant 3R cultures harvested at $OD_{600} = 0.2$. Hybridization of the RNA with a DIG-labelled RNA probe derived from plasmid L11 and consisting of a 680 bp fragment specific for the *sum* gene, indicated that *sum* was independently transcribed (Fig. 4.5). As revealed by the single specific hybridization signal, the wild type transcript was approximately 1.2 kb in size (lane a). This is in agreement with the estimated transcript size of 1.1 kb which includes the sequence between the putative promoter region and the ATG start, as well as the inverted repeat sequence of the potential transcription terminator. The *sum* transcript was also produced in mutant 3R, but was slightly truncated i.e. approximately 1.1 kb in size (lane b). Truncation at the site of the transposon insertion would result in a transcript of approximately 0.9 kb. Presumably, the mutant transcript is larger than predicted because of read-through into the transposon sequence. Since the 3' junction of the transposon insertion has not been sequenced, the position of the first in-frame transcription termination codon in the transposon cannot be determined. Results (not shown) of an additional Northern hybridization experiment performed using RNA isolated from wild type cells in mid-exponential and stationary growth phases, suggest that the *sum* gene is constitutively transcribed.

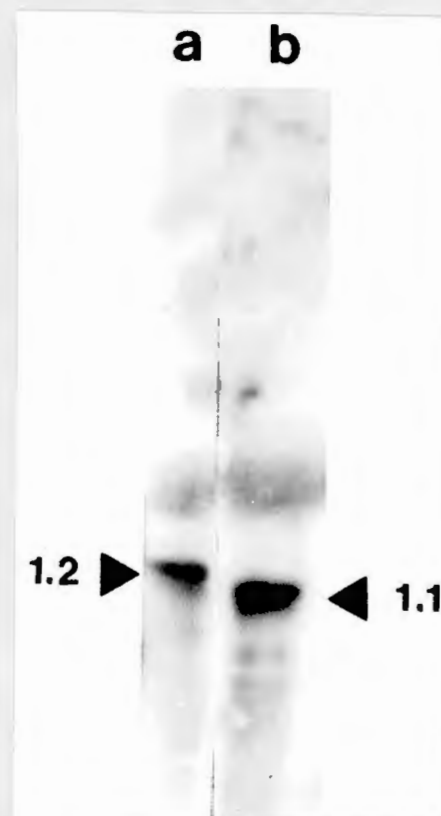


Fig. 4.5. Hybridization of the *sum*-specific DIG-labelled RNA probe to total RNA extracted from the *C. acetobutylicum* wild type (lane a) and mutant 3R (lane b). The probe was derived from plasmid L11. The arrows indicate the position and size (kb) of the *sum* transcripts.

Fig. 4.6. Autoradiogram of [^{35}S] methionine-labelled proteins of pBluescript SK control (lane a), pWT1 (lane b), pWT3 (lane c) and pWT2 (lane d) synthesised in an *in vitro* coupled transcription-translation assay. The putative *sum* translation product (38 kDa) is indicated with an arrow.



4.3.6 Identification of gene product by *in vitro* transcription-translation

The *sum* gene product was identified in a coupled transcription-translation assay (Fig. 4.6). To confirm that the intact *sum* gene was contained within a 1.5 kb *EcoRI* restriction fragment as predicted by the sequence data, this 1.5 kb DNA fragment was subcloned from plasmid pWT1 into Bluescript vector SK, to generate plasmid pWT3 (see Fig. 4.2). A protein of the predicted size for the *sum* gene product (37.8 kDa) was synthesised *in vitro* from plasmids pWT1 (lane b) and pWT3 (lane c) compared with the pSK control (lane a). Plasmid pWT2 which contains the *sum* gene truncated at the 3' end by the *HindIII* restriction site, produced several translation products in the 38 kDa range (lane d). Presumably these polypeptides were generated by transcriptional read-through into the Bluescript SK vector sequence.

4.4 Discussion

The metronidazole resistant phenotype of mutant 3R was shown to be caused by a single insertion of the Tn925::Tn917 cointegrate within a structural gene, designated *sum*.

Sequence analysis of the wild type DNA region corresponding to the disrupted locus in mutant 3R, enabled identification of the complete target site (5'-TCTTTTTTGATTCAAAAAAAA-3') as defined by Trieu-Cuot *et al.* (1993). This is a 20 bp sequence consisting of a 7 bp T-rich segment separated from a 7 bp A-rich segment by a 6 bp spacer region (underlined).

The *sum* gene is 1005 bp in length and encodes a 334 amino-acid protein with a predicted molecular weight of 37.8 kDa. Transcription and translation products of the sizes predicted for *sum* were detected by Northern hybridization and *in vitro* protein synthesis. The *sum* gene is independently transcribed and therefore represents a monocistronic operon.

The *sum* product displayed significant homology (36% identity, 58% similarity) to a PFL-activase homologue, designated *pfla*, identified in the anaerobic methanogen *T. litoralis* (Kletzin *et al.*, 1995). This *pfla* homologue was identified on the basis of sequence similarity with the *act* and *pflC* genes encoding two different activating enzymes of the two *E. coli* pyruvate formate lyases (Rödel *et al.*, 1988; Reizer *et al.*, 1995). Although homology between the *sum* product and the *E. coli* PFL-activases is low (21% identity, 45% similarity), the cluster of cysteine residues which constitutes the putative Fe(II)-binding domain (Wong *et al.*, 1993) is conserved.

PFL is the key enzyme in the anaerobic carbon metabolism of *E. coli*. It is the anaerobic counterpart of the pyruvate dehydrogenase complex of aerobic metabolism and catalyzes the non-oxidative conversion of pyruvate to formate and acetyl-CoA (see Stewart, 1988) (Fig. 4.7). Breakdown of pyruvate via this pathway is a non-redox reaction because the reducing equivalents of cleavage are maintained as formate which can either be exported from the cell or converted to carbon dioxide and hydrogen by the formate-hydrogen lyase (Stewart, 1988; Sauter and Sawers, 1990). PFL is non-functional in the absence of the PFL-activating enzyme. The PFL-activase is responsible for post-translational modification of PFL by a novel hydrogen atom abstraction process which generates the active site glyceryl radical (Gly⁷³⁴) i.e. PFL-activase converts PFL to its active free radical enzyme form (Knappe *et al.*, 1984; Wagner *et al.*, 1992). Pyruvate is a positive allosteric effector of the activation process and the reaction requires *S*-adenosylmethionine (SAM), reduced flavodoxin, ferrous iron, and dithiothreitol (DTT) (Conradt *et al.*, 1984). The activated PFL

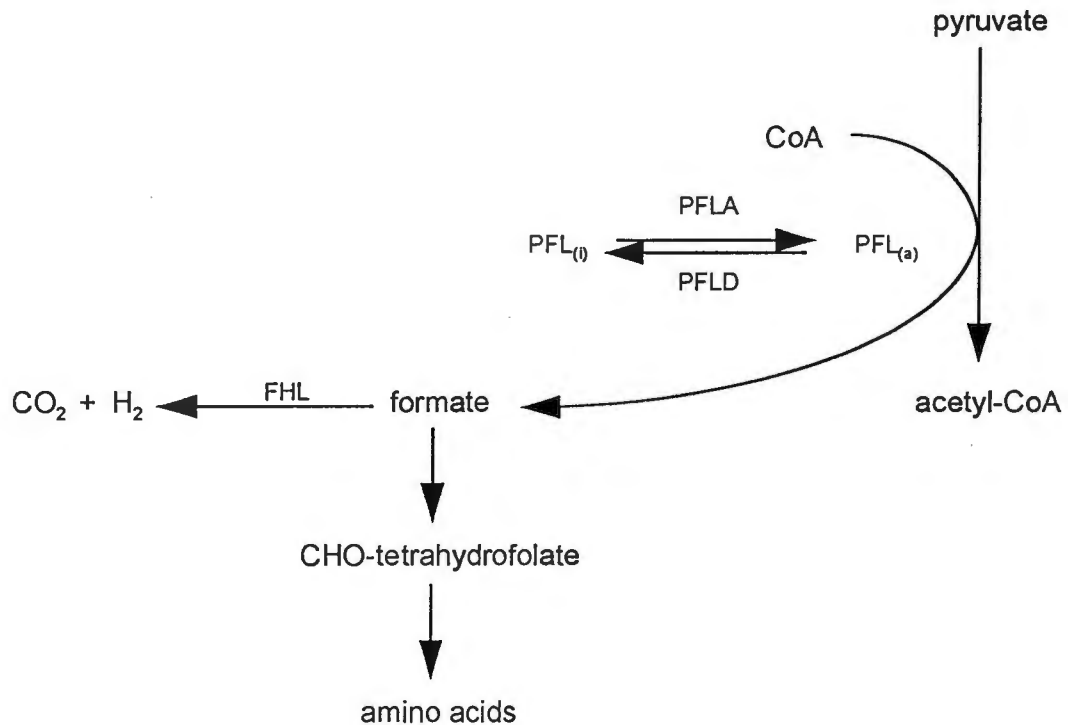


Fig. 4.7. Diagrammatic representation of the PFL reaction and the formate degradation pathway in *E. coli* (and possibly *C. acetobutylicum*). The PFL-activase (PFLA) and the PFL-deactivase (PFLD) convert PFL to its active (PFL_(a)) and inactive (PFL_(i)) forms, respectively. The PFL reaction is reversible in the clostridia i.e. pyruvate is synthesised from formate and acetyl-CoA (Thauer *et al.*, 1972). In the formate degradation pathway, formate is converted to H₂ and CO₂ by the formate hydrogen lyase (FHL) system. The clostridial pathway of C₁-unit synthesis, in which CHO-tetrahydrofolate serves as the reaction intermediate, is included (Thauer *et al.*, 1972).

can be converted back to its native nonradical form by a PFL-deactivase which is one of three redox functions encoded by the polyfunctional *adhE* gene (Kessler *et al.*, 1991; 1992) (Fig. 4.7). Pyruvate is the negative effector of the deactivation process and the reaction is Fe²⁺-, NAD- and CoA-dependent. The reversible formation of the active site radical is tightly regulated by the activating and deactivating enzymes in response to the metabolic status of the cell (Kessler *et al.*, 1992).

PFL activity has been demonstrated in several clostridial species, viz. *Clostridium kluyveri*, *Clostridium butylicum* and *C. butyricum* (Thauer *et al.*, 1972). In contrast to *E. coli*, the clostridial PFL reaction is readily reversible i.e. pyruvate is synthesised from acetyl-CoA and formate. Furthermore, the physiological role of PFL in clostridia is mainly anabolic.

Formate serves as the key intermediate for C₁-unit synthesis, whereas in *E. coli* PFL has a catabolic function, leading to the production of ATP via acetyl-CoA (Fig. 4.7). However, in *C. butyricum*, a close relative of *C. acetobutylicum* P262, formate is also an end-product of the fermentation i.e. PFL function is both catabolic and anabolic (Thauer *et al.*, 1972). The activation-inactivation behaviour of the *C. butyricum* enzyme is similar to that of *E. coli* (Thauer *et al.*, 1972; Wood and Jungermann, 1972), and this implies regulation of PFL activity by an analogous activase-deactivase system. The homology between the *C. acetobutylicum* DSM 792/ATCC 824 *adhE/aad* gene (Fischer *et al.*, 1993; Nair and Papoutsakis, 1994) and the *E. coli* *adhE* gene (Kessler *et al.*, 1991) is significant in this respect. The 96 kDa AdhE protein of *E. coli* has two catalytic functions besides that of the PFL-deactivase, viz. acetaldehyde-CoA dehydrogenase and alcohol dehydrogenase, both of which are responsible for ethanol formation. The 96 kDa AdhE/AAD protein of *C. acetobutylicum* is active in butanol formation and demonstrates both aldehyde and alcohol dehydrogenase functions, but has not been tested for PFL-deactivase activity (Nair and Papoutsakis, 1994). The PFL-deactivase functional unit of the *E. coli* AdhE protein is undefined. It does not seem to be encoded by a continuous amino acid sequence as is the alcohol dehydrogenase function (residues 450-762), and its activity may depend on interaction between the N-terminal half of the polypeptide (residues 1-499) and the C-terminal end (residues 763-890) (Kessler *et al.*, 1992). It is very likely that in the solventogenic clostridia, the AdhE/AAD protein harbours this PFL-deactivase function, which together with the Sum protein, constitutes the PFL activase-deactivase system.

There are three ways in which the PFL-activase might contribute to metronidazole susceptibility. The first possibility is that as a reductase, the PFL-activating enzyme is responsible for direct enzymatic reduction of the drug, although presumably its reducing activity is highly specific. The second possibility is that formate, the end product of the PFL reaction, acts as the reductant of metronidazole via the formate degradation pathway. In *E. coli*, formate is the inducer of the formate-hydrogen lyase system which catalyses the degradation of formate to hydrogen and carbon dioxide (Fig. 4.7). The components of this system are the formate dehydrogenase (FDH), unidentified electron carriers, and a hydrogenase (Stewart, 1988). FDH has been shown to be active with methyl viologen and benzyl viologen as artificial electron acceptors (Stewart, 1988). These same electron

carriers are able to couple the enzymatic reduction of metronidazole by the *C. pasteurianum* hydrogenase (Church *et al.*, 1988). The third possibility is that formate, as a reduced product, alters the redox potential of the cell such that sensitivity to metronidazole is increased. This option is elaborated on in Chapter 7. It is interesting that the presence of excess formate (as sodium formate) in the culture medium has been observed to increase susceptibility of *E. coli* strains to metronidazole (Santangelo, 1991). It is also interesting that the cloned *C. acetobutylicum* P262 flavodoxin gene confers metronidazole sensitivity in *E. coli* F19 (Santangelo *et al.*, 1991). Reduced flavodoxin is required in *E. coli* for the conversion of PFL from the inactive to the active form (Conradt *et al.*, 1984). It is postulated that overexpression of flavodoxin might optimise the conversion of PFL to its active form via the PFL-activase, resulting in accelerated breakdown of pyruvate to acetyl-CoA and formate, which in turn stimulates the formate hydrogen lyase system (Santangelo, 1991).

In conclusion, the *sum* product displays homology to the PFL-activase, an unusual organic radical activating enzyme. This provides indirect evidence for the existence of a formate-producing pathway in *C. acetobutylicum* P262 and implies a direct/indirect role for the PFL-activase in metronidazole activation. It must be stressed however, that the functionality of the *T. litoralis pfla* gene, with which *sum* shares homology, has not been confirmed. In *E. coli*, the *pfl* and *act* genes are adjacent on the chromosome although they are independently expressed (Rödel *et al.*, 1988). The *E. coli pflD* and *pflC* genes encoding homologues of PFL and its activating enzyme are also juxtaposed, and are located within a novel phosphotransferase system gene cluster (Reizer *et al.*, 1995). In *T. litoralis*, the *pfla* gene is flanked by genes encoding a pyruvate kinase and a tungsten-containing formaldehyde:ferredoxin oxidoreductase (Kletzin *et al.*, 1995). The following chapter describes sequence analysis of the DNA regions flanking the *sum* gene.

Chapter 5

Cloning and nucleotide sequencing of the DNA regions flanking the *sum* gene on the *C. acetobutylicum* P262 chromosome

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Chapter 5

Cloning and nucleotide sequencing of the DNA regions flanking the *sum* gene on the *C. acetobutylicum* P262 chromosome

5.0 Summary

DNA fragments overlapping the 5' and 3' ends of the *sum* gene on plasmid pWT1, were cloned and sequenced. The sequence of ORFB, which was identified upstream of the *sum* gene from the initial sequencing data (Chapter 4), was completed. ORFB is convergent and in the same reading frame as *sum*. The deduced hydrophobic amino acid sequence shared 24% identity and 54% similarity with a 38 kDa unidentified protein from the replication origin of *B. subtilis*. These two homologous proteins also shared the same eight-transmembrane-segment hydropathy profile. Sequence analysis of the DNA region downstream of the *sum* gene revealed five open reading frames (ORFs1-5), all transcribed in the opposite direction relative to *sum*. These ORFs represent a stationary phase gene cluster. ORF1 displayed substantial homology (60% identity, 74% similarity) with the spore-cortex lytic enzyme (SCLE) of *C. perfringens*. The C-terminal end of this putative spore-cortex lytic enzyme featured a unique sequence, LPKTG, corresponding to the consensus motif of cell wall sorting signals. These signals have been identified in the surface proteins of many Gram-positive bacteria, but have not yet been reported for *Clostridium*. The deduced amino acid sequence of ORF2 contained three regions with significant similarity to the sequences involved in the formation of the active site of the serine proteases of the subtilisin family. The ORF2 product may be responsible for proteolytic processing of SCLE. ORF3 and ORF4 both encoded low molecular weight proteins. The ORF3 product displayed significant sequence homology to a number of regulatory proteins, most notably, the Cro-like protein of the *B. subtilis* bacteriophage ϕ 105 (29% identity, 52% similarity) and Sin, a multi-functional regulatory protein of *B. subtilis* (22% identity, 39% similarity). Both the primary and secondary structure of a 20 amino acid sequence within the N-terminus of ORF3 conformed to the requirements for a helix-turn-helix structural motif. The predicted amino acid sequence of the C-terminal end of ORF5 displayed high homology with peptidyl-prolyl-isomerases which are implicated in protein folding.

5.1 Introduction

The isolation and molecular characterization of the *sum* gene, a putative PFL-activating enzyme was described in Chapter 4. Disruption of this gene in *C. acetobutylicum* transconjugant 3R caused the mutant to acquire resistance to metronidazole. This chapter documents the cloning and nucleotide sequencing of the DNA regions flanking the *sum* gene. The aim was to define *sum* in the context of its neighbouring genes on the chromosome, and gain further insights as to the *sum* gene function. Both the *E. coli* genes encoding the two different PFL-activases are adjacent to the genes encoding the two PFL enzymes (Rödel *et al.*, 1988; Reizer *et al.*, 1995). The *T. litoralis* PFL-activase homologue is flanked by genes encoding a pyruvate kinase and a formaldehyde:ferredoxin oxidoreductase (Kletzin *et al.*, 1995).

5.2 Materials and methods

5.2.1 Bacterial strains, plasmids and culture conditions

E. coli strains LK111 (Zabeau and Stanley, 1982), JM105 (Yanisch-Perron *et al.*, 1985) and JM109 (Yanisch-Perron *et al.*, 1985) were used interchangeably as the cloning hosts for pBluescript SK-based DNA constructs. Plasmids pWT1, pWT2 and p3R are described in Chapter 4. *E. coli* cultures were grown at 37°C in 2xYT broth or on 2xYT agar (Messing, 1983) supplemented with ampicillin (100 µg/ml) for plasmid selection. Genomic DNA was extracted from *C. acetobutylicum* P262 (Jones *et al.*, 1982) which was grown anaerobically at 34°C in buffered CBM (Allcock *et al.*, 1982). The origins of the plasmid constructs generated in this study are illustrated in Fig. 5.1.

5.2.2 DNA preparation

Plasmid DNA was isolated either by the alkaline hydrolysis method of Ish-Horowicz and Burke (1981) or from Nucleobond[®] columns using the kit purchased from Machery-Nagel. *C. acetobutylicum* P262 chromosomal DNA was prepared by the method of Marmur (1961) as modified by Zappe *et al.* (1986).

5.2.3 Construction of size-selected genebanks

Partial genebanks were prepared from *C. acetobutylicum* P262 chromosomal DNA. DNA (50 µg) was digested to completion with the appropriate restriction enzymes and the

resulting fragments were gel-fractionated. Size-selected DNA fragments were extracted from agarose gels, ligated to appropriately digested Bluescript SK vector, and transformed into *E. coli*.

5.2.4 Colony hybridization

Colonies harbouring recombinant plasmids with *C. acetobutylicum* DNA inserts were processed for hybridization as described in Chapter 2, Section 2.2.8.

5.2.5 Nucleotide sequencing

The DNA sequence of the 0.5 kb insert of plasmid pUP2 (Fig. 5.1) was obtained by sequencing in from each end of the fragment using the M13/pUC 1212F and 1201R primers (BioLabs). The 5.8 kb DNA insert of plasmid pDO1 (see Section 5.3.3) was sequenced on one strand only. An overlapping set of deletion clones was constructed from the left side of the 5.8 kb DNA insert by exonuclease III digestion of *ApaI*-*ClaI*- and *ApaI*-*SalI*-restricted fragments according to the method of Henikoff (1984). Double-stranded plasmid DNA templates were sequenced by the dideoxy-chain termination method of Sanger *et al.* (1977) using [³⁵S] dATP and the Sequenase[®] version 2 kit (Biochemical Corp.) as specified by the manufacturer. The 1212F primer was used for the sequencing reactions. The sequence data were analysed on a VAX 6000-330 computer using the Genetics Computer Group Inc. sequence analysis software package (Devereux *et al.*, 1984). Amino acid homology searches were carried out against the cumulative non-redundant updated protein database which was accessed via the BLAST network service (Altschul *et al.*, 1990).

5.3 Results

5.3.1 Chromosome walking to complete the ORFB sequence upstream of the *sum* gene

Chapter 4 described the nucleotide sequencing of a 1944 bp DNA region which revealed an incomplete open reading frame, ORFB, upstream of the *sum* gene. ORFB (286 aa) resided on the 2.4 kb *XbaI*-*HincII* DNA insert of plasmid pWT1 and was truncated at the *XbaI* site (Fig. 5.1). A 2.5 kb *EcoRV* fragment that overlapped most of the insert of plasmid pWT1 and included 0.5 kb of DNA upstream of the *XbaI* site, was identified by Southern hybridization using the 0.6 kb *XbaI*-*EcoRI* DIG-labelled fragment of plasmid pWT1 as a probe (results not shown). Using the same probe, this fragment was isolated by colony hybridization from a size-selected genebank constructed from *EcoRV*-digested

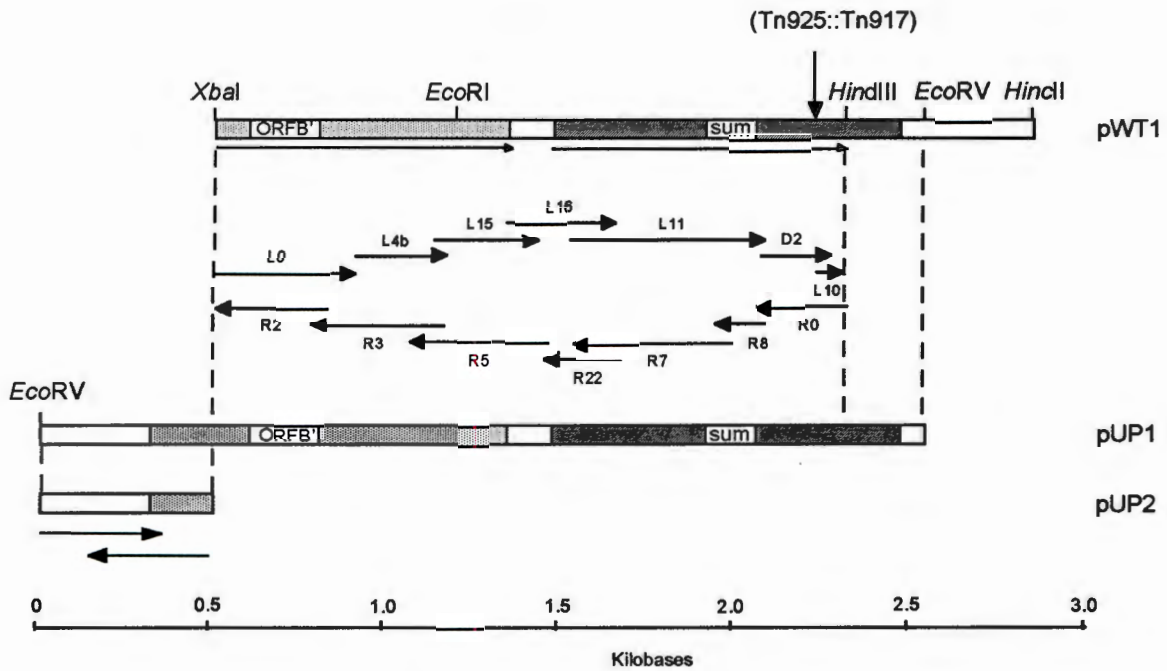


Fig. 5.1. Sequencing strategy for ORFB. Shortened constructs were derived from plasmid pWT2 (see Chapter 4). A 0.5 kb *EcoRV*-*Xba*I fragment, containing the 5' end of ORFB, was subcloned from plasmid pUP1 to generate pUP2. The DNA fragment was sequenced from both ends.

C. acetobutylicum chromosomal DNA. Two clones containing the DNA of interest were identified from the 400 recombinants screened. Restriction analysis revealed that both constructs were identical with respect to the orientation of the insert DNA. One of these plasmids, designated plasmid pUP1 (Fig. 5.1), was selected for subsequent manipulation. The 0.5 kb *EcoRV*-*Xba*I DNA fragment was subcloned into Bluescript pSK, to generate plasmid pUP2 (Fig. 5.1), and was sequenced from either end (approximately 300 bp in both directions) to complete the ORFB open reading frame.

5.3.2 Sequence analysis of ORFB

The gene encoded by ORFB presumably initiates at a TTG codon at nucleotide 328 and terminates in TAA at nucleotide 1387 (Fig. 5.2). There is an ATG codon approximately 160 bp downstream of the TTG codon, at position 490, but TTG was considered the most likely start for the following reasons. First, TTG is a common initiation codon in Gram-positive bacteria (Hager and Rabinowitz, 1985). Second, a putative SD sequence 5'-AGGGGG-3' is positioned 8 bp from the TTG codon and is identical to the sequence identified upstream of the *C. acetobutylicum* endo- β -1,4-glucanase gene (Zappe *et al.*, 1988), whereas no SD-like

```

1   TTTTCCCATCCAAAATTATTCATATTTCTCTCTAGTCTAAGTACATATACACAATGATATTAGATTATTATTTTGGTATCTACATAATTAATTACTTA   100
101  TTTAATTGTACATTGTAATAATTCATAATGTACATTGTTGAATAACATTCTGCATCGTTAATTCATTCTCTCTACGCCTTAAGTGTAATAATTACCTAAAT   200
201  ACTACATATCAATATAATTACTACCCATTATTTCTAATTTATAATACATAAAATTAATATTACAAGAATAAAATACAATGTAAAGCTGTATATTAATACA   300
301  TAGGAATGTTTTAAGGGGGGATAATAAATGAAATCGGAATTAACNTAAATATTCAAGTTGCCACAGTCTTCATTGGAACAATTGTTGGTGCCTGCCTTG   400
      N I S S C H S L H W N N C W C C L A
401  CCTCTGGAAAGGAAATTACTGAATTCCTTACTCAATATGGAATTAAGCTTCTTGGGAATTGTAGCATGTTGGTATTTCTATATAATTTAGAGTCCAT   500
      S G K E I T E F F T Q Y G I K S F L G I V A C G I F Y I I M S S I
      XbaI
501  AATTCAAAGATAAGTATTGAACATAATCTAGATTTCATAGTGATGTTATAACATAATAAGCCCTAATGTTATAGGAAATTTACTGGCTTTATAACT   600
      I S K I S I E H N L D S Y S D V I N I I S P N V I G K F T G F I T
601  AACTGTTCTTATTCTAGTGTCTCAATAATCTTGCCTGGAAGTGGAGCTTTAATACATCAGTTTTTTGGAAATACCAAAGATAATCGGTTTCATTAATAA   700
      T L F L I S S A S I I L A G S G A L I H Q F F G I P K I I G S L I M
701  TGATTATAAGTCTTATTTCTTATTTCTCGCGGTACTGAAGGTTGATCGAAGTAAATCTTTCATTGTTCTCGGATTAGTACTTACAATAACTTTAAAT   800
      I I I A L F F L F R G T E G L I E V N S F I V P G L V L T I T L I
801  CACAGTTTATACTTCTGTTTTGTAAAAACATAGTTTCATTAAATAATGTTTGAATTTTCCCGCACAAAAAAGTGGGATTGCATTATCAACAATATTA   900
      T V L Y F C F C K N I V S F N N V C N F P A Q K T G I A L S T I L
901  TATGCAGGATATAACTCTTTTCTAGCATCAGGCGTAATAGTCCACTAAGTAATGAAATGAAAAAACAAGTTATGGTAATGGCATTATATGCGGTG   1000
      Y A G Y N T L S A S G V I V P L S N E M K K T K V M V I G I I C G A
1001 CTGTTGGTCTTACTTTGCTTTGCTCTATGATAAATTTATTATTAACAATTAACCAACCATATATATCAATATGAAATTCCTCTGCTTTTGTAGCAAA   1100
      V G L T L L C S M I N L L L T I N Q P Y I Y Q Y E I P L L F V A N
1101 CAGATTGGAATGTAATACAAGCTGTTTACTTATTATATATGGCTGAAATGTTCTCAACCGAAGTATCTGATGATACTCAATAAGTAAACTTTA   1200
      R F G N V I Q A V L L I I I W L E M F S T E V S D V Y S I S K T L
1201 GAACAATCTTTAATATTGAATTCAAAACAGCTATTTCCAGTATTAGCAGTAGCATTATAATCTCTCAATTTGGTTTTGGCAACTTAATAACAAGC   1300
      E Q S F N I E F K T A I F P V L A V A F I I S Q F G F G N L I N K L
1301 TATATCCTATGTTGGATTGTTAAGTTTATTATTATATCTCAATGCATAATATTCTTTATTAACATAGAAATATCTACAATAAATAAACATCAGATT   1400
      Y P M F G L L S L L F I S Q C I I F F I K H R N I Y N K *
1401 CTTATTTATACAATGAATTTCAAATTTTATATTCTGCATA 1440

```

Fig. 5.2. Nucleotide and deduced amino acid sequence of ORFB. The -35 and -10 regions of two possible promoter sequences (underlined) are double underlined, and the putative Shine-Dalgarno sequence is indicated in boldface type.

sequence precedes the ATG codon. Third, TESTCODE analysis predicted a coding region of approximately 1060 bp as opposed to 900 bp in length. (The TESTCODE program identifies protein coding sequences independently of the reading frame by plotting the nonrandomness of the composition at every third base). Since this DNA region was sequenced from one strand only, a misplaced stop codon (not shown) occurs within the coding sequence of ORFB, near the putative TTG start. Therefore the exact size of the coding sequence (approximately 350 amino acids with a predicted molecular weight of 40 kDa) cannot be determined.

ORFB is transcribed in the same direction and reading frame as the *sum* gene, and two sequences displaying reasonable similarity to the consensus sequences for vegetative promoters are indicated in Fig 5.2. Although *sum* is transcribed independently of ORFB (see Chapter 4 for *sum* mRNA transcript analysis), there is no inverted repeat sequence in the intergenic region which can be implicated in transcription termination of ORFB; presumably some alternative termination signal exists.

The deduced amino acid sequence of ORFB was very hydrophobic (a characteristic feature of membrane-bound proteins) and a hydropathy plot predicted eight membrane-spanning domains for the ORFB protein. An amino acid homology search revealed significant sequence similarity between the ORFB product and a hypothetical 38 kDa protein of *B. subtilis* (N. Ogasawara and H. Yoshikawa, unpublished data, Swiss Prot accession number P37520). Comparison of the two sequences using the GAP alignment program indicated that they shared 24% identity and 54% similarity (Fig. 5.3). Hydropathy analysis of the *B. subtilis* hypothetical protein revealed an eight transmembrane segment profile similar to the one obtained for ORFB (Fig. 5.4).

5.3.3 Cloning and nucleotide sequencing of the DNA region downstream of the *sum* gene

Based on the results of a Southern hybridization experiment described in Chapter 4, a 5.5 kb *Hind*III restriction fragment, which overlapped the 3' end of the *sum* gene on the insert of plasmid pWT1, was identified. This fragment was isolated by colony hybridization from a size-selected genebank prepared from *Hind*III-digested *C. acetobutylicum* DNA,

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Ca ORFB : NISSCHSHQ.....NNWCCLASGKETIEFFITQYGIKSFICIVACGHEIIMSSISKISIEHN : 60
Bs 38   : .MLWRAGKMLLILGSLIGAGYASGQEIWQFF...CAESGLAIVLFTLMEIFSSYIMKISKVQ : 62

Ca ORFB : LDSYSDVNIISPNVIGREIGFITTITLSSASTIAGSEAIHIFPGIKIIESLINIITAFFE : 126
Bs 38   : STHELPLVLEHMGPWLAIRYDVIIIEYLRSTIMVMHAG.GGILDMYKEEFWGCITLICTIVCLE : 127

Ca ORFB : FRGTEGTEVNSFIVEGLV..ITITITIVLYFCCKNIYSANNVNEPAOKTGIALSTILMAGYNT : 190
Bs 38   : LADVKGILSINSITIEVLVAGILYALISFQSTHHHTWIDLSQQANQPA.....SITITSLMI : 185

Ca ORFB : LSASGVIVPLENEMRKTQVMVITILCCAYGETILRSINILLITINQPVYIYVEIPIEFVANRFGNV : 256
Bs 38   : LSVVAILESSVGRKEMRGLGEARKISVASGEIEGVLSFYNETLVELAGSESOFEIPIEAVLLEGAPYF : 251

Ca ORFB : IQAVELIITWEMESTEVSDYYSKTLTQSENFTEFKTAEPPVAVAFIISQFGEYILINKLYPK : 322
Bs 38   : FLCHTAVLCLATYITIVAGLLQSSPLMAFTHPRWMIYVILVLMVPEITSEGEYDLIAFLYPK : 317

Ca ORFB : GLISLLEISQCTFFIKHRNITYK*.. : 347
Bs 38   : GMLNDYELV.CREELY....PPLSRWK* : 339

```

Fig. 5.3. Amino acid sequence homology comparison of ORFB from *C. acetobutylicum* (Ca ORFB) and the hypothetical 38 kDa protein identified in *B. subtilis* (Bs 38; N. Ogasawara and H. Yoshikawa, unpublished data cited in the SwissProt data library, accession no. P37520). Identical amino acids are indicated by a black background and conservative amino acid substitutions are shaded.

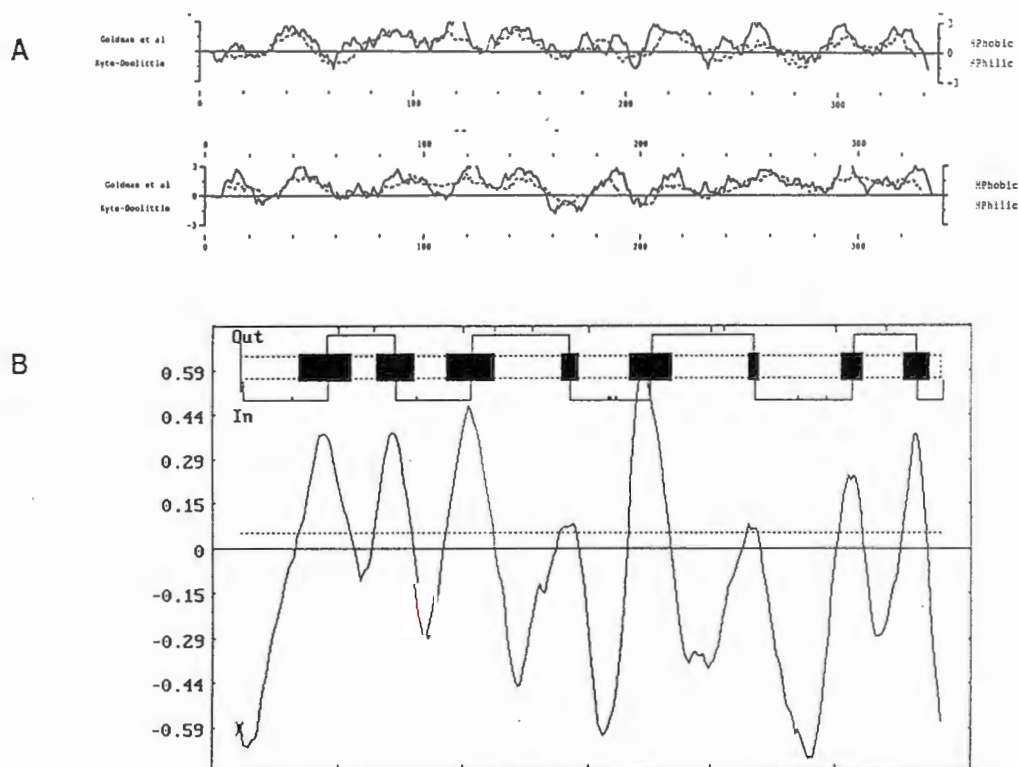


Fig. 5.4. Comparison of hydropathy profiles of ORFB from *C. acetobutylicum* P262 (A) and the unidentified 38 kDa protein from *B. subtilis* (B). Profiles were obtained using the GCG program PEPLOT. Membrane topology profile of ORFB (C) was obtained using the software package Antheplot (Deléage *et al.*, 1988).

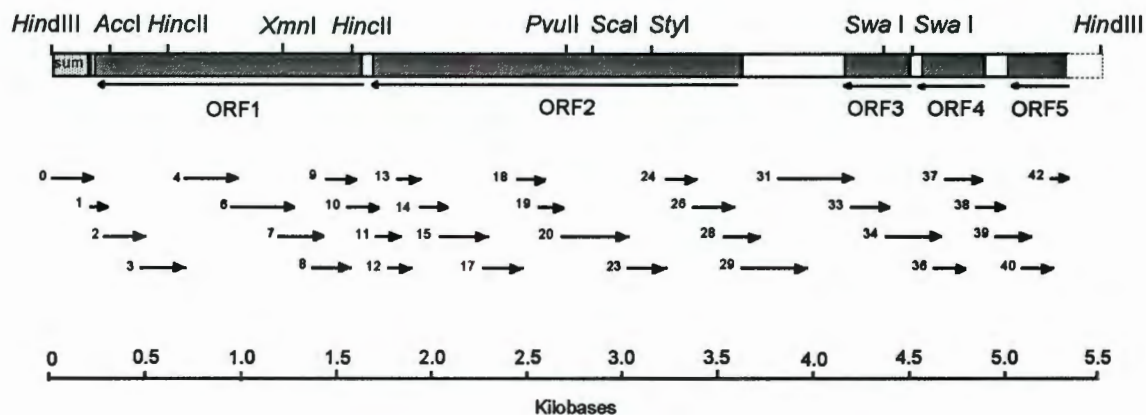


Fig. 5.5. Physical map of the 5.5 kb *C. acetobutylicum* DNA insert of plasmid pDO1, overlapping the 3' end of the *sum* gene. The direction of transcription of ORFs1-5 is indicated with arrows. The sequencing strategy is outlined below the map. The numbered arrows show the extent and direction of sequence obtained from the individual clones generated by exonuclease III digestion.

using the 0.3 kb *HindIII-EcoRI* DIG-labelled fragment of plasmid p3R as a probe. One positive clone, pDO1, was identified from the 450 recombinants screened. Restriction analysis established that the DNA insert of plasmid pDO1 had the same orientation in the Bluescript SK vector as the insert of plasmid pWT1. The majority (5056 bp) of this DNA insert was sequenced to reveal five open reading frames, one of which was incomplete. The sequence was determined from one stand only from a range of overlapping fragments generated by exonuclease III digestion from the left side of the insert; the sequencing strategy is shown in Fig. 5.5. The ORFs numbered 1-5, are transcribed in the same direction with respect to each other and are convergently transcribed relative to ORF6 and the *sum* gene. The nucleotide sequence and deduced amino acid sequence of these ORFs is presented in Fig. 5.6.

5.3.4 Sequence analysis of ORF1, a putative spore-cortex-hydrolase gene

ORF1, the *sum* proximal ORF, initiates at one of two GTG codons (positions 3464 or 3497), each of which is preceded by a potential SD sequence, and extends 1335 or 1368 bp in length to the TAA stop codon at position 4829 (Fig. 5.6). No promoter-like sequences were identified upstream of ORF1. Located 29 bp downstream of the TAA stop codon is an imperfect inverted repeat which overlaps the TAG stop codon of the *sum* gene.

ORF5

1 ATGATCAAGGAGGATGTCCGNAGGCACCTGGAATGGGTGGTCCAGGATATCTATTAAAGGTGAATTACATCAAAATGGATTTAATAATACATTAAC 100
M I Q G G C P X G T G M G G P G Y S I K G E F T S N G F N N T L K H

101 ACTCAAGAGGTGTATTATCAATGGCTAGAGCTATGCATCCAGACTCAGCAGGAAGTCAATCTTTGTTATGGTAGCAGATGCTCCACACTTAGATGGTCA 200
S R G V L S M A R A M H P D S A G S Q F F V M V A D A P H L D G Q

201 ATATGCATCATTGGTAAAGTAATTGAAGGAATGGAAGTGGCTGATAAAATGTAGCTCAAAAACCTGANGATATGACAGACCTTATGAAGATCAAAAAATA 300
Y A S F G K V I E G M E V L I K C S S K N *

ORF4

301 AAGAGTGTGTAGTTGATATGCAAGGTGAAGAAATTAAGAACCAGAAATAATAGAAGAATAATATTCATAAAATAAATAGAAAAGAGGTGTTTATATG 400
M

401 GCAATAAATATCAATGTATATTAGTATATGGATTAACGCAAGGAACTCGACAAGATACAAAAGAGAAGATAAAAGTGAAGAAGTAACTAATGAGA 500
A I N Y Q C I L V Y G L T Q E E L D K I Q K R R L K V K E V T N E N

501 ATGCTTCAATGAGAATAGTAGATATTTGTGCGAAGNAAGCAAAGAAATACTTATGCTGAATTGCCAGTAAATGAAAAGGCACCTATTTCATGGATA 600
A S M R I V D I L C E X S K E N T Y A E L P V N E K A L I F N G Y

601 TAATGATAAAGAATTAAGAAATGCCATTAATTTATTAGAACGTTTATAGAAGGTGGAGTATTGGCTGTAGTTACAGNACAATCTAGTAAATGGACGTTA 700
N D K E L R N A I K F I R T F I E G G V L A V V T X Q S S K W T L

ORF3

701 AGGACTTAATGGAACCTCTAATAGAAGAAAGAGAATGGTACAAAGCAAAGGGGCAAATTAAGTGAAGTCGNGTAGGAGAAAATATAAAACAAGCTAGAGN 800
R T * V S R V G E N I K Q A R X

801 AAAAAATGGAATGACAGTTAAAGTTTACGNAAAAAGTTGGNGTGGCAGNAAAAATTTAAATGAAGTTGAAATGGGAAGAAAAGTTGCACCTGNATCA 900
K N G M T V K V L A K K L G V A X K Y L N E V E M G R K V A P X S

901 TTTATAGATAGACGCTAAACTTTTAAAGGCCGATTTAAATGATATAAGTATGGTAGTTACAGATGAGGCATTAATGGAGGAAAAGAAAACCTTAAAGAG 1000
F I D R A A K L L K A D L N D I S M V V T D E A L M E E K K T L R E

1001 AACTTCCAAAGAAAAAGTAGAAAGTTCAGAAGTTGGACAGATGCTTTTCATCAGTCTTTAGAAGTGTCTTTTCTCCATTAATGCCTCATAATAAA 1100
L P K K K V E S S E V W T D A F S S V L R S V L F L H *

1101 AAGGATTTAAAGAAATGCCTGTTCATTCAAACAAAATAGAAGGTATCCGAGGATAAGGTTTTCTATGTAGAAATAGAAGATGATGAAATGAATGGATT 1200

1201 TAGAATGTTAAAGGTGAGTAGCATTGCGCACACTGTAAAAGAAGTAAGTAATAATGGATTTTCTTAATTGACTATAAGGACAAAAGAAAGATAAGACA 1300

1301 AGTAAAAGTTTTAGAAATTCANAAGTGCTTTTAGTTAGCAAATGCAGGAAATTTACTTACTGAGACTATGGAAGTAAAAGAGTTAAAGTAATCGCTAAAT 1400

1401 AGAAAAAGTTGAAATTAACCTTAATAATATATAGCCAAAATAAATTTGTAAGTTTGACTTATTATTTATTTCATAGCTAATAATAACTAGATATAAC 1500

1501 AGACTGTTCAAAAATTATATTTTGAACAGTCTATTTAATTTATAAAAATTTGCTTAATGACAATTAGATTTTTGTTATGTGAAAAATTTAATTG 1600

ORF2

1601 CATAATATAGTAGTGTATATAAATAAAGGTTGTGGTTATTATGCCAAATTCAGTTGAAGAATTAGCATCTAAAATAGATACATCATTAGGTTTGCTTA 1700
M P N S V E E L A S K I D T S L G L L T

1701 CTAATGTACCTAAAGCAATATTAGATAATTTAGGTTATAAGTTAATGTGGGAAAAGAAGAAAATATCATCGAAGTTACAATTTTATATAGGGATACTCC 1800
N V P K A I L D N L G Y K F N V G K E E N I I E V T I L Y R D T P

1801 AGAAAATATGAAAACCGTAGTGAAGGTTGGGAGGAGTATTTGAGGATTTAGGATTTAATTTTGAATAGTAGATTACCCTTAGACAAATGGATGAT 1900
E N M K S V V E G L G G V F E D L G F N F G I V D L P L D K L D D

ORF2 (cont.)

1901 TTAGCTAGAAGCAAAGTGTGCAATATATAGAATTACCTAAAAATTGTATGAAGCAGATGCAGCAAGTAATATAGCGTCTTGTGTCCAGATGTAGTTT 2000
L A R S K S V Q Y I E L P K N L Y E A D A A S N I A S C V P D V V S

2001 CTAATTATAAAGTTTCAGGAAAAGGAGGTTAATTGGATTGTGTGATTGAGGAATAGATTATACCCATCCAGCATTATGAATAACCAAGGAACGACAAG 2100
N Y K V S G K G G L I G F V D S G I D Y T H P A F M N N Q G T T R

2101 AATAGAATACATTATGACTTGAGTGATGGGGAAAACCTTTATAATAAGCAAACCTATCAACGAAGCAATAAAGCACCAAAATCCATTCTCAATAATACCA 2200
I E Y I Y D L S D G G K L Y N K Q T I N E A I K A P N P F S I I P

2201 CTACAGCTTCAGCTATGAACTCATGTGATGGAGATGCTGTGCTGGAGAAATATAATCCGGAATATAAAGGAGCAGCACCAGATGCATCAATAGCAA 2300
L Q L S A M N S C D G D A C A G G N I N P E Y K G A A P D A S I A M

2301 TGGTTAAAGCATCAAGAGGTGTTTCAATATTAAGTTCACAATAATGAAAGGTCTTAGATTTTTAGTAGAGAAAAGTAAAGAGTTGAATATGCCATTGGT 2400
V K A S R G V S I L S S Q I M K G L R F L V E K S K E L N M P L V

2401 TGTAGTCTTAGTTAAGTACTAATAATGGAGCACATAATGGAAGTAGTCTATTAGAACAATATATTAGGACAGTGGCAAATTTAGAGCGAGCATCTATT 2500
V S L S L S T N N G A H N G S S L L E Q Y I R T V A N L E R A S I

2501 GTTATTGCAGCTGGAATGAAGCGATGCAGGACNTCATACAAGTGGTATTTAAATCAATCCAAATCAAACATTTAATATAGCAAGTATGAACTG 2600
V I A A G N E G D A G X H T S G I L K S N P N Q T F N I A S D E T A

2601 CAATTGTTATGAATTTATATAAGCCTATTTTACCGGAAATAACTCTTACTATAATTAATCTACAGGGAAGCNGTGGAGTGATAAAGATACAAGAAGG 2700
I V M N L Y K P I L P E I T L T I I N P T G K S X G V I K I Q E G

2701 TTATATCAAGGTAAATNGCAAAGATAGATTGATATATATGTATCAGGTGCAGCCATTCATTAGAGAGTGAATTCAAATAATAATTTCTTCACCA 2800
Y I Q G K X A K D R F D I Y V S G A A I Q L E S E I Q I I I S S P

2801 TTAGGATATTTAGCTGAGGGGTATGACTTTAACAATAGTGTACTAATAATTACGATGGGGAATATCTATTTGGCTTCCGGTATCTGAAGGACTTA 2900
L G Y L A E G V W T L T I G V T N N Y D G E Y S I W L P V S E G L N

2901 ATCTCAAACCTAGGTTTTTAGAACCAATTACATTAATACTCTAGGAATACCAGCAACTGTTGATAATATAAATGCAGTTGGAAGTTATAATCCTATATT 3000
P Q T R F L E P I T F N T L G I P A T V D N I I A V G S Y N P I L

3001 AAATAATATTTCTTCATTAGTGGAAAGAGGAAAGCAAGATTCTGGAGGTAGAGTAAGACCAGATTAGTTGCGCCAGGAGAAAATATAATGGGACCAGTT 3100
N N I S S F S G R G K Q D S G G R V R P D L V A P G E N I M G P V

3101 CCTAATGGAAGCTATGATAGTAAACAGGAACATCTATGGCTGCTCCACAAGTAGCTGGGATTTGTGCNTAATATGGAATGGGGAATAATTAAGGAA 3200
P N G S Y D S K T G T S M A A P Q V A G I C A L I M E W G I I K G N

3201 ATGATCCATATCTATTTGGTCAAAGACAAAAATATTATCTAATAAGCCAGCAAGCAACACGATTAGACGTAGTTTATCCTAATCCATCATGGGGATA 3300
D P Y L F G Q R Q K Y Y L I K P A K R T R L D V V Y P N P S W G Y

3301 TGGACAAGTATGCGNGCTTGATAGTCTAAACTTAATGAAGAGGATTTAAGTGCCATATTAAGTGGGGAATAAGCGATTTTATGATGCCAATGCACCA 3400
G Q V C X L *

ORF1

3401 GTAACAAATCAATATATGATAAATAAATATATATAGAAAAATATTAAGGAGAAAATACGGTGAATATAAACACTAGGCAAACTACAGCTAATGTGG 3500
V N I N T R Q T T A N V G

3501 GAAGATTAAGTTCAATGCTTTAAAGTATGGGTATATCCAGTTGACGGAACCTAAAGTAACTGTAAGAACATCAGTCAATCCGATAATGAAAATAG 3600
R L K V Q C F K S D G Y I P V D G T K V T V R T S V Q S D N V N S

3601 CATTGAATTAGTACAAATGTATCTGGATTAACACAGGAAATAGAGTTGCAAGCACCACCAATAGAATATCCTTAGATGAAAATAGTAATCAAACACCA 3700
I E L V T N V S G L T Q E I E L Q A P P I E Y S L D E N S N Q T P

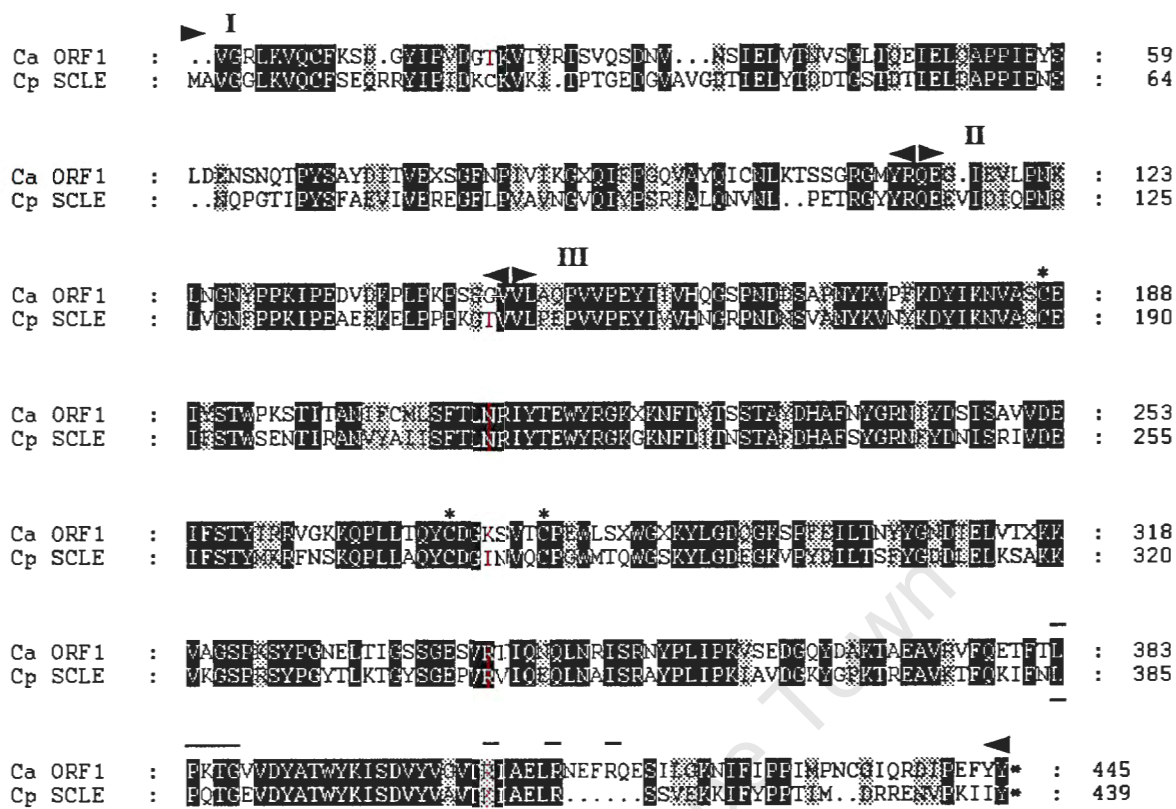


Fig. 5.7. Amino acid homology comparison of ORF1 from *C. acetobutylicum* (Ca ORF1) and the spore-cortex-lytic enzyme of *C. perfringens* (Cp SCLE; Miyata *et al.*, 1995a; accession no. D45024). The two proteins consist of a prepro-sequence (I), a pro-sequence (II) and the mature enzyme (III). The extent of each of these three regions is indicated by arrowheads. Identical amino acids are indicated by a black background and conservative amino acid substitutions are shaded. One or more of the three cysteine residues, indicated by asterisks, are implicated in enzyme activity. The LPXTG motif is underlined and overlined. Three arginine residues which may constitute the charged tail in ORF1 are overlined.

This sequence is capable of forming a stem-loop structure with 1 mismatch ($\Delta G = -12.1$ kcal/mol) and may function as a bidirectional terminator for both ORF1 and the *sum* gene. The deduced 444 amino acid sequence of ORF1 (taken from the second GTG start codon) was highly homologous (60% identity, 74% similarity) to the spore-cortex lytic enzyme (SCLE) of *C. perfringens* (Miyata *et al.*, 1995a) (Fig. 5.7). This enzyme is encoded by the *sleC* gene and is responsible for degradation of spore peptidoglycan during spore germination. Like SCLE, ORF1 is presumably synthesised as an inactive precursor and the deduced amino acid sequence can be divided into three parts: 1) an acidic N-terminal prepro-sequence (113 aa), 2) a hydrophilic pro-sequence (34 aa), and 3) the mature enzyme

Ca ORF2	:	KA	SI	AG	NE	GD	AG	XH	TS	SI	KS	SI	QI	TF	IA	SE	TA	IM	LL	RP	IL	PE	IT	TI	IN	PI	GK	SK	GV	:	347
Cp ORF1	:	KL	TS	SS	SE	EL	IE	PL	TY	GG	KN	LA	VE	NG	IN	IY	EG	TI	:	43								
Ca ORF2	:	IK	IQ	EG	YI	QK	XK	AD	RS	VS	GA	AI	EE	SI	QI	SS	PL	GY	AE	TI	ET	GV	IN	YD	SI	:	412				
Cp ORF1	:	YD	SD	EL	IG	ER	IR	AA	Q	NI	KP	ER	RE	TG	QH	IT	FD	DA	:	89						
Ca ORF2	:	PV	SE	GL	NP	QI	PF	LE	PI	TN	TL	GI	PA	TV	NT	IA	GS	YI	PI	LN	IS	FF	GR	KQ	DS	GR	VR	DE	VA	:	477
Cp ORF1	:	LQ	KE	LL	AE	GI	FL	IS	SP	TT	LI	IP	GA	AK	MI	TV	AS	YN	QEN	TI	LS	SG	RG	YARN	II	QI	TE	AM	:	153	
Ca ORF2	:	PE	EN	IG	PV	EN	SY	DE	KI	TI	MA	EQ	VAG	IA	NE	GI	RG	ND	FY	SG	QK	YI	PA	KR	TL	:	542				
Cp ORF1	:	GE	VA	AT	IN	NK	KE	KV	VS	GE	NA	IV	AG	CC	AL	EG	GI	YN	DE	TL	VA	KL	QY	LI	GV	N	TE	:	217		
Ca ORF2	:	EV	Y	EN	PS	GY	Q	Y	C	X	:	559																		
Cp ORF1	:	ED	Y	EN	RE	GY	ET	DM	EN	V	F	N	S	L	R	T	*	:	243												

Fig. 5.9. Amino acid homology comparison of ORF2 from *C. acetobutylicum* (Ca ORF2) and the truncated *orf1* located upstream of the *sleC* gene of *C. perfringens* (Cp ORF1; Miyata *et al.*, 1995a; accession no. D45024). Identical amino acids are indicated by a black background and conservative amino acid substitutions are shaded.

conserved pentapeptide LPXTG motif, followed by a C-terminal hydrophobic domain and a charged tail, both of which are variable in length and sequence (Schneewind *et al.*, 1993). The charged tail presumably prevents secretion of the protein into the surrounding medium and the LPXTG motif is the site of proteolytic cleavage and cell wall linkage (Navarre and Schneewind, 1994). The nature of the chemical linkage is still to be elucidated. The amino acid sequence following the LPKTG motif of ORF2 is not extensively hydrophobic, but three arginine residues (Arg⁴⁰⁶, Arg⁴¹¹ and Arg⁴¹⁵) may constitute the charged tail.

5.3.5 Sequence analysis of ORF2, a putative serine protease gene

ORF2 is located immediately upstream of ORF1 (Fig. 5.6). ORF2 presumably initiates at the ATG codon at nucleotide 1643 (although there is an alternative TTG start codon 42 bp upstream). A putative SD sequence 5'-AGGTTG-3' precedes the ATG codon, whereas the sequence 5'-ATGTGA-3' 11 bp upstream of the TTG codon would constitute an unconventional ribosome binding site. The stop codon has not been identified for ORF2, but alignment of this ORF with the unidentified truncated *orf1* upstream of *sleC* (Fig. 5.9) indicates that it extends approximately 1700 bp and encodes a protein of roughly 560 amino

acid residues. Analysis of sequences upstream of the presumptive translational start site revealed two sequences similar to σ^A consensus promoter sequences. A 14 bp inverted repeat is located between the two putative promoter sequences and overlaps the ORF2 proximal promoter-like sequence. This inverted repeat has the potential to form a stem-loop structure ($\Delta G = -18.5$ kcal/mol) which may be involved in transcriptional regulation of ORF2.

The ORF2 product displayed 42-56% sequence similarity to a number of serine proteases of *Streptococcus* and *Bacillus*. According to GAP alignment, amino acid sequence comparisons identified three regions within ORF2 with significant homology to the sequences surrounding the active site of serine proteases of the subtilisin family (Fig. 5.10), suggesting that ORF2 encodes a serine protease. The most prominent region of homology spanned 40 amino acid residues at positions 472-511 of the ORF2 sequence and corresponded to a region in subtilisins containing the serine active site. A second region, positions 103-150, showed less extensive, but significant sequence similarity to the sequences surrounding the catalytic asparagine residue of the subtilisins. The reactive Ser²²¹ and Asp³² residues (numbering according to the *B. mesentericus* subtilisin), together with the conserved His⁶⁴ residue, constitute the charge relay system that is essential for enzyme activity (Kraut, 1977). The third region of homology occurred between amino acid residues 253-303 and corresponded to the sequences involved in the formation of the S1 specificity crevice which accommodates the P1 side chain of the protein substrate when it is bound to the enzyme. Amino acids Ser¹²⁵-Leu¹²⁶-Gly¹²⁷ of the *B. mesentericus* subtilisin form one side of the subtilisin pocket, whereas residues Ala¹⁵²-Ala¹⁵³-Gly¹⁵⁴ make up the other side of the crevice. Included in this region is the highly conserved Asn¹⁵⁵ residue which is required for stabilization of the reaction intermediate formed during proteolysis (Kraut, 1977).

5.3.6 Sequence analysis of ORF3, a putative regulatory gene

ORF3 is situated approximately 550 bp upstream of ORF2 (Fig. 5.6). On the basis of amino acid homology comparisons, the GTG codon at position 763 was selected as the most appropriate translation start site for ORF3, rather than the ATG codon at position 811. ORF3 extends 324 bp to the TAA stop codon at position 1084 and encodes a 107 amino acid protein with a predicted molecular weight of ~12 kDa. A potential SD sequence

Ca ORF2	:	YEADAASIA [*] SCIDV [*] ENYK [*] SGKGLIGFVD [*] GGYTHAFM [*] INQG	:	150
Bs Subt	:PLKADK [*] QAQ [*] .E [*] GAIVK [*] GI [*] D [*] IGAA [*] SH [*] TK [*] ..TV	:	45
Bm Subt	:SQK [*] ALH [*] EQ [*] .E [*] TGS [*] V [*] VA [*] D [*] SG [*] S [*] H [*] AN [*] ..VR	:	45
Bs ISP1	:KVL [*] AE [*] MW [*] AK [*] .E [*] V [*] GN [*] IV [*] AL [*] D [*] IGC [*] TH [*] EL [*] KN [*] OII	:	65
Sc Prot	:	YPTDAKALSMAN [*] QAS [*] W [*] ENYK [*] GE [*] IV [*] S [*] LD [*] GG [*] P [*] TH [*] K [*] M [*] R [*] L [*] S [*] SD	:	232
Sp SCP	:	KATIRDLDP [*] SQ [*] K [*] Y [*] Q [*] E [*] K [*] AL [*] .E [*] G [*] AT [*] IV [*] VA [*] VD [*] AG [*] FN [*] HE [*] AW [*] RL [*] TK	:	145
Ca ORF2	:	VSL [*] STNNGAHNGSS [*] LLE [*] QY [*] IRT [*] VEN [*] LERA [*] SI [*] IA [*] GN [*] EDA [*] XH [*] SGI	:	303
Bs Subt	:S [*] GG [*] PS [*] .STAL [*] KQ [*]E [*] RAYA [*] IV [*] VA [*] LAG [*] NS [*] SS [*] ...ONTI	:	164
Bm Subt	:S [*] GG [*] PT [*] .STAL [*] KT [*]V [*] RAYA [*] IV [*] VA [*] LAG [*] NS [*] SS [*] ...STV	:	165
Bs ISP1	:	I [*] S [*] S [*] GG [*] ...DVPE [*] EE [*]K [*] NAV [*] KN [*] VL [*] C [*] AGN [*] ...UG [*] DER [*] EEL	:	190
Sc Prot	:	L [*] S [*] SS [*] SD [*] ...NQT [*] EP [*] ELA [*] Q [*] Q [*] ANE [*] TAA [*] IS [*] AGN [*] SI [*] ...ALEV	:	394
Sp SCP	:S [*] F [*] NAALAYAN [*] DP [*] ET [*] KK [*] AF [*] Y [*] E [*] KS [*] K [*] V [*] SI [*] TS [*] AGN [*] DS [*] F [*] GK [*] R [*] ..	:	304
Ca ORF2	:	RE [*] DLV [*] AP [*] GEN [*] IM [*] GP [*] VE [*] NG [*] SY [*] DS [*] KT [*] GT [*] SM [*] ...FO [*] AG [*] IC [*] LL	:	511
Bs Subt	:	EE [*] VM [*] AP [*] GV [*] SV [*] Y [*] YS [*] NT [*] Y [*] TS [*] NG [*] TSM [*] ...SP [*] AG [*] AA [*] LL	:	233
Bm Subt	:	EE [*] VM [*] AP [*] GV [*] S [*] Q [*] TE [*] LG [*] TY [*] AY [*] NG [*] TSM [*] ...TP [*] AG [*] AA [*] LL	:	234
Bs ISP1	:	IE [*] DLV [*] AP [*] GEN [*] IL [*] EL [*] PK [*] Y [*] GK [*] IG [*] TSM [*] ...PI [*] SG [*] LL	:	259
Sc Prot	:	K [*] PE [*] TA [*] P [*] GG [*] IV [*] ST [*] Q [*] N [*] NG [*] Y [*] TN [*] MS [*] GT [*] SM [*] ...SP [*] FI [*] GS [*] QL	:	633
Sp SCP	:	K [*] PE [*] TA [*] P [*] GQ [*] IL [*] SV [*] AN [*] K [*] Y [*] AK [*] SG [*] TSM [*] ...PL [*] AG [*] IM [*] GL	:	525

Fig. 5.10. Sequence homology at the active site among streptococcal and *Bacillus* serine proteases compared with the homologous regions of ORF2 from *C. acetobutylicum* P262 (Ca ORF2). The proteases are as follows: subtilisin from *B. subtilis* (Bs Subt; Nedkov *et al.*, 1986; accession no. P00781), subtilisin from *Bacillus mesentericus* (Bm Subt; Svendsen *et al.*, 1986; accession no. P07518), the major intracellular serine protease from *B. subtilis* (Bs ISP1, Koide *et al.*, 1986; accession no. P11018), a wall-associated protease from *Streptococcus cremoris* (Sc Prot; Kok *et al.*, 1988; accession no. P16271), and the C5a peptidase from *Streptococcus pyogenes* (Sp SCP; Chen and Cleary, 1990; accession no. P15926). Asparagine and serine residues of the catalytic triad are marked with asterisks. Residues that constitute the S1 specificity crevice of subtilisin are overlined and the asparagine residue that is important for stabilizing the reaction intermediate during proteolysis is indicated by #. Identical amino acids for all six sequences are indicated by a black background. Identical amino acids for two sequences and three to five sequences are indicated by light and dark shading, respectively. Adapted from Chen and Cleary, 1990.

5'-AAGGGG-3' is located 10 bp from the GTG start codon. No typical conserved sequences for promoters could be identified upstream of the coding sequence. Although there is no inverted repeat sequence downstream of ORF3 which can be implicated in transcription termination, two 25 bp repeats located within the C-terminal end of the coding sequence may be of some significance. These repeats are positioned 80 bp apart with one of them overlapping the TAA stop codon.

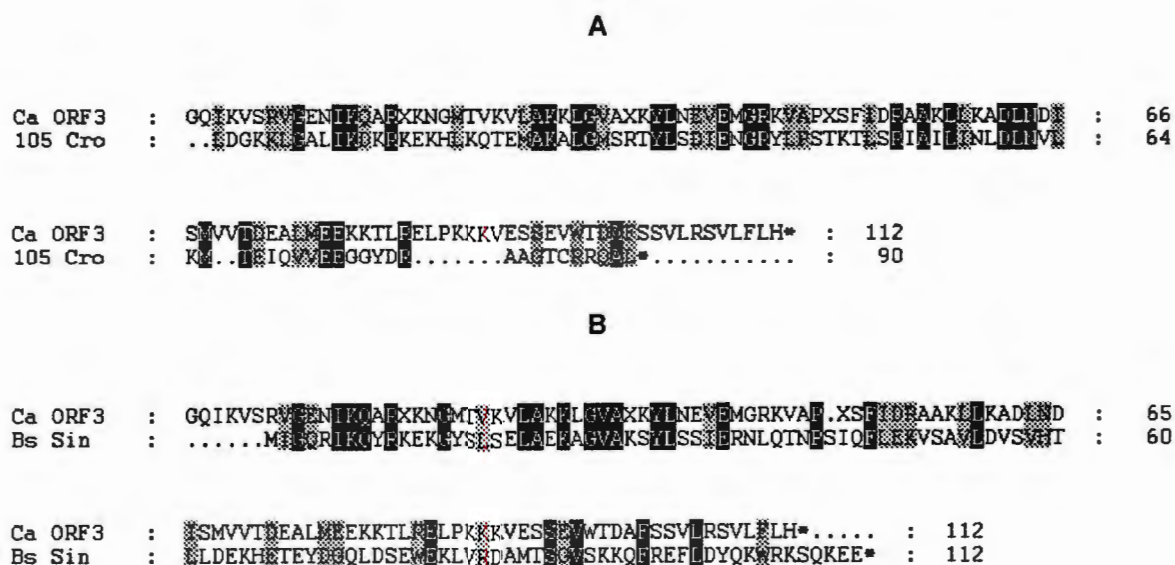


Fig. 5.11. Amino acid homology comparison between ORF3 from *C. acetobutylicum* (Ca ORF3) and the bacteriophage ϕ 105 Cro-like protein (105 Cro; Van Kaer *et al.*, 1987; accession no. P13772) (A) and Sin of *B. subtilis* (Bs Sin; Gaur *et al.*, 1986; accession no. P06533) (B). Identical amino acids are indicated by a black background and conservative amino acid substitutions are shaded.

The deduced amino acid sequence of ORF3 displayed homology to a variety of bacteriophage and bacterial regulatory proteins. Using the BLASTP program (Altschul *et al.*, 1990), the highest segment pairing score was obtained against an 89 amino acid polypeptide located in the *Eco*RI-F immunity control region (*immF*) of the *B. subtilis* phage ϕ 105 (Van Kaer *et al.*, 1987). This protein is the topological equivalent of λ Cro. GAP alignment revealed 29% identity and 52% similarity between this putative regulator of ϕ 105 lysogeny and ORF3 (Fig. 11A). The second most significant match for ORF3 was obtained against the Sin protein of *B. subtilis* (Gaur *et al.*, 1986) and *B. licheniformis* (Sekiguchi *et al.*, 1990). Sin is both a negative and positive regulator of many late growth developmental processes in *Bacillus*. GAP alignment indicated that ORF3 shares 22% identity and 39% similarity with the Sin protein of *B. subtilis* (Fig. 11B).

GAP alignment of the amino acid sequence of ORF3 with those of other regulatory proteins such the *Pvu*II restriction endonuclease regulator of *Proteus vulgaris* (Tao *et al.*, 1991), a putative regulator of the fructosyltransferase gene of *Streptococcus mutans* (Shiroza and

Common to the N-terminus of ORF3 and these regulatory proteins is a stretch of 20 amino acids with sequence consensus to the helix-turn-helix motif of DNA-binding proteins (Pabo and Sauer, 1984). Amino acid sequence comparison revealed a number of highly conserved residues in this region (Fig. 5.12). Chou-Fasman analysis of the amino acid sequence of ORF3, predicted that this region contained two alpha helices connected by a turn at the conserved glycine residue (Chou and Fasman, 1978). Furthermore, the predicted amino acid composition of ORF3 is rich in charged amino acids (33%); this is a feature of RNA polymerase sigma subunit proteins (Burton *et al.*, 1981) and presumably other DNA-binding proteins.

5.3.7 Sequence analysis of ORF4, a hypothetical gene and ORF5, a putative peptidyl-prolyl-isomerase gene

ORF4 is located approximately 50 bp upstream of ORF3 and consists of a coding sequence of 312 bp, starting at an ATG codon at position 398 and ending in a TAA codon at position 707 (Fig. 5.6). The ATG codon is preceded by a potential SD sequence 5'-AAGAGG-3' and a σ^E consensus promoter sequence (Rather *et al.*, 1986). No inverted repeat sequence was identified downstream of ORF4. The deduced amino acid sequence of ORF4 contains 103 amino acid residues with a predicted molecular weight of ~12 kDa. No obvious sequence similarity was detected between the ORF4 product and any of the protein sequences available in the protein databases.

Situated approximately 130 bp upstream of ORF4 is an incomplete open reading frame (267 bp in length) which lacks an initiation codon and is designated ORF5. No inverted repeat sequences were identified downstream of the TGA stop codon of ORF5. The predicted 88 amino acid sequence of ORF5 displayed strong homology with the corresponding sequences for the peptidyl-prolyl-isomerases (PPIases) of e.g. *E. coli* (Tran *et al.*, 1990) and *B. subtilis* (Sorokin *et al.*, 1993) (Fig. 5.13). Like molecular chaperones, the highly conserved PPIases are involved in protein folding, can be induced by shock treatments, are ubiquitous in prokaryotic and eukaryotic cells, and have been found associated with various signalling molecules e.g. receptors, kinases and transcription factors (Rutherford and Zuker, 1994). It has been suggested that chaperones and PPIases, as members of the cellular protein folding machinery, promote structural changes required for

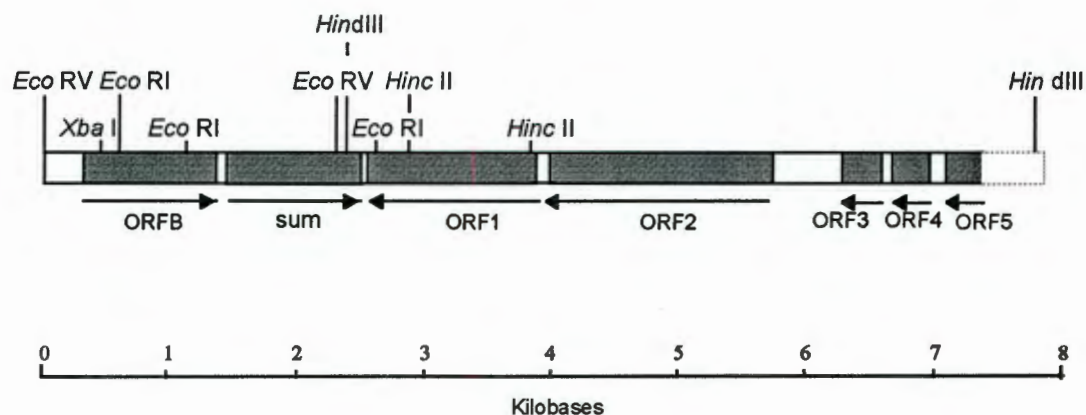


Fig. 5.14. Physical map of the complete *C. acetobutylicum* DNA region sequenced (described in Chapter 4 and this chapter). Arrows indicate the direction of transcription of the genes.

gene is independently transcribed. ORFB encoded a ~350 amino-acid protein with a predicted molecular weight of ~40 kDa. The ORFB product shared sequence similarity with an unidentified 38 kDa protein of *B. subtilis*, the coding sequence of which is located in the replication origin of the chromosome. The deduced amino acid sequences of ORFB and its presumptive *B. subtilis* homologue are extensively hydrophobic. The hydropathy profile of ORFB predicted the conservation of eight hydrophobic regions that span the cytoplasmic membrane, and the *B. subtilis* homologue shares this transmembrane topology. It is worth mentioning that an unidentified ORF encoding a 224 amino acid hydrophobic protein is located upstream of the *pfl* gene (encoding the pyruvate formate-lyase) in *E. coli* (Sawers and Böck, 1989). Possible functions of this putative membrane-bound protein are formate extrusion, pyruvate transport or environmental signal transduction (Sawers and Böck, 1989).

Five ORFs were identified downstream of the *sum* gene. These ORFs are all convergently transcribed relative to *sum* and constitute an interesting stationary phase gene cluster. The predicted amino acid sequence of ORF1, the *sum* proximal ORF, displayed a high degree of homology to the spore cortex lytic enzyme (SCLE) of *C. pasteurianum* (Miyata *et al.*, 1995a). Cortex hydrolysis is considered to be a key event in spore germination (Foster and Johnstone, 1990). Although no promoter-like sequence was identified upstream of ORF1, a

σ^G promoter-like sequence precedes the *C. perfringens sleC* gene which indicates that SCLE is produced in the forespore. The LPXTG motif identified in the ORF1 and SCLE proteins corresponds to the signature sequence for cell wall sorting signals of Gram-positive bacteria. The complete signal for cell wall anchoring consists of the conserved pentapeptide motif, a C-terminal hydrophobic domain and a charged tail. The sequence following the LPKTG motif of the ORF1 product is only moderately hydrophobic, compared to homologous sequences identified in many Gram-positive surface proteins. However, the degree of hydrophobicity required in the C-terminal hydrophobic domain, in order for cell wall sorting to occur, has not been established. What is important, is the spacing between the LPXTG motif and the charged tail; the first positively charged amino acid is positioned 28-31 residues from the leucine residue of the LPXTG motif (Schneewind *et al.*, 1993). In the ORF1 product, the first arginine (R) residue of the positively charged tail is located 28 residues from the start of the motif. Navarre and Schneewind, (1994) have demonstrated that cleavage occurs between the threonine (T) and glycine (G) residues of the pentapeptide motif and propose a model for cell wall linkage similar to the penicillin-sensitive transpeptidation reaction of bacterial cell wall synthesis. Schneewind *et al.* (1993) have suggested that the cell wall anchoring signals of Gram-positive bacteria may be as common as the signal sequences for protein export. Nevertheless, this appears to be the first example of such a sequence identified in *Clostridium*.

SCLE probably occurs in three forms: 1) an inactive 50 kDa precursor with a N-terminal prepro-region of 113 amino acid residues which directs the inactive enzyme to an appropriate site in the dormant spore; 2) an inactive 36 kDa proenzyme which is non-covalently attached to the outside of the cortex layer; and 3) a mature 31 kDa enzyme (Miyata *et al.*, 1995a). Presumably the pro-enzyme (36 kDa) is activated during germination by proteolytic cleavage between Val¹⁴⁹ and Val¹⁵⁰ (numbering according to ORF1 of *C. acetobutylicum*) to release the 34 amino acid pro-sequence. This is similar to the activation mechanism proposed for the spore cortex-lytic enzyme of *B. megaterium* (Foster and Johnstone, 1990).

ORF2 may very well encode the SCLE activating protease. The deduced amino acid sequence of ORF2 (~ 560 amino acids) was homologous to the unidentified incomplete *orf1*

upstream of *sleC* (Miyata *et al.*, 1995a), and displayed 42-56% sequence similarity to the serine proteases of *Streptococcus* and *Bacillus*. Homology did not extend over the entire length of the polypeptide, but was confined to three regions. These included the two sequences surrounding the reactive Asp³² and Ser²²¹ residues of the catalytic centre of subtilisins (numbering according to the *B. mesentericus* subtilisin), as well as the sequence spanning the S1 specificity crevice. Like the cell envelope-located proteases PI, PII and PIII of *Lactococcus lactis* (Kok *et al.*, 1988; Kiwaki *et al.*, 1989), ORF2 lacks the histidine active site signature of subtilisins. According to the information on serine proteases provided in the MOTIFS program, the presence of at least two of the three active site signatures in a given protein guarantees its classification as a serine protease. On the basis of this definition, ORF2 apparently specifies such an enzyme. One of the two vegetative promoter-like sequences identified upstream of ORF2 is located in a region with possible stem-loop structure which may constitute the binding site for a regulatory protein. Similar regions of dyad symmetry have been identified upstream of the genes encoding the *S. cremoris* proteinase (Kok *et al.*, 1988), the *B. subtilis* subtilisin E protease (Wong *et al.*, 1984) and the streptococcal C5a peptidase (Chen and Cleary, 1990).

Presumably, ORF2 is subject to regulation by ORF3. The ORF3 product displayed homology to a number of bacteriophage and bacterial regulatory proteins. Sequence similarity was most highly conserved at the N-terminus, within a 20 amino acid sequence conforming to the helix-turn-helix configuration of DNA-binding proteins. The Cro-like protein in the immunity control region (*immF*) of *B. subtilis* phage ϕ 105 (Van Kaer *et al.*, 1987) and Sin, a dual-function regulatory protein of *B. subtilis*, produced the highest-scoring matches with ORF3. The ϕ 105 *immF* region consists of the ϕ 105 repressor gene and the Cro-like gene, under control of the P_m and P_r promoters, respectively. P_m directs transcription of the ϕ 105 repressor gene, whereas P_r presumably initiates the lytic pathway. Differential regulation of these two divergent genes is mediated by recognition of a 14 bp operator sequence which occurs as three direct repeats (Van Kaer *et al.*, 1987). Two copies of this operator sequence are juxtaposed in the non-transcribed region between the respective promoters, the third copy is located at the 3' end of the Cro-like gene. It is interesting that homologues of the ϕ 105 immunity repressor and the ϕ 105 Cro-like protein (displaying 20% and 35% identity, respectively) have been identified in the *B. subtilis*

genome on the *skin* element (Takemaru *et al.*, 1995) which is presumed to be a cryptic remnant of an ancestral temperate phage.

The 111 amino acid sequence of Sin predicts a leucine zipper dimerization motif, flanked by two helix-turn-helix motifs (Gaur *et al.*, 1991). Sin is a transition-state regulator - a term given to a group of proteins which controls the expression of functions associated with the transition between exponential growth and the stationary phase in *B. subtilis* (Perego *et al.*, 1988; Strauch and Hoch, 1993). These functions include the development of competence, motility and sporulation, and the production of antibiotics and degradative enzymes such as subtilisin - all of which are adaptive responses to ensure survival of the cell in a nutrient-depleted environment. The role of transition state regulators is to prevent inappropriate and potentially detrimental expression of these functions during active growth. Sin is only one of four transition state regulators (AbrB, Hpr, Pai, Sin) which inhibits subtilisin/extracellular alkaline protease (*aprE*) gene transcription during vegetative growth (Honjo *et al.*, 1990). Binding of Sin to the *aprE* gene has been demonstrated to occur in a region more than 200 bp upstream from the transcription start site (Gaur *et al.*, 1991).

The large 550 bp intergenic region between ORF2 and ORF3 suggests that like *aprE*, ORF2 (encoding the putative protease) might be regulated by a number of proteins. ORF3 is obviously not a Sin homologue since it lacks the structural leucine zipper motif and the C-terminal helix-turn-helix motif predicted from the Sin amino acid sequence, but on the basis of amino acid homology with the N-terminal domain of Sin, it is highly likely that ORF3 regulates the activity of the putative protease. Like Sin, ORF3 may also possess additional regulatory functions.

The *sin* gene is part of a dicistronic operon (Gaur *et al.*, 1986). The first gene of the operon, *sinI*, encodes a protein of 57 amino acids (Isin) which regulates Sin activity. Without transcription analysis, speculation is limited, but it is possible that ORF3 and the unidentified ORF4 (104 amino acids) constitute a dicistronic operon. No transcription and translation initiation signals were identified upstream of ORF3, whereas putative SD and σ^E promoter consensus sequences were identified upstream of ORF4. Perhaps ORF4 controls ORF3 activity and both these genes are under developmental control? Furthermore, ORF5,

the putative PPIase, might be involved in protein maturation or export of any of one these proteins.

Although the identification of the spore cortex-lytic enzyme homologue, complete with putative proteolytic processing enzyme and regulatory gene region in itself is interesting, there is no obvious connection with the *sum* gene. ORFB, because of its location relative to the *sum* gene, is more likely to be associated with *sum* function. Unfortunately, the ORFB product, a putative membrane protein, is homologous to an, as yet, unidentified protein in *B. subtilis*. It is interesting that except for this membrane-binding protein, the physical organization of the *sum* gene region does not reflect the arrangement of the PFL-activase gene regions in *E. coli* nor *T. litoralis*. Detailed physiological characterization of *C. acetobutylicum* mutant 3R was therefore undertaken to further investigate the function of *sum*.

Chapter 6

Physiological characterization of *C. acetobutylicum* P262 mutant 3R and effects of the *sum* gene cloned on a multicopy vector in *C. acetobutylicum* NCIMB 8052

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Chapter 6

Physiological characterization of *C. acetobutylicum* P262 mutant 3R and effects of the *sum* gene cloned on a multicopy vector in *C. acetobutylicum* NCIMB 8052

6.0 Summary

Physiological examination of *C. acetobutylicum* P262 mutant 3R revealed that inactivation of the *sum* gene in the chromosome resulted in a pleiotropic phenotype. Relative to the *C. acetobutylicum* P262 wild type, vegetative cells of mutant 3R exhibited increased autolysin activity and two characteristics often associated with an autolytic phenotype, namely an increased rate of cell division and enhanced motility. In addition, mutant 3R showed a substantial reduction in clostridial stage formation, a moderate decrease in solvent production, complete resistance to the toxic tripeptide analogue, bialaphos, and was defective in sporulation. The counterpart of the *sum* gene which encodes a putative PFL-activating enzyme, was detected in *C. acetobutylicum* NCIMB 8052 by low-stringency Southern hybridization. Attempts to generate a *sum*-homologue disruption mutant via homologous recombination in *C. acetobutylicum* NCIMB 8052 were unsuccessful. However, the *sum* gene was introduced into *C. acetobutylicum* NCIMB 8052 by electroporation using the *B. subtilis*/*C. acetobutylicum* shuttle vector, pFNK1. The presence of *sum* in multiple copies inhibited cell division and autolysin activity in the host strain.

6.1 Introduction

As described in Chapters 2-4, conjugative transposon mutagenesis and the metronidazole resistance selection system were used to target and isolate a gene implicated in carbon metabolism, namely *sum*, a putative PFL-activase homologue. Transposon mutagenesis is not only useful as a cloning procedure, however, but is also important for the investigation of metabolic pathways and the analysis of gene function and regulation. Presumably, altered carbon metabolism in mutant 3R, due to disruption of the formate-producing pathway, would not only result in a change in end product ratios, but would also be reflected in the growth patterns and morphology of the mutant.

Ideally, the effects of *sum* gene disruption should be confirmed by reconstituting the wild type phenotype in mutant 3R via introduction of an intact gene copy. An alternative approach is to generate an equivalent mutant by targeted integration of the *sum* gene in *C. acetobutylicum* P262. At this stage, however, integrational plasmid technology is not applicable to the P262 strain, and it lacks a system for gene transfer. Furthermore, due to stability of the Tn925::Tn917 insertion (see Chapter 2), the wild type phenotype could not be restored in mutant 3R by induction of transposon excision. *C. acetobutylicum* NCIMB 8052 was therefore chosen as an alternative host for this aspect of the study. Genes have been transferred to this species by electroporation (Oultram *et al.*, 1988), conjugation (Williams *et al.*, 1990) and homologous recombination (Wilkinson and Young, 1994). Before attempting targeted integration of the *sum* gene in a heterologous host, it was first necessary to confirm the presence of the *sum* gene homologue in *C. acetobutylicum* NCIMB 8052.

This chapter details the physiological effects of the *sum* mutation in *C. acetobutylicum* P262 mutant 3R and reveals the pleiotropic nature of the mutant. The other metronidazole resistant transconjugant, mutant 30R (described in Chapter 2) was included for purposes of comparison. Targeted integration of the *sum* gene in *C. acetobutylicum* NCIMB 8052 was attempted, and multicopy effects of *sum* and ORF3, a putative regulatory gene within the neighbouring stationary phase gene cluster (described in Chapter 5), were analysed in *C. acetobutylicum* NCIMB 8052.

6.2 Materials and methods

6.2.1 Bacterial strains and plasmids

The *C. acetobutylicum* P262 wild type strain (Jones *et al.*, 1982), mutants 3R and 30R, and *C. acetobutylicum* NCIMB 8052 (laboratory stock) were used in these studies. *E. coli* JM105 and JM109 (Yanisch-Perron *et al.*, 1985) were used interchangeably as intermediate hosts for integrational plasmid constructs, and *B. subtilis* 1A46 (BGSC, Ohio State University) served as the intermediate host for pFNK1-based DNA constructs. *E. coli* CA448, containing the heat-sensitive λ prophage and the kanamycin resistant IncP plasmid R702 with transfer functions, was obtained from S. Wilkinson and M. Young, (Institute of Biological Sciences, University of Wales). This strain served as the conjugal donor for plasmid pCTC1 and integrational plasmid pMTL30 derivatives. Plasmid pCTC1 and plasmid pMTL30 (Appendix D) both contain mobilization functions and ampicillin and erythromycin resistance markers (Williams *et al.*, 1990). Plasmid pMTL30 contains the *lacZ'* region and lacks a Gram-positive origin of replication. The *B. subtilis/C. acetobutylicum* shuttle vector pFNK1 (Appendix D) contains the MLS resistance marker and the pIM13 origin of replication (Mermelstein *et al.*, 1992) and has a moderate copy number of eight in *C. acetobutylicum* ATCC 824 (Lee *et al.*, 1993).

6.2.2 Media and growth conditions

C. acetobutylicum strains were grown anaerobically at 37°C in CBM or 2xYT broth supplemented with 1% glucose. Tryptone yeast glucose medium (TYGM) (Hongo *et al.*, 1968), containing 6% glucose was used for batch fermentation analysis of *C. acetobutylicum* P262 and NCIMB 8052 strains. (TYGM proved to be a suitable medium for non-degenerate growth of *C. acetobutylicum* NCIMB 8052). *E. coli* and *B. subtilis* were grown aerobically at 37°C in 2xYT medium. For the conjugation procedure, the *E. coli* CA448 donor was grown aerobically in brain heart infusion broth (BHIB) (Difco) at 30°C, and the *C. acetobutylicum-E. coli* conjugation mixture was plated on reinforced clostridial medium (RCM) (Difco) and incubated at 42°C. Media were supplemented with antibiotics where appropriate in the following concentrations: ampicillin (100 $\mu\text{g/ml}$), kanamycin (50 $\mu\text{g/ml}$) and erythromycin 10 and 2 $\mu\text{g/ml}$ for *C. acetobutylicum* and *B. subtilis*, respectively.

6.2.3 Growth and morphology

A Zeiss photomicroscope fitted with phase- and interference-contrast optics was used to

visualise cells. Total cell numbers were determined using a Thoma counting chamber (Weber Scientific International, UK). Cells were examined for the presence of granules, and flagella by staining with iodine and Gray's flagella stain, respectively (Doetsch, 1981). Motile cell numbers were estimated as a percentage of the sample population. Viable cells were quantified by plating for colony forming units (cfu).

6.2.4 End product analysis

The production of acetate, butyrate, acetone, butanol and ethanol during the course of the batch fermentation was measured by gas chromatography, using a Hewlett Packard 5880A gas chromatograph fitted with a flame ionisation detector. A glass column (1.86 m x 4 mm) packed with 10% diethylene glycol adipate on 80-100 mesh chromosorb-W-HP (Supelco) was used with 2-propanol (isopropanol) as an internal standard. The column temperature was a linear gradient from 60 to 180°C at 20°C per min and the detector and injector temperatures were set at 300 and 250°C, respectively. The nitrogen carrier gas was set at a flow rate of 30 ml/min.

Lactate and formate concentrations were not measured, since database information identifying the *sum* gene as a putative PFL-activase, only became available at the conclusion of this work. Formate cannot be detected by gas chromatography on the column that was used (Supelco, Inc., Buletin 748F), and lactate is detected by gas chromatography only as a methylated derivative (Smibert and Krieg, 1981).

6.2.5 Bialaphos susceptibility assay

Cultures (100 µl) in early exponential growth phase were spread onto an agar plate. Bialaphos (130 µg) was spotted onto a square piece of filter paper placed in the centre of the plate. Plates were examined for zones of inhibition after overnight incubation. Bialaphos was obtained from M. Spencer (Discovery Research Plant Genetics, DEKALB, Connecticut) and had been purified from Herbiace (Meiji Seika Kaisha, Ltd.).

6.2.6 Autolysis assay

Cellular autolysis of vegetative cells was determined according to Allcock *et al.* (1981). Cells (10 ml) in mid-exponential growth phase were harvested by membrane filtration or centrifugation, washed once with distilled water and resuspended in 3 to 8 ml of 0.04 M

sodium phosphate buffer, pH 6.3. Autolysis at 37°C was monitored turbidimetrically at OD₆₀₀. One unit of cellular autolysis was defined as the loss of 0.001 optical density unit per h. To compare the autolysis of different samples, results are expressed in units of autolysis per 1.0 OD unit of cell suspension. One unit is then equivalent to a loss of 1% of the initial turbidity per h.

6.2.7 DNA isolation

C. acetobutylicum chromosomal DNA was prepared by the method of Marmur (1961) as modified by Zappe *et al.* (1986). Plasmid DNA was extracted from *C. acetobutylicum* NCIMB 8052 according to Mermelstein *et al.* (1992), from *B. subtilis* according to Errington (1990) and from *E. coli* according to Ish-Horowicz and Burke (1981).

6.2.8 PCR

Complete and internal ORF3 DNA fragments were isolated from plasmid pDO1 (see Chapter 5) by PCR amplification. The reaction mixtures (100 µl) contained template DNA (10 ng), primers (200 ng each), dNTPs (25 µM each), 50 mM KCl, 10 mM Tris-HCl pH 7.5, 1.5 mM MgCl₂ and Taq DNA polymerase (Promega; 1 U), overlaid with mineral oil. The samples were submitted to 30 amplification cycles consisting of a 1 min denaturation period at 93°C, a 1 min annealing period at 42°C, and a 1 min extension period at 72°C. The synthetic oligonucleotides primers 5'-AGCTGCAGAAAAAATGGAATGAC-3' (with 2 mismatches to generate a *Pst*I restriction site) and 5'-TCTAGGCCTCCATTAATGCCTC-3' (with 3 mismatches to generate a *Stu*I restriction site) were used for amplification of a 0.2 kb DNA fragment internal to ORF3. Primers 5'-GAGAATTCGCTGTAGTTACAG-3' (with 2 mismatches to generate an *Eco*RI restriction site) and 5'-TTCTGCAGTGTGCGCAAATGC-3' (with 2 mismatches to generate a *Pst*I restriction site) were used for amplification of a 0.6 kb DNA fragment containing the complete ORF3 gene region. The restriction sites in the primer sequences are underlined. The primers were synthesised by E. Botes (Department of Biochemistry, University of Cape Town).

6.2.9. Southern hybridization

Southern hybridization was performed as described in Chapter 2, Section 2.2.9 but low stringency conditions were used. Hybridization and washes were performed at 50°C and SDS concentrations were reduced to 0.01% in wash solutions.

6.2.10 Bacterial transformation

Integrational plasmids were transformed into *E. coli* strain CA448 by the conventional CaCl_2 method, but heat-shock was performed at 30°C to prevent induction of the lambda prophage harboured by this strain. Plasmid pFNK1-based constructs were initially established in *B. subtilis* which was transformed by the protoplast procedure described by Errington (1990). Electroporation of *C. acetobutylicum* NCIMB 8052 was performed according to Oultram *et al.* (1988). Cells (100ml) were harvested at $\text{OD}_{600} = 0.6$, washed, and resuspended in 5 ml of ice cold electroporation buffer (270 mM sucrose, 1 mM MgCl_2 , 7 mM NaHPO_4 , pH 7.4). Cell samples (0.8 ml) containing plasmid DNA (0.5 μg) were subjected to a 2.0 kV pulse using a Bio-Rad Gene Pulser™ set at maximum capacitance (25 μF), giving time constants between 3.8 and 6.9 ms. The cells were diluted in 10 ml 2xYT broth containing glucose (1%) and incubated at 37°C for 2 h prior to plating on selective medium.

6.2.11 Conjugation procedure

Conjugative mobilization of pCTC1 and pMTL30-based integrational plasmids from *E. coli* CA448 to *C. acetobutylicum* NCIMB 8052 was performed as described by Williams *et al.* (1990) and Wilkinson and Young (1994). Donor strains of *E. coli* were grown aerobically overnight at 30°C in BHIB containing kanamycin (50 $\mu\text{g}/\text{ml}$) and ampicillin (100 $\mu\text{g}/\text{ml}$). The *C. acetobutylicum* recipient was grown to an OD_{600} of 2.7. Donor cultures (200 ml) and recipient cultures (20 ml) were mixed and pelleted by centrifugation, resuspended in 2 ml CBM and plated on RCM. Mating bacteria were incubated at 30°C overnight. The following day, bacterial growth on each plate was resuspended in 0.7 ml CBM and subsequently plated onto selective medium. Transconjugants were selected anaerobically at 42°C on CBM containing erythromycin (10 $\mu\text{g}/\text{ml}$). Donor and recipient cell numbers were obtained via counterselection by aerobic incubation on 2xYT medium at 30°C and anaerobic incubation on CBM at 42°C, respectively. Frequencies of plasmid transfer were expressed as the number of transconjugants per recipient at the end of the mating period.

6.3 Results

6.3.1 Growth profiles

Synchronous batch fermentations of *C. acetobutylicum* wild type, mutant 3R and mutant 30R in TYGM were monitored over a 58 h period. Exponential-phase CBM cultures were used to inoculate TYGM, resulting in an initial cell concentration of approximately 10^4 cells/ml and hence a very short lag period for all three cultures (Fig. 6.1). During the exponential growth phase (3 to 10 h), the mutant 3R culture displayed a shorter doubling time compared to the wild type and mutant 30R cultures. This indicated that in the initial growth phase, mutant 3R cells underwent an increased rate of cell division. In the stationary growth phase, total cell numbers of the wild type and mutants 3R and 30R stabilized at 10^9 cells/ml. After 34 h, however, the growth curves for mutants 3R and 30R showed a steady decrease in viable cell numbers, resulting in a loss of 3 to 4 log units of cell viability at the end of the batch fermentation. The mutant 3R growth profile was therefore characterized by an increased doubling time in the exponential growth phase and loss of cell viability in the mid-late stationary growth phase.

6.3.2 Morphology

The sequence of morphological changes during the course of the batch fermentation was monitored for the wild type, mutant 3R and mutant 30R cultures and is represented in Fig. 6.2 and Fig. 6.3. The exponential growth phase of the wild type was characterized by highly motile phase-dark vegetative cells. Mutant 3R cells displayed enhanced motility (higher percentage motile cells and more frenetic movement) relative to the wild type, whereas mutant 30R cells were completely non-motile. In addition, mutant 3R vegetative cells were shorter and smaller than wild type cells. Cessation of active growth at about 10 h corresponded with a decrease in motility in the wild type. However, motility was prolonged in 50% of the cell population of mutant 3R (Fig. 6.2). Persistence of peritrichous flagella in mutant 3R relative to the wild type is illustrated in Fig. 6.3 A, B. Only a small percentage of mutant 3R and 30R cells developed into phase-bright clostridial forms, indicating that the differentiation process was impaired (Fig. 6.2 and Fig. 6.3 C, D). Furthermore, mutant 3R clostridial stage cells appeared to undergo degradative changes: clostridial forms were misshapen and often curved, with irregular walls (Fig. 6.3 E, F). The interior of these cells had a "beaded" or striped appearance which is indicative of an autolytic phenotype.

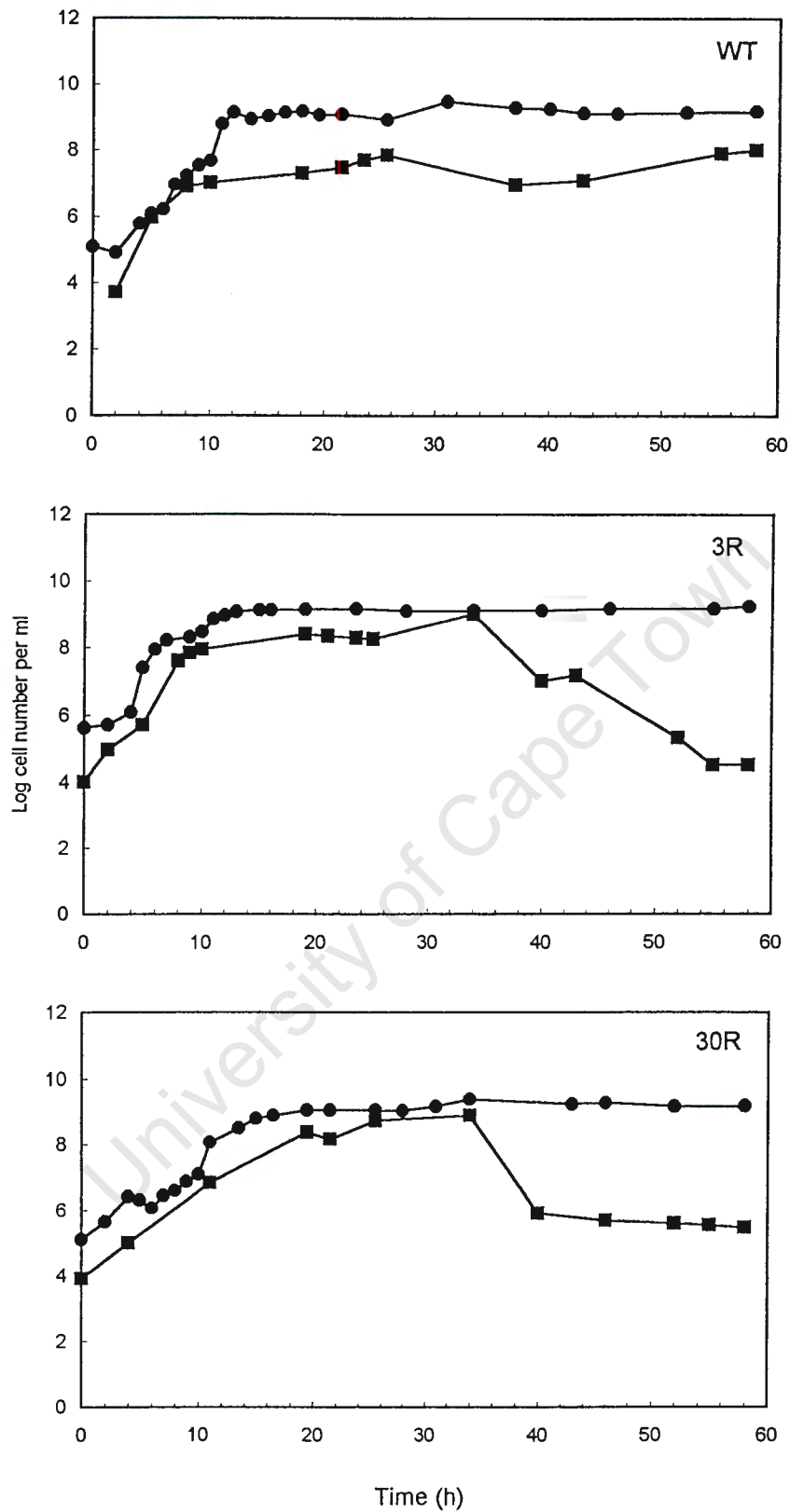


Fig. 6.1. Growth profiles of the *C. acetobutylicum* P262 wild (WT), mutant 3R (3R) and mutant 30R (30R) cultures in TYGM. Total cell numbers (●) are compared with viable cell numbers (cfu/ml) (■).

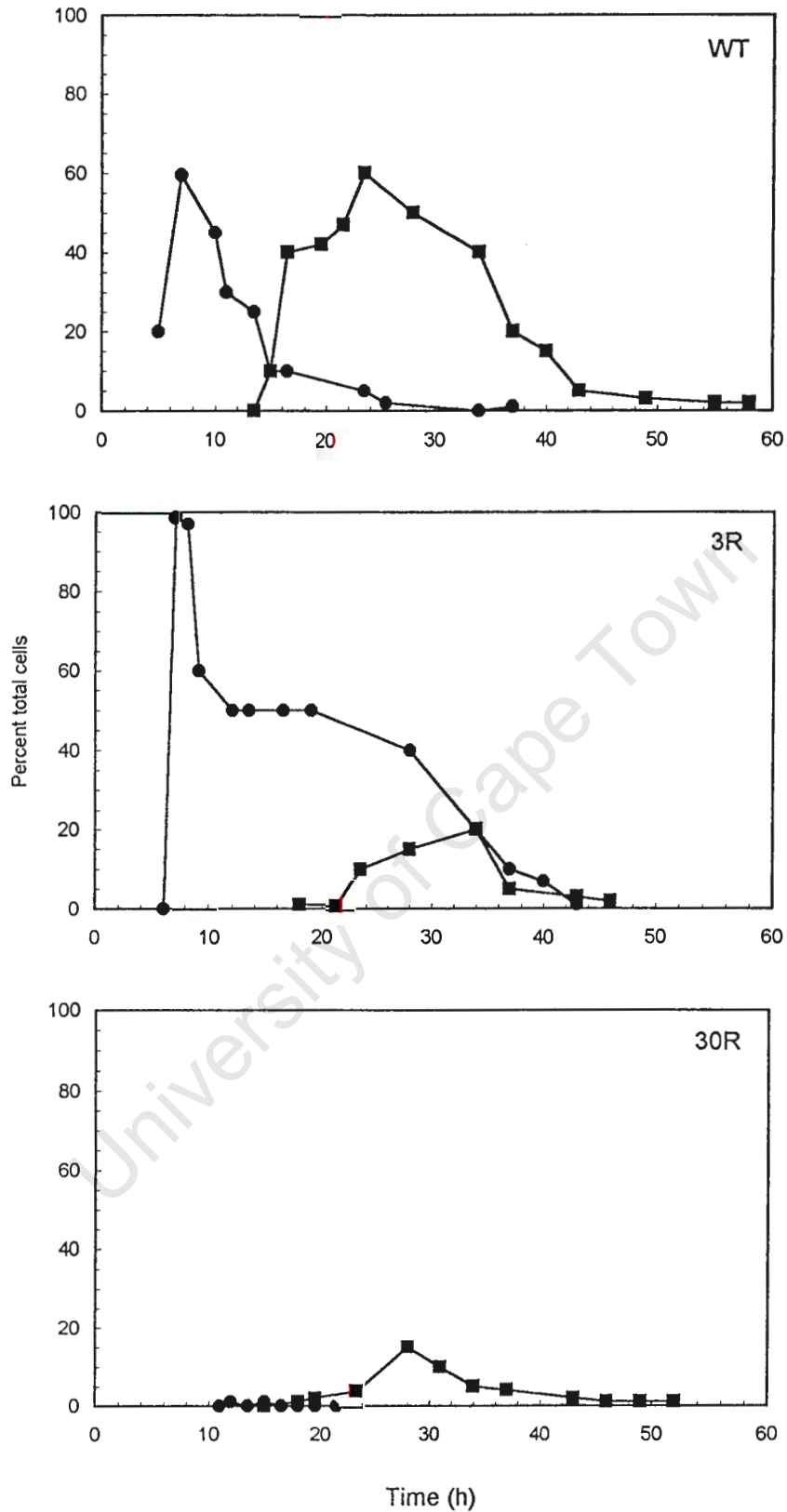
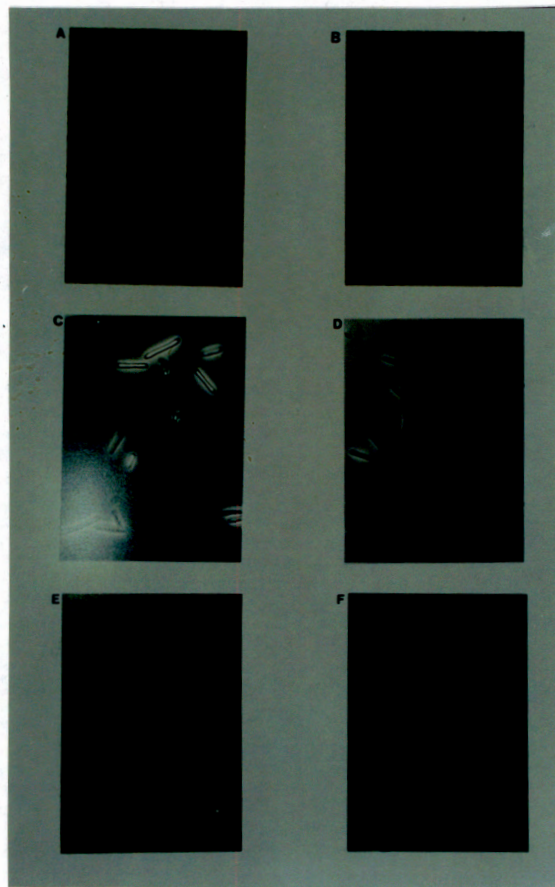


Fig. 6.2. Motile (●) and clostridial cell (■) numbers of *C. acetobutylicum* P262 wild (WT), mutant 3R (3R) and mutant 30R (30R) estimated a percentage of the total cell population.



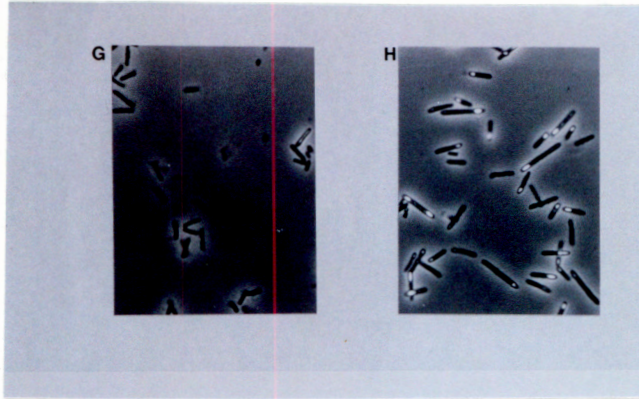


Fig. 6.3. (See previous page also) Comparison of *C. acetobutylicum* P262 wild type (A, C, E, G) and mutant 3R (B, D, F, H) cells during the following stages in growth: early exponential phase (A, B), early stationary phase (C, D), mid stationary phase (E, F) and late stationary phase (G, H).

Copious amounts of polysaccharide material were visible as an aggregate in the wild type culture, but this polysaccharide was virtually absent in the mutant 3R and 30R cultures. The synthesis of a clearly defined extracellular capsule is associated with the development of vegetative rods into clostridial forms. Wild type clostridial forms developed phase-dark forespores (stage II) which proceeded through the developmental sequence, culminating in the production of mature spores (Fig. 6.3 E, G). Only a small percentage of mutant 3R and mutant 30R cells developed forespores (Fig. 6.3 F). These developed into mature spores in mutant 30R which was therefore classified as oligosporogenous. In mutant 3R, however, forespore development generally did not proceed beyond spore engulfment (stage III). Oligospores were non-viable and for this reason the mutant was classified as asporogenous (Fig. 6.3 G).

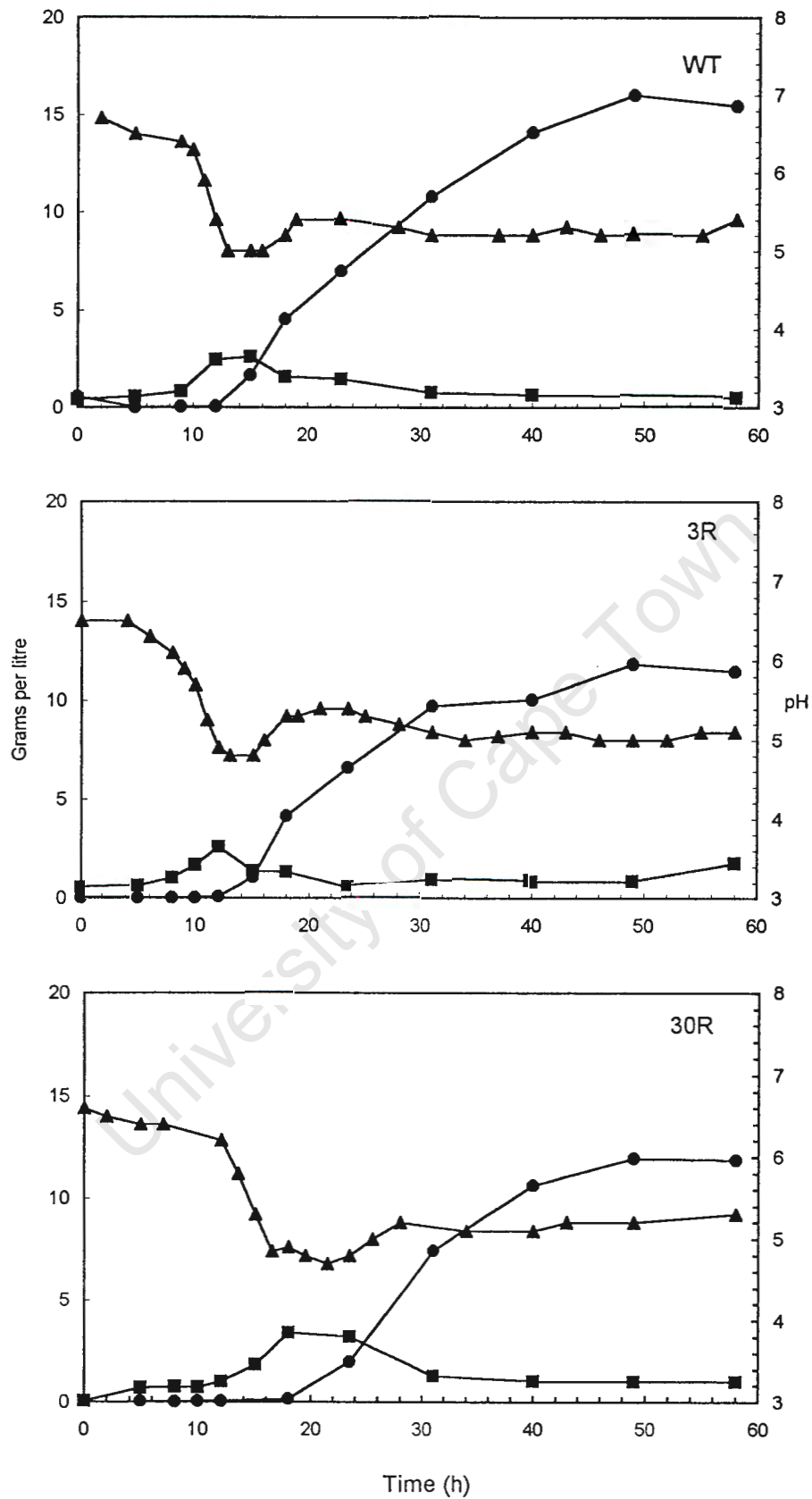


Fig. 6.4. Fermentation profiles of *C. acetobutylicum* P262 wild type (WT), mutant 3R (3R) and mutant 30R (30R) in TYGM showing pH (▲), concentration of acetate and butyrate (■) and concentration of acetone, butanol and ethanol (●).

6.3.3 Fermentation profiles

The pH profiles and levels of acid and solvent formation in the wild type and mutants 3R and 30R during the course of the batch fermentation are shown in Fig. 6.4. The accumulation of acids corresponded with a decrease in pH. The pH break point (pH 5.0, pH 4.8 and pH 4.7 for the wild type, mutant 3R and mutant 30R fermentations, respectively) occurred when the total acid concentration reached a maximum (3 to 4 g/l). There were no significant differences in the acetate:butyrate ratios. Relative to the wild type and mutant 3R, the pH breakpoint of the mutant 30R fermentation was delayed and prolonged in duration, corresponding with an extended peak in acid production. The initiation of solvent production at the pH break point was accompanied by a decrease in acid concentration and an increase in pH. Total solvent levels reached a maximum of 16, 12 and 12.5 g/l for the wild type, mutant 3R and mutant 30R cultures, respectively. There was a slight difference in the acetone:butanol ratio between mutant 3R (2.6:1.0) and the wild type and mutant 30R (2.0:1.0).

6.3.4 Autolytic activity

Increased autolysin activity is frequently associated with an autolytic phenotype and affects characteristics such as motility and cell division (Ayusawa *et al.*, 1975; Fein and Rogers, 1976). Having obtained morphological and physiological evidence of an autolytic phenotype in mutant 3R, the wild type and both mutants were assayed for differences in autolysin activity (Fig. 6.5). Exponential-phase cells of mutant 3R displayed enhanced cellular autolysin activity (905 U) relative to the wild type (378 U) and mutant 30R (384 U). The autolysin activity of mutant 3R was therefore more than double that of the parent strain (140%).

6.3.5 Bialaphos resistance

A link has been established between a sporulation-deficient (Spo0) phenotype and defective oligopeptide transport in a *B. subtilis* mutant (Perego *et al.*, 1991), demonstrating that peptide uptake plays a role in the initiation of sporulation. The possibility that there might be some connection between the asporogenous phenotype of mutant 3R and reduced oligopeptide uptake, was investigated indirectly. Bialaphos is a toxic tripeptide analogue with an intracellular target, namely glutamine synthetase (Kondo *et al.*, 1973). Resistance to

bialaphos has been demonstrated in *B. subtilis* mutants with disrupted oligopeptide transport systems (Perego *et al.*, 1991) and arises due to failure to transport the compound into the cell. In filter "disc" assays, the wild type *C. acetobutylicum* showed sensitivity to bialaphos (zone of inhibition measured ~10 mm), whereas mutant 30R displayed intermediate resistance (zone of inhibition measured ~5 mm) and mutant 3R was completely resistant (no zone) (Fig. 6.6).

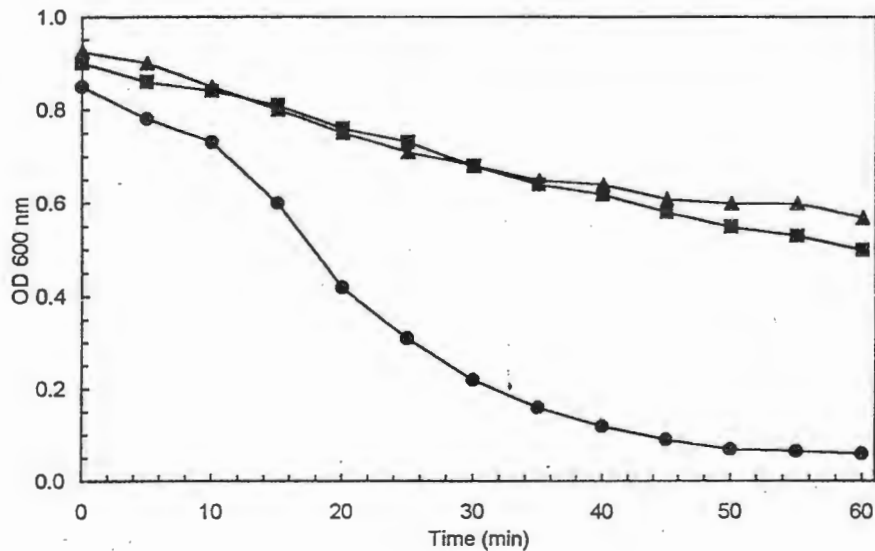


Fig. 6.5. Autolytic activity of *C. acetobutylicum* P262 wild type(■), mutant 3R (●) and mutant 30R (▲) measured as a loss in optical density at 600nm. Results are representative of a trend observed in three experiments.

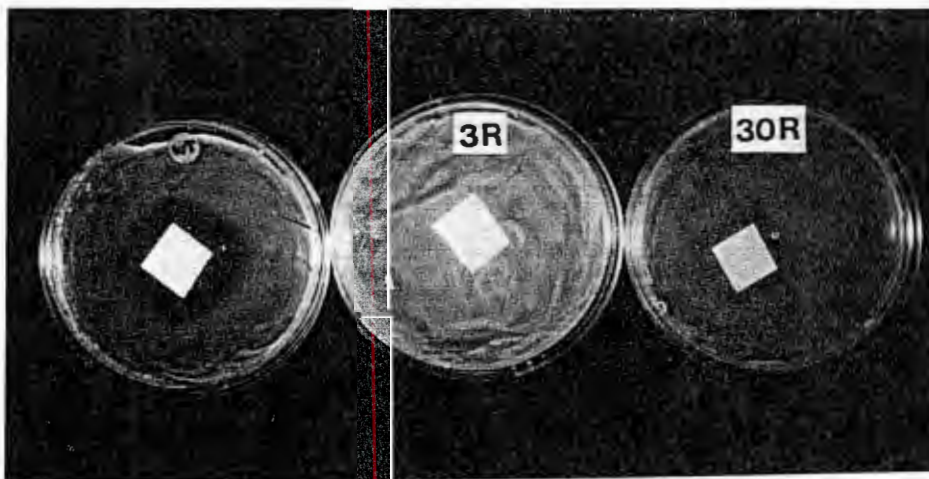
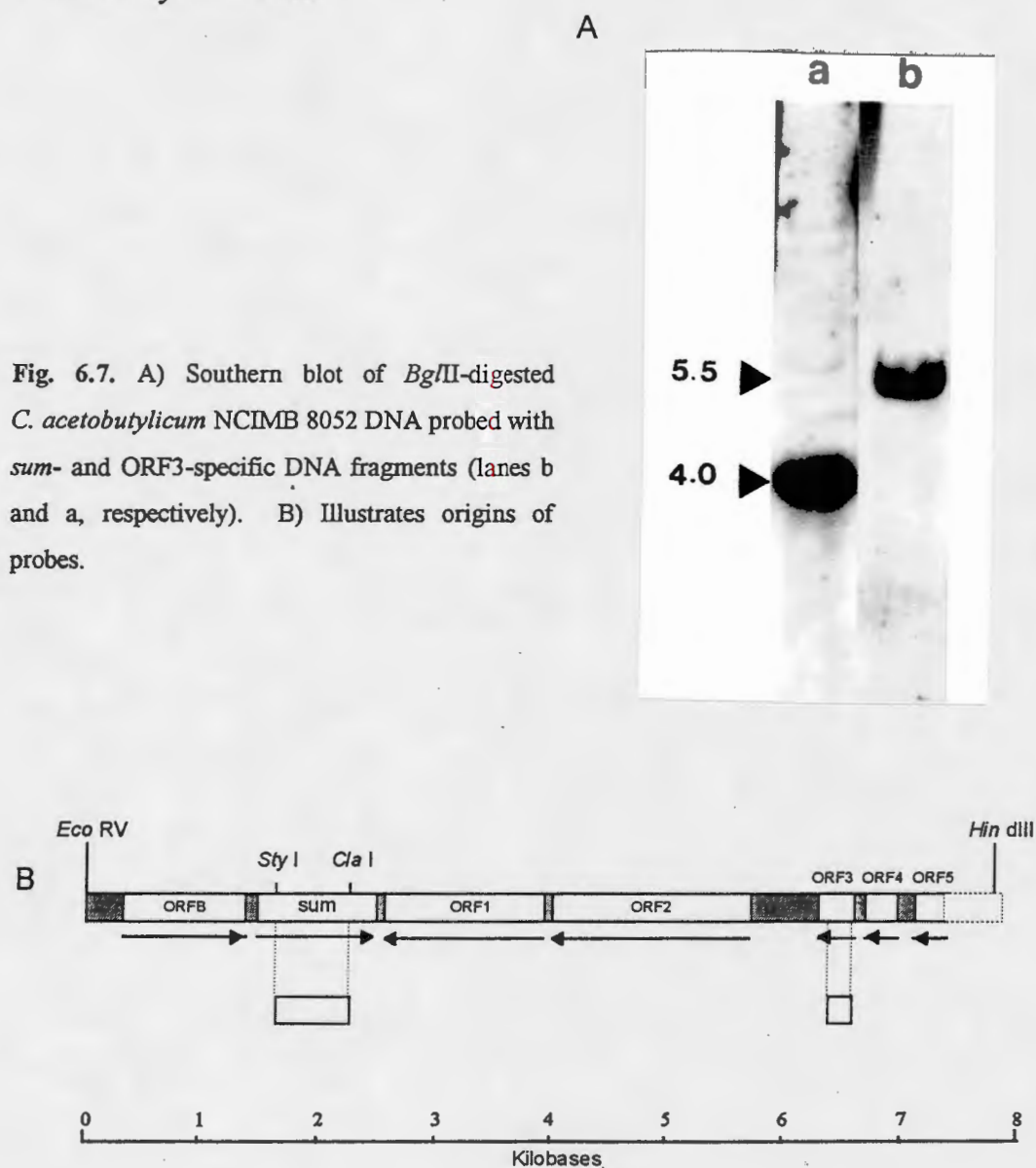


Fig. 6.6. Comparison of bialaphos susceptibility in the *C. acetobutylicum* wild type, mutant 3R and mutant 30R.

6.3.6. *C. acetobutylicum* P262 versus *C. acetobutylicum* NCIMB 8052 cross-hybridization

Attempts in our laboratory to effect conjugative plasmid transfer from *E. coli* to *C. acetobutylicum* strain P262 have been unsuccessful. This has been corroborated by Williams *et al.* (1990). However, *C. acetobutylicum* NCIMB 8052 has proved to be a suitable recipient for gene inactivation (Wilkinson and Young, 1994), and in an attempt to inactivate the *sum* and ORF3 genes in this strain, *C. acetobutylicum* NCIMB 8052 chromosomal DNA was probed for *sum* and ORF3 gene homologues. ORF3 was included in these experiments because it appears to represent an interesting regulatory gene in *C. acetobutylicum* P262.



DIG-labelled probes, specific for the *sum* and ORF3 genes were prepared from a 0.63 kb *StyI-ClaI* DNA fragment from plasmid pWT2 and a 0.2 kb PCR product amplified from plasmid pDO1, respectively (Fig. 6.7 B). The *sum*-specific probe included the DNA region encoding the putative iron-binding domain of the PFL-activase, and the ORF3-specific probe was designed to incorporate most of the helix-turn-helix motif (see Chapter 5). Preliminary Southern hybridization confirmed the chromosomal origin and the specificity of the probes (data not shown). For the Southern blot presented in Fig. 6.7 A, *C. acetobutylicum* NCIMB 8052 chromosomal DNA was digested with *BglIII* endonuclease and hybridized at low stringency with the two probes. The *sum*-specific probe hybridized to a 5.5 kb DNA fragment (lane b) and the ORF3-specific probe hybridized to a 4 kb DNA fragment (lane a). Another probe overlapping the 3' end ORF1 and the 5' end of ORF2, hybridized to both these DNA fragments (data not shown). It was therefore concluded that *C. acetobutylicum* NCIMB 8052 contains both the *sum* and ORF3 gene homologues and that the spatial relation of these homologues is conserved.

6.3.7 Attempt to generate *sum* and ORF3 gene-disruption mutants in *C. acetobutylicum* NCIMB 8052

In an attempt to inactivate the *sum* and ORF3 gene homologues in *C. acetobutylicum* NCIMB 8052 by integrative recombination, the following *C. acetobutylicum* P262 DNA fragments were cloned into the replication-deficient plasmid pMTL30. A 0.65 kb *XmnI-HindIII* internal fragment of *sum* from plasmid pWT2 was inserted into *StuI-HindIII*-digested pMTL30 DNA to generate plasmid pMTLsum, and a PCR-amplified 0.2 kb ORF3-specific fragment was cloned into *XbaI-PstI*-digested pMTL30 DNA to generate plasmid pMTLorf3 (Fig. 6.8). *E. coli* CA448 derivatives harbouring these integrational plasmids were conjugated with the *C. acetobutylicum* NCIMB 8052 recipient in two large-scale experiments. *E. coli* CA448 donors containing the replication-proficient plasmid, pCTC1 and the replication-deficient plasmid, pMTL30 served as positive and negative controls, respectively. Representative results are shown in Table 6.1.

The transfer frequencies for the pCTC1-containing donor strain were 10^{-6} per recipient, but no transconjugants were obtained for the donors containing pMTL30 and the integrational constructs. Since the frequency of integration of replication-defective plasmids was reported

as two to three orders of magnitude less than that of the replication-proficient control i.e. 0.1 to 1% integration by homologous recombination (Wilkinson and Young, 1994), it was concluded that the experiments probably failed because the DNA inserts were of heterologous origin. It has been shown that components of the methyl-directed mismatch repair system in *E. coli* prevent heteroduplex formation between two DNA fragments that have diverged by only a few percent at the sequence level (Modrich, 1994).

Table 6.1. Frequency of pCTC1 transfer to *C. acetobutylicum* NCIMB 8052 and limit of detection frequency for plasmid integration

Plasmid	Replication	Frequency per recipient
pCTC1	Rep ⁺	1.1 x 10 ⁻⁶
pMTL30	Rep ⁻	< 2.8 x 10 ⁻¹⁰
pMTLsum	Rep ⁻	< 6.2 x 10 ⁻⁹
pMTLorf3	Rep ⁻	< 7.0 x 10 ⁻⁹

6.3.8 Cloning of the *sum* and ORF3 genes in *C. acetobutylicum* NCIMB 8052

To evaluate the effect of the *sum* and ORF3 genes cloned in multicopy in *C. acetobutylicum* NCIMB 8052, these genes were separately cloned from *E. coli* plasmids into the *B. subtilis/C. acetobutylicum* shuttle vector, pFNK1. A 2 kb *ScaI-PstI* DNA fragment containing the intact *sum* gene and upstream putative promoter region (including the 3' end of ORFB), was cloned from plasmid pWT1 into *PvuII-PstI*-digested pFNK1 to yield plasmid pFNKsum (Fig.6.8). The ORF3 gene was amplified by PCR as a 600 bp fragment from plasmid pDO1, and cloned into *PstI-EcoRI*-digested pFNK1 to yield plasmid pFNKorf3 (Fig. 6.8). The recombinant plasmids pFNKsum and pFNKorf3 were initially established in *B. subtilis* 1A46. Plasmid DNA extracted from the *B. subtilis* transformants was then used to electrotransform *C. acetobutylicum* NCIMB 8052.

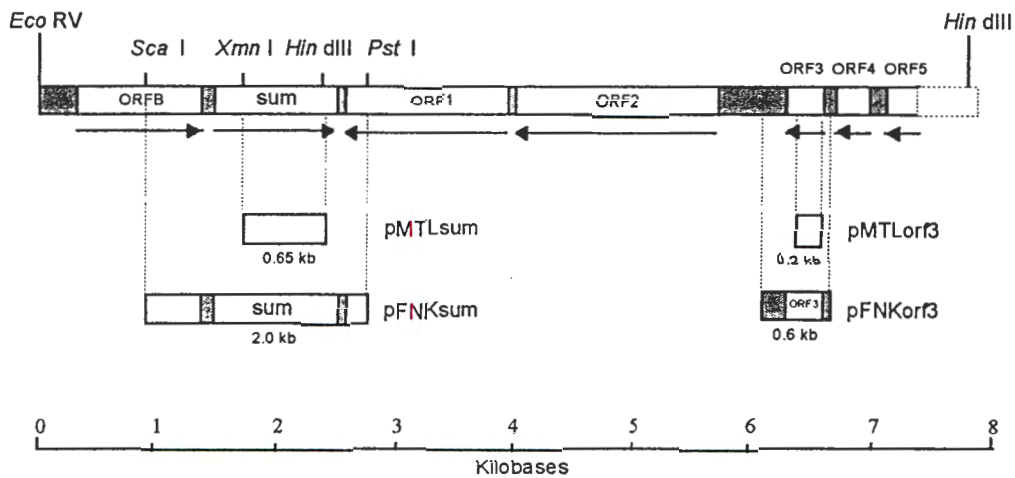


Fig. 6.8. Diagrammatic representation of the origins of pMTL- and pFNK1-based constructs.

6.3.9 Physiological characterization of *C. acetobutylicum* NCIMB 8052 transformants

The *C. acetobutylicum* NCIMB 8052 strains containing pFNK1, pFNKsum and pFNKorf3 were assayed for differences in susceptibility to metronidazole, UV radiation and bialaphos, as well as for differences in cell division, motility, autolytic activity, solvent production and sporulation (all phenotypes identified in mutant 3R). The presence of multiple copies of the *sum* and ORF3 genes had no effect on most of these characteristics (data not shown). However, vegetative cells of strain NCIMB 8052 (pFNKsum) were spectacularly elongated compared to vegetative cells of control strain NCIMB 8052 (pFNK1) (Fig. 6.9).

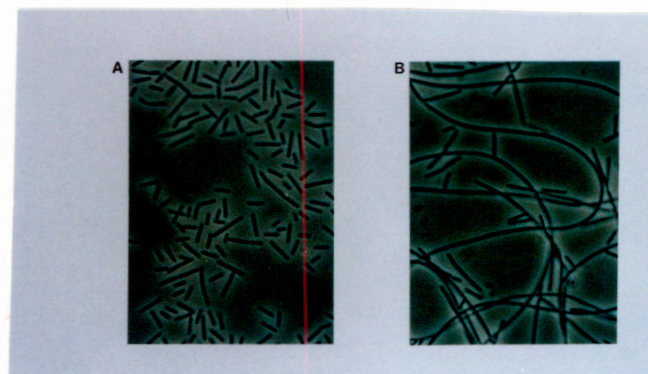


Fig. 6.9. Photomicrograph of vegetative cells of *C. acetobutylicum* NCIMB 8052 strains containing the control plasmid pFNK1 (A) and plasmid pFNKsum. Magnification 400x.

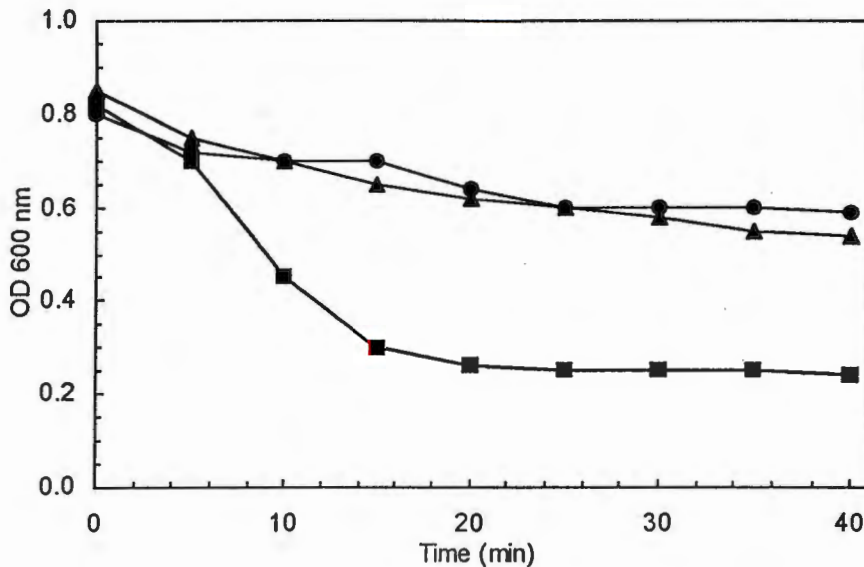


Fig. 6.10. Autolysin activity of *C. acetobutylicum* NCIMB 8052 strains containing the control plasmid pFNK1 (■), plasmid pFNKsum (●) and plasmid pFNKorf3 (▲).

Furthermore, both strains harbouring pFNKsum and pFNKorf3 showed a reduction in autolysin activity compared to the control strain (Fig. 6.10). The autolysin activity for NCIMB 8052 (pFNK1), NCIMB 8052 (pFNKsum) and NCIMB 8052 (pFNKorf3) was calculated at 721, 396 and 383 U, respectively. This represents a ~45% reduction in autolysin activity in the latter two strains.

6.4 Discussion

The inclusion of mutant 30R in the physiological study described above, was useful for assessing the pleiotropic phenotype of mutant 3R. Although mutants 3R and 30R contained one and four distinct transposon insertions, respectively, and did not appear to have a common metronidazole resistant mutation (Chapter 2), they both shared a number of physiological characteristics. Mutant 30R and 3R batch fermentation cultures produced only a limited number of clostridial forms, few or no spores, respectively, and slightly reduced levels of solvents (approximately 12 g/l). Moreover, a decrease in cell viability was observed for both mutants at the end of the stationary growth phase. Conceivably, this phenomenon is related to impaired clostridial form development. It is interesting that the ~70% reduction in clostridial cell numbers in both mutant cultures relative to the wild type was not proportional to the reduction in solvent formation (~25%). These shared features

of mutants 3R and 30R are reminiscent clostridial strain degeneration - a general term used to describe the loss of ability to sporulate and produce solvents. The molecular mechanisms responsible for degeneration have not yet been characterized, but single transposon-insertion degeneration-resistant mutants of *C. acetobutylicum* NCIMB 8052 have been isolated (Kashket and Cao, 1993; 1995), and the capacity of degenerate-susceptible NCIMB 8052 cells to revert to a non-degenerate phenotype at low frequency implicates a global regulatory locus (Woolley and Morris, 1990).

Despite the above-mentioned similarities, mutant 3R was also distinct from mutant 30R (and the wild type) in the followings ways. Vegetative cells were smaller in size, divided more actively during the exponential growth phase and displayed enhanced motility. One or more of these characteristics can probably be attributed to the elevated levels of autolysin activity observed in the 3R mutant, since autolysins are implicated in physiological processes such as cell division, motility and turnover of wall polymers (Ayusawa *et al.*, 1975; Fein and Rogers, 1976). The addition of partially purified autolysin to a *B. subtilis* filamentous mutant has been shown to convert the filaments to short cells (Fan, 1970); and it has been suggested that autolytic activity is required for flagella assembly, since localised peptidoglycan hydrolysis allows for insertion of basal-body structures of the flagellar apparatus into or through the Gram-positive cell wall. The decrease in cell viability in mutant 3R may also be associated with enhanced autolysin activity, as artificial reduction of autolysis in *C. acetobutylicum* ATCC 824 has been shown to correlate with an extension of cell viability (Zhou and Traxler, 1992).

In addition, mutant 3R was resistant to bialaphos, which might explain its asporogenous phenotype. It is postulated that intracellular accumulation of cell-wall oligopeptides as they are released from the peptidoglycan during growth, may play a signalling role in the initiation of sporulation in *B. subtilis* (Perego *et al.*, 1991). Mutations within the *spo0K* operon which encodes an ATP-dependent oligopeptide permease system generate sporulation-deficient, bialaphos-resistant mutants (Perego *et al.*, 1991). If, as for the *B. subtilis* mutants, resistance in mutant 3R was due to the inability to transport bialaphos into the cell, the asporogenous phenotype of mutant 3R may be associated with a defective oligopeptide uptake system.

The pleiotropic phenotype of mutant 3R was caused by chromosomal inactivation of the *sum* gene which encodes the putative PFL-activating enzyme. This implies that the formate-producing pathway contributes directly/indirectly to clostridial stage differentiation, optimal solventogenesis, sporulation and peptide uptake, and has a negative effect on motility, autolysin production and cell division. Confirmation of the inhibitory effects of the Sum protein on the latter two characteristics was obtained by multicopy expression of the *sum* gene in *C. acetobutylicum* NCIMB 8052, which resulted in filamentous growth and decreased autolysin production in the host strain. Overexpression of the gene had no apparent effect on sporulation, motility and peptide uptake.

The complexity of gene regulation in a differentiating organism like *C. acetobutylicum* makes it difficult to interpret the behaviour of the *sum*-disruption mutation in molecular terms. A pleiotropic phenotype in *C. acetobutylicum* is not uncommon e.g. a number of *C. acetobutylicum* NCIMB 8052 cysteine auxotrophic mutants containing a single transposon insertion are impaired in three related stationary phase responses, viz. sporulation, granulose accumulation and solventogenesis (Wilkinson *et al.*, 1995a). Furthermore, a number of pleiotropic regulatory genes conferring similar phenotypes to those described for mutant 3R, have been isolated from *Bacillus* (reviewed by Klier *et al.*, 1992; Smith, 1993; Strauch and Hoch, 1993).

Unfortunately, molecular evidence that the *sum* gene encodes a PFL-activating enzyme has not been tested physiologically, and assaying for PFL activity and formate levels in mutant 3R is a priority for future work. Nevertheless, this study has served to demonstrate the physiological significance of the Sum protein, a putative regulator of the formate-producing pathway which appears to contribute to the cellular differentiation process in *C. acetobutylicum* P262.

Results presented in Chapters 1-6, are consolidated in Chapter 7; the implications of these findings and proposed direction for future research are discussed.

Chapter 7

General Conclusions

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Chapter 7

General Conclusions

The direction of electron flow controls the direction of carbon flow in the *C. acetobutylicum* fermentation (Kim and Zeikus, 1985; Meyer *et al.*, 1986; Jones and Woods, 1986; Vasconcelos *et al.*, 1994; Girbal *et al.*, 1995), and some of the electron transport proteins which play a key role in electron distribution in the cell, e.g. the ferredoxin and the hydrogenase, are responsible for the reductive activation of metronidazole in the clostridia (reviewed by Church and Laishley, 1995). The aim of this study was to isolate electron transport gene(s) by selection for transposon-induced metronidazole resistance, with a view to manipulation of metabolic pathways in *C. acetobutylicum* P262. In the process, the feasibility of transposon mutagenesis of strain P262, which lacks conventional systems for DNA delivery, was assessed, and more insight was gained into the mechanisms of metronidazole resistance in anaerobic microorganisms.

7.1 Transposon mutagenesis and mutational cloning of the *sum* gene

Using the transposon cointegrate Tn925::Tn917, it was demonstrated that transposon mutagenesis in *C. acetobutylicum* P262 is practicable (Babb *et al.*, 1993). Two metronidazole resistant mutants, referred to as 3R and 30R were isolated. Southern hybridization and sequence analysis established that mutants 3R and 30R harboured one and four transposon insertions in the chromosome respectively, and that they did not share a common metronidazole resistant mutation. Sequence analysis of three left end transposon-chromosome junction fragments indicated that transposon integration in the *C. acetobutylicum* P262 chromosome was suitably "random", although in each case, the thymine-rich end of the transposon had inserted next to a stretch of adenine residues in the target site. The multiple transposon copy number of mutant 30R made it impractical to define the metronidazole resistant mutation, whereas mutant 3R was a suitable candidate for further characterization. The intact wild type gene region corresponding to the disrupted gene region in mutant 3R was cloned and sequenced, and the precise transposon target site, (5'-TCTTTTTGATTCAAAAAAAA-3') as defined by Trieu-Cuot *et al.* (1993) was

determined (the 6 bp spacer region is underlined). Mutational cloning resulted in isolation of the *sum* gene.

7.2 Sum, a putative PFL-activase

The *sum* gene encoded a 334 amino-acid protein which displayed homology (36% identity and 58% similarity) to a PFL-activase homologue identified in the anaerobic archaeobacterium, *T. litoralis* (Kletzin *et al.*, 1995). Interestingly, there is already a precedent for high amino acid sequence conservation between some proteins derived from the hyperthermophilic archae and the clostridia e.g. glutamine synthetase (Brown *et al.*, 1994b, Kletzin *et al.*, 1995; F. Robb, personal communication). Although the *sum* product shared low homology (21% identity, 45% similarity) with the two *E. coli* PFL-activating enzymes, the cluster of cysteine residues, thought to constitute the iron-binding domain (Wong *et al.*, 1993), was conserved. It is possible that the strong sequence similarity between the *sum* product and the PFL-activase homologue of *T. litoralis*, reflects a variant of this enzyme specific for strict anaerobic systems.

The PFL-activating enzyme of *E. coli* is a flavodoxin-dependent reductase which is responsible for post-translational modification of PFL. In its activated free-radical form, PFL catalyses the CoA-dependent cleavage of pyruvate to formate and acetyl-CoA (Knappe, 1987). The PFL reaction has been demonstrated in several clostridial species, including *C. butyricum*, a close relative of *C. acetobutylicum* P262 (Thauer *et al.*, 1972; Keis *et al.*, 1995). The function of the PFL reaction in *C. butyricum* is both anabolic and catabolic. These pathways are likely to be in competition with the phosphoroclastic reaction and the lactate dehydrogenase in *C. acetobutylicum*, and are represented in Fig. 7.1. Under conditions of stress, lactate is a major fermentation product in *C. acetobutylicum* (Bahl *et al.*, 1986). Lactate and formate serve as alternative electron sink products and the lactate dehydrogenase cloned from *C. acetobutylicum* strain B643 in *E. coli* appears to compete with the *E. coli* PFL for pyruvate (Clark, 1989; Contag *et al.*, 1990).

The Sum protein has been identified as a PFL-activase only on the basis of sequence similarity with the putative PFL-activase homologue from *T. litoralis*, also identified only by sequence homology. It would be important to confirm that *sum* does indeed encode a PFL-

activase or the functional equivalent. This could be done by 1) functional complementation in *E. coli*; 2) attempting to rescue the mutant 3R phenotype in the presence of extraneous formate; and 3) comparing formate levels and PFL activity in the wild type and mutant 3R, as well as assaying for differences in acetyl-CoA and lactate concentrations. It would also be useful to establish the physiological function (i.e. catabolic and/or anabolic) of the PFL reaction in *C. acetobutylicum* P262.

7.3 Proposed mechanism of metronidazole resistance in mutant 3R

Disruption of the *sum* gene in mutant 3R resulted in a metronidazole resistant phenotype. The *sum* product, on the basis of amino acid homology to the PFL-activase, could be responsible for metronidazole susceptibility in one of three possible ways. First, as a reductase, the PFL-activating enzyme might be capable of direct reduction of metronidazole (Fig. 7.1 I), although presumably, its reducing activity is extremely specific. Metronidazole is activated via reduction of the nitro group, whereas the PFL-activase is responsible for removal of a hydrogen atom from the PFL enzyme. The second possibility is that activation of the drug occurs via the formate degradation pathway (Fig. 7.1 II). In *E. coli*, formate is converted to carbon dioxide and hydrogen by the formate-hydrogen lyase system, which consists of a formate dehydrogenase, a hydrogenase, and unidentified electron carriers (Stewart, 1988). The formate dehydrogenase is induced by formate and is active with methyl viologen and benzyl viologen as artificial electron acceptors. The isolation of a tungsten-selenium-containing NADP-dependent formate dehydrogenase from *Clostridium thermoaceticum* (Yamamoto *et al.*, 1987) is a precedent for the existence of the formate degradation pathway in the clostridia. The third possibility is that formate, as an electron sink product, reduces the redox potential of the cell such that sensitivity to metronidazole is increased (Fig. 7.1 III).

The following experimental evidence enables us to eliminate the first two options. The observation that the *C. acetobutylicum* NCIMB 8052 strain containing the *sum* gene on a multicopy plasmid, did not display increased sensitivity to metronidazole rules out a direct role for the PFL-activase in drug reduction (Fig. 7.1 I). Instead, it implies that in the presence of excess activating enzyme, PFL synthesis is the rate-limiting step in formate production and that drug sensitivity depends on formate levels. If this is the case, one might

anticipate that *C. acetobutylicum* would display increased sensitivity to metronidazole in the presence of excess formate. There is no indication of a decreased rate of metronidazole reduction in mutant 3R relative to the parent strain. It is therefore unlikely that decreased drug susceptibility is due to reduced activity of the formate-hydrogen lyase system in response to decreased levels of formate (Fig 7.1 II). Instead, physiological evidence indicates that the metronidazole resistant phenotype of mutant 3R arises from a redox-responsive increased capacity for DNA repair, since the mutant displays decreased susceptibility to the physical and chemical DNA damaging agents, UV radiation and bleomycin, respectively.

Thus, the third option is favoured. Our hypothesis is that disruption of the formate production pathway causes a redox imbalance which induces an oxidative stress response, such that the overall DNA repair capacity of the *C. acetobutylicum* mutant is enhanced. A redox stress response can be induced in *E. coli* by depletion of cellular NAD(P)H pools for example (Kappus and Sies, 1981), and is partially independent of the hydrogen peroxide stress response (Greenberg and Demple, 1989). This would explain why the *C. acetobutylicum* wild type and mutant 3R showed no difference in susceptibility to hydrogen peroxide treatment (data not shown). Although there have been no reports of metronidazole resistance occurring via an increased capacity to repair metronidazole-induced DNA damage, the reverse phenomenon, in which *E. coli* mutants with defective DNA repair systems show increased susceptibility to metronidazole, has been demonstrated (Jackson *et al.*, 1984; Yeung *et al.*, 1984).

It is interesting that the suggested way in which the cloned *C. acetobutylicum* P262 flavodoxin gene renders the *E. coli* F19 host susceptible to metronidazole (Santangelo *et al.*, 1991; Woods and Santangelo, 1993) reflects a common theme. These authors postulate that since the post-translational activation of PFL requires reduced flavodoxin, the cloned flavodoxin may enhance the conversion rate of pyruvate to acetyl-CoA and formate, which in turn might induce the formate dehydrogenase. The subsequent reduction in the overall redox potential of the cell could result in increased sensitivity to metronidazole, as has been demonstrated in *E. coli* (Onderdonk *et al.*, 1979).

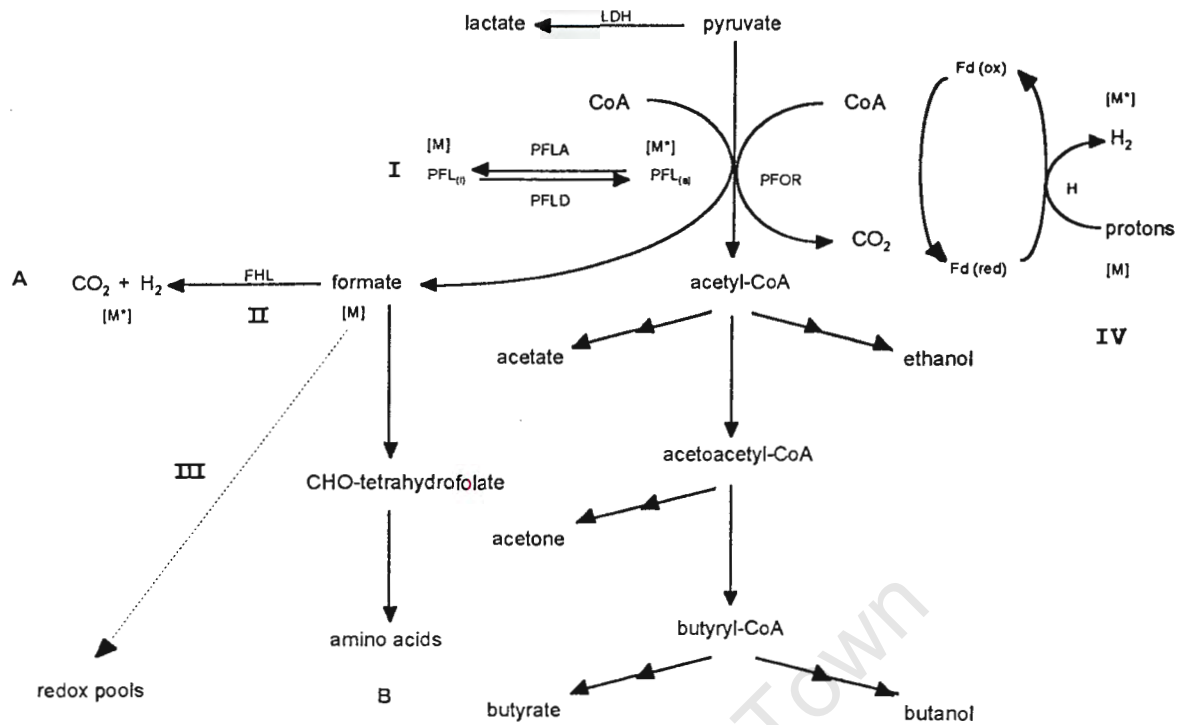


Fig. 7.1. Proposed formate pathway in *C. acetobutylicum* showing the pyruvate-formate lyase reaction (I), and catabolic (A) and anabolic (B) processing of the formate product. The PFL-activase (PFLA) and the PFL-deactivase (PFLD) interconvert PFL between its active (PFL_(a)) and inactive (PFL_(i)) forms, respectively. Presumably, the pyruvate-formate lyase (PFL) competes with the lactate dehydrogenase (LDH) and the pyruvate:ferredoxin oxidoreductase (PFOR) of the phosphoroclastic reaction. Acetyl-CoA is the primary intermediate for the acidogenic (acetate and butyrate) and solventogenic (ethanol, acetone and butanol) pathways. The formate pathway may contribute to metronidazole susceptibility in *C. acetobutylicum* in one of three possible ways. I) The PFL-activase may be responsible for direct reduction of metronidazole, converting the drug from its inactive (M) to its active (M*) form. II) Metronidazole may be reduced by components of the formate-hydrogen lyase (FHL) system via the formate degradation pathway. III) Formate may contribute to the redox potential of the cell such that sensitivity to metronidazole is increased. The main route for metronidazole activation in *C. acetobutylicum* is presumably via the ferredoxin-linked hydrogenase (H) coupled to the phosphoroclastic reaction (IV).

7.4 Metronidazole susceptibility of the *C. acetobutylicum* wild type

It is assumed that DNA is the main target for metronidazole cytotoxicity in *C. acetobutylicum* P262, because surprisingly, there was no evidence for death by rapid cell lysis following treatment with a high concentration of metronidazole, as reported for *C. pasteurianum* (Church *et al.*, 1991). Another important observation was that the sensitivity of *C. acetobutylicum* P262 wild type varies with the position of the culture in the

growth cycle. Vegetative cells in the early exponential growth phase displayed maximum sensitivity to metronidazole (loss of almost four log units of viability) whereas stationary phase clostridial cells were resistant to metronidazole treatment (loss of less than one log unit of viability). This correlates with the *C. acetobutylicum* hydrogenase activity profile which has been shown to drop significantly in the shift to solventogenesis (Kim and Zeikus, 1985), and substantiates the report that metronidazole reduction in *C. pasteurianum* copurifies with hydrogenase activity (Church *et al.*, 1988). It is possible that the clostridial hydrogenase functions not only as a component of the phosphoroclastic reaction, but is also active in the formate degradation pathway. Methyl viologen and benzyl viologen can substitute for reduced ferredoxin in the hydrogenase-mediated reduction of metronidazole in *C. pasteurianum* (Church *et al.*, 1988), and these same electron carriers are able to react with the *C. thermoaceticum* formate dehydrogenase (Ljungdahl and Andreesen, 1978). Moreover, addition of a clostridial hydrogenase preparation to an extract from a hydrogenase-deficient *E. coli*-like strain is capable of reconstituting the formate-hydrogen lyase reaction (Gest and Peck, 1955). The hydrogenase has been shown to act as a multifunctional enzyme in *C. pasteurianum* (see Church and Laishley, 1995; Yanke *et al.*, 1995).

7.5 Physiological effects of disrupted *sum* gene function in *C. acetobutylicum*

Insertional inactivation of the *sum* gene in *C. acetobutylicum* mutant 3R resulted in a number of physiological effects, presumably arising from a block in the formate-producing pathway. These pleiotropic characteristics are summarised in Table 7.1.

We are not able to consolidate all these phenotypes in a model, but some of them can possibly be accounted for. Presuming the physiological function of the PFL reaction in *C. acetobutylicum* P262 is both anabolic and catabolic as for the closely-related *C. butyricum* (Thauer *et al.*, 1972), disruption of the formate-producing pathway, would probably result in a decrease in acetyl-CoA, formate and CHO-tetrahydrofolate pools, and an increase in pyruvate levels (refer to Fig. 7.1). A decrease in the acetyl-CoA concentration might explain the 25% reduction in solvent levels. A decrease in CHO-tetrahydrofolate levels could conceivably result in decreased amino acid biosynthesis. Increased motility has

Table 7.1. Comparison of phenotypes observed in the *C. acetobutylicum* P262 wild type, *C. acetobutylicum* P262 mutant 3R, and a *C. acetobutylicum* NCIMB 8052 strain containing the *sum* gene on a multicopy plasmid

Phenotype	P262 wild type	P262 mutant 3R	8052 (pFNKsum)
Metronidazole susceptibility	S	R	S
UV susceptibility	S	R	S
Bleomycin susceptibility	S	R	ND
Bialaphos susceptibility	S	R	S
Cell division	0	+	-
Motility	0	+	0
Autolysin activity	0	+	-
Clostridial differentiation	0	-	0
Sporulation	0	-	0
Solvent production	0	-	0

S, sensitive; R, resistant; 0, wild type phenotype; +, increased relative to wild type phenotype; -, decreased relative to wild type phenotype; ND, not done.

been observed in nitrogen starved cultures of *C. acetobutylicum* P262 (L. Brown, personal communication), and so nutrient limitation might account for the enhanced motility of mutant 3R cells. Furthermore, autolysin-deficiency is often associated with the loss of flagella and motility (Ayusawa *et al.*, 1975; Yoneda and Maruo, 1975; Fein and Rogers, 1976; Fein, 1979), and therefore the increased autolytic phenotype of mutant 3R correlates with enhanced cell motility. Autolysin activity has been observed to parallel cell growth (Allcock *et al.*, 1981; Soucaille and Goma, 1986) and this may explain the higher rate in cell division observed in mutant 3R during the exponential growth phase. When *sum* is present on a multicopy number plasmid in *C. acetobutylicum* NCIMB 8052, cell division and autolytic activity are inhibited.

The mutant 3R phenotypes can also be interpreted in the context of clostridial degeneration and acid stress. Mutant 3R is partially degenerate, in that solventogenesis is reduced and cellular differentiation and sporulation are impaired. Kashket and Cao (1995) have demonstrated that small increases in the growth rate can cause excess acid production, resulting in a pH below the pH breakpoint value which is therefore toxic to the cell. The pH break point of the mutant 3R batch fermentation is lower than that of the wild type. It is interesting that an oligosporogenous butanol-deficient strain of *C. saccharoperbutyl-acetonicum* which is impaired in NADH generation (and consequently suffers a redox imbalance) has been identified as a degenerate mutant (Hayashida and Yoshino, 1990; Kashket and Cao, 1995).

The pleiotropic phenotype of mutant 3R also shows similarities with the phenotypes conferred by a number of regulators in *Bacillus*. These include the transition state regulators which control late growth developmental functions such as the synthesis of degradative enzymes, the development of competence and motility, and the production of autolysin - all of which are temporally linked to the initiation of sporulation, yet dispensable for the sporulation process. Inactivation of the *sin* gene in the *B. subtilis* chromosome, for example, results in filamentous growth, increased exoenzyme activity and loss of competence, motility and autolytic activity, whereas amplification of *degT* encoding a putative membrane sensor protein results, *inter alia*, in loss of motility and autolysin synthesis.

The apparent block in peptide uptake in mutant 3R could also be responsible for the impaired differentiation process and defective sporulation. The *spo0K* operon of *B. subtilis* consists of five genes highly homologous to the oligopeptide transport (Opp) system of Gram-negative bacteria and is required for sporulation and genetic competence (Rudner *et al.*, 1991). It is interesting to note that in *B. subtilis*, the competent state is accompanied by a derepression of SOS repair functions such as *recA* and the *din* genes (Dubnau, 1993), and this correlates with the enhanced DNA repair phenotype observed in mutant 3R. In terms of our interpretation of the increase in DNA repair arising from a redox stress response, it is also interesting that ClpC/MecB, a stress response-related ATPase in

B. subtilis, controls cell division, competence and degradative enzyme synthesis (Krüger *et al.*, 1994; Msadek *et al.*, 1994).

Although all these physiological functions are biochemically distinct, they share many aspects of their regulation and are also subject to multiple control. The above discussion serves to illustrate the interdependence of these pathways and the complexity of the mutant 3R phenotype. A stationary phase gene cluster implicated in the expression of late sporulation functions is located downstream of the *sum* gene.

7.6 A stationary phase gene cluster

The 5 kb DNA region downstream of the *sum* gene contains an interesting stationary phase gene cluster. Five ORFs have been provisionally identified (the DNA fragment was sequenced from one strand only), all of which are transcribed in the opposite direction relative to *sum*. ORF1 encoded a protein with high sequence similarity (74%) to the spore-cortex-lytic enzyme (SCLE) of *C. perfringens* (Miyata *et al.*, 1995a) which is implicated in spore germination. SCLE presumably exists as an inactive 36 kDa pro-enzyme, covalently attached to the outside of the cortex layer, and is processed by proteolytic cleavage to release the active 31 kDa enzyme during germination (Miyata *et al.*, 1995a). A putative cell wall binding motif, LPXTG (Navarre and Schneewind, 1994), was identified at the C-terminal end of the *C. acetobutylicum* and *C. perfringens* SCLE protein sequences. Deletion studies would confirm whether this motif is required for linkage of SCLE to the peptidoglycan of the spore. ORF2, located immediately upstream of ORF1, encoded a serine protease, and is therefore a likely candidate for the SCLE-processing enzyme. This has assisted Miyata and co-workers in preliminary identification of the incomplete *orf1* upstream of the *sleC* gene in *C. perfringens* (Miyata *et al.*, 1995a). ORF3 displayed homology to a number of small regulatory proteins such as Sin, an ambivalent regulator of late developmental growth processes in *Bacillus* (Smith, 1993), and the Cro-like protein implicated in the regulation of phage ϕ 105 lysogeny (Van Kaer *et al.*, 1987). A helix-turn-helix motif has been identified in the predicted amino acid sequence of the ORF3 product, and gel retardation studies would show whether this protein binds within the 500 bp intergenic region upstream of ORF2, the putative serine protease. ORF4 remains to be identified, but together with ORF3, is likely to constitute a regulatory operon. The partial

amino acid sequence of ORF5, lacking the N-terminal end, shared convincing sequence similarity with peptidyl-prolyl-isomerases which are involved in protein folding and the regulation of signalling pathways (Rutherford and Zuker, 1994). Transcription analysis and regulatory studies of this stationary phase gene cluster should prove rewarding.

7.7 Concluding remarks

Three significant findings have emerged from this study which contribute to our knowledge of complex anaerobic metabolism and cellular differentiation in *C. acetobutylicum*, and lay the groundwork for three areas of research, outlined as follows. First, sequence similarity between the *sum* product and the PFL-activating enzyme homologue identified in *T. litoralis* (Kletzin *et al.*, 1995) provides indirect evidence for the existence of the formate-producing pathway in *C. acetobutylicum* P262. This is substantiated by the homology between the AAD/AdhE protein of *C. acetobutylicum* strains ATCC 824 and DSM 792 (Fischer *et al.*, 1993; Nair and Papoutsakis, 1994) and the trifunctional AdhE protein of *E. coli* which harbours, *inter alia*, a PFL-deactivase activity. This activity, however, does not appear to be encoded by a continuous amino acid sequence within the AdhE protein (Kessler *et al.*, 1992). Although the AAD/AdhE and Sum proteins have not been tested for their respective deactivase and activase activities, they can be tentatively identified as the two elements responsible for interconversion of PFL between its inactive and active forms. The formate-producing pathway has been neglected in *C. acetobutylicum* research, but as judged from the phenotype of *C. acetobutylicum* P262 mutant 3R, it appears to be essential for clostridial differentiation, sporulation and optimal solventogenesis.

Second, the apparent role of the PFL-activase or the formate degradation pathway in metronidazole susceptibility is of medical interest as twelve percent of all clinical isolates of *Clostridium* are resistant to metronidazole (V. Abratt, personal communication). The metronidazole-susceptible trichomonads lack PFL and formate dehydrogenase activity (Lindmark and Müller, 1973), but the formate pathway appears to contribute to metronidazole sensitivity in anaerobic bacteria such as *C. acetobutylicum*. Presumably, the ferredoxin-linked hydrogenase reaction is the predominant mechanism for metronidazole activation and susceptibility in *C. acetobutylicum* (Fig.1.7 IV), as has been demonstrated in *C. pasteurianum* (Church *et al.*, 1988), but loss of this important metabolic system is liable

to be lethal to the cell. This explains why both mutants 3R and 30R harbour low-level (but viable) metronidazole resistant mutations. Characterization of defined metronidazole-induced mutations is a valuable exercise, since "unlabelled" drug resistant mutations in clinical isolates are difficult to characterize at the molecular level, as evidenced by only one such report to date (Quon *et al.*, 1992).

Identification of a stationary phase gene cluster downstream of the *sum* gene is the third important finding. This cluster includes genes encoding a spore-cortex-lytic enzyme (SCLE), a putative SCLE-activating protease, and an interesting regulatory protein with N-terminal homology to the bacteriophage ϕ 105 Cro-like protein and Sin, a *Bacillus* transition state regulator. This adds to the increasing number of sporulation gene homologues recently identified in *C. acetobutylicum*.

Appendix A

Characterization of *E. faecalis* donor strain: experimental evidence

The *E. faecalis* OG1RF(pINY1275) donor strain containing the Tn925::Tn917 composite transposon was used in the conjugation experiments described in Chapter 2. This strain was originally misidentified as *E. faecalis* OG1SSp(pCF10) which contains Tn925. Therefore the initial strategies for molecular investigation of the *C. acetobutylicum* mutants were based on the understanding that the transposon element harboured by these transconjugants was Tn925. Experimental data revealing the true identity of the *E. faecalis* donor are presented in this Appendix.

A Southern hybridization experiment was performed to determine the transposon copy number of mutants 3R and 30R (Fig. A.1). Chromosomal DNA from *C. acetobutylicum* wild type (lane b), mutant 3R (lane c) and mutant 30R (lane d) was digested with *Hind*III endonuclease and probed with DIG-labelled *Hinc*II-digested pTet plasmid DNA. Plasmid pTet DNA digested with *Hinc*II (lane a) served as the positive control. Transposon Tn925 contains a single *Hind*III restriction site (Christie *et al.*, 1987) and so it was expected that every two hybridization bands would represent one transposon insertion. However, three hybridization bands (an odd number) were detected for mutant 30R, and a 5.3 kb band was common to both mutants. The same anomalous 5.3 kb fragment was detected by Strätz *et al.* (1990), and interpreted to mean that Tn925 contained an additional *Hind*III site.

This 5.3 kb *Hind*III DNA fragment was cloned from mutant 30R to generate plasmid p30R0 (see Chapter 2, Fig. 2.2). The DNA insert of plasmid p30R0 did not hybridize with wild type chromosomal DNA (results not shown) and sequence analysis of the left transposon end of the insert revealed high homology with Tn917, indicating that the mutants harboured the Tn925::Tn917 cointegrate. This was confirmed by testing the transconjugants and the p30R0 plasmid for erythromycin resistance, since Tn917 carries an erythromycin resistant marker. Thus the hybridization results presented in Fig. A.1 were re-interpreted as follows: each band excluding the internal 5.3 kb band represents a distinct transposon insertion. As shown in Chapter 2, both the >7 kb bands detected for mutant 30R represent doublets. For

equal quantities of chromosomal DNA, the hybridization signal for the 5.3 kb band in each mutant sample would be proportional to the transposon copy number. These results have been reported (Babb *et al.*, 1993).

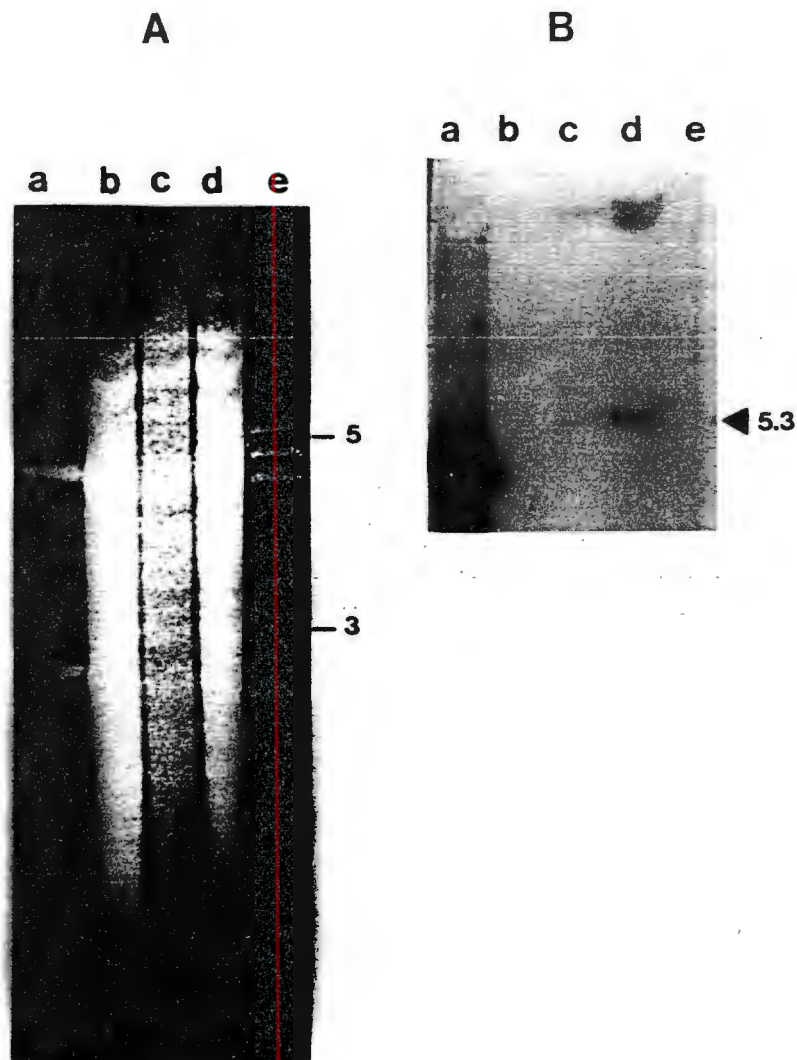


Fig. A.1. A) Agarose gel and B) corresponding autoradiograph of *HincII*-digested pTet plasmid DNA (lane a), and *HindIII*-digested *C. acetobutylicum* chromosomal DNA from the wild type (lane b), mutant 3R (lane c), and mutant 30R (lane d) probed with DIG-labelled *HincII*-digested DNA from plasmid pTet. Lane a contains *PstI*-digested DNA as size-markers.

Appendix B

E. coli strains, genotypes and references

Strain	Genotype	Reference
JM105	<i>thi pps endA sbcB15 hspR4 Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^qZΔM15</i>]	Yanisch-Perron <i>et al.</i> , 1985
JM109	<i>recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 λ₊Δ(lac-proAB)</i> [F' <i>traD36 proAB lacI^qZΔM15</i>]	Yanisch-Perron <i>et al.</i> , 1985
LK111	<i>thr1 leuB6 thi1 supE44 tonA21 r_k⁻ m_k⁺ lacY⁺ lacI^qZΔM15</i>	Zabeau and Stanley, 1982

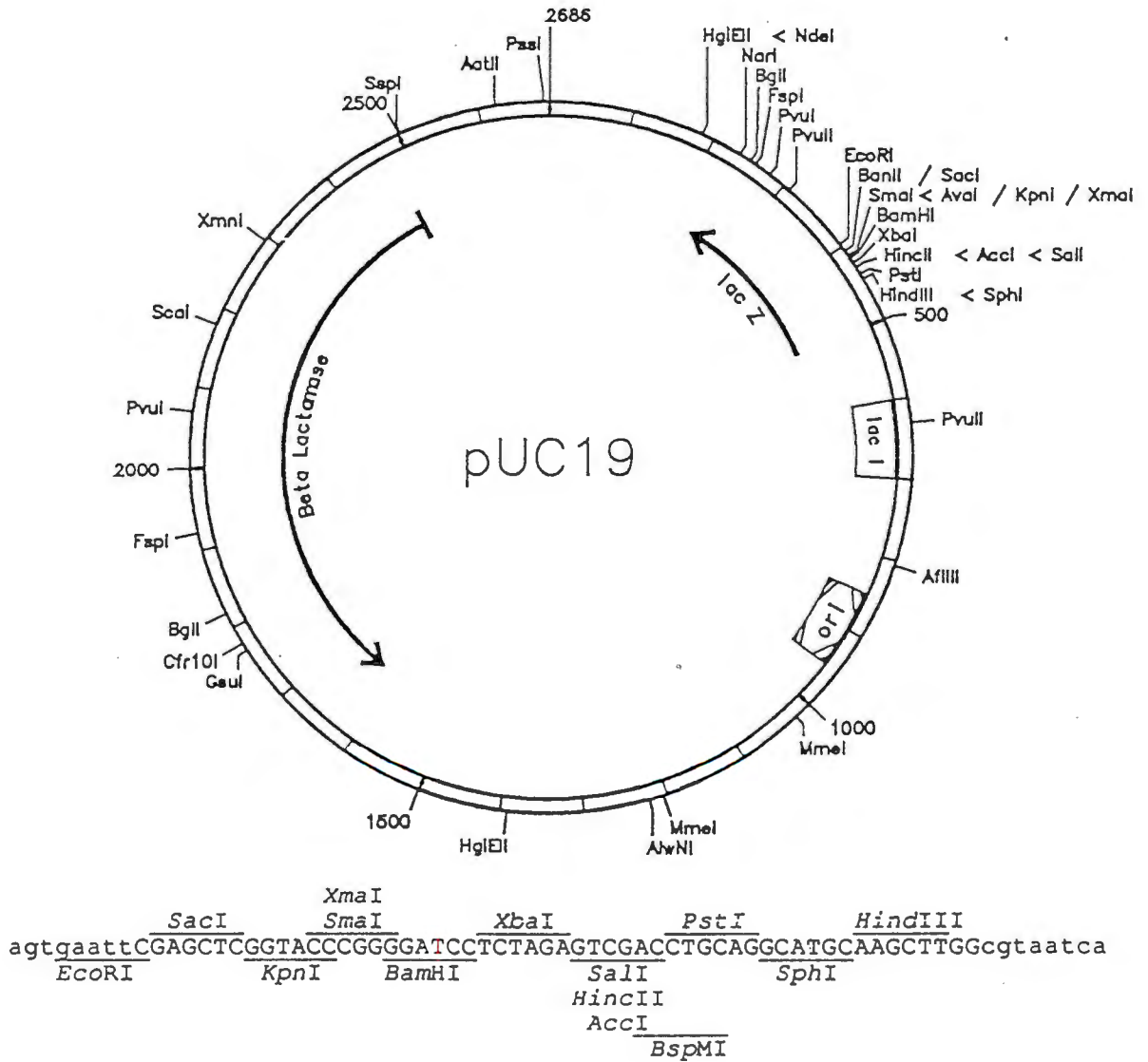
Appendix C

One- and three letter codes used for amino acids

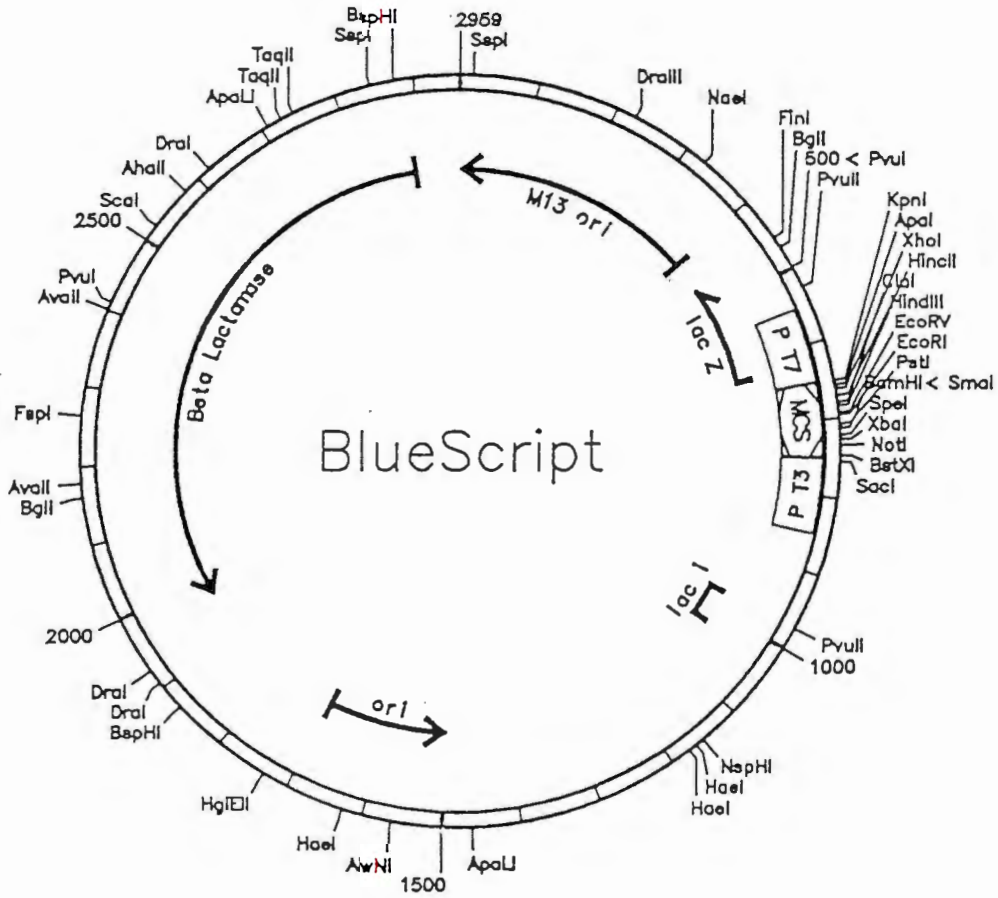
Amino acid	Codes	Code	Amino acid
Alanine	Ala A	A	Alanine
Arginine	Arg R	C	Cysteine
Asparagine	Asn N	D	Aspartic acid
Aspartic acid	Asp D	E	Glutamic acid
Cysteine	Cys C	F	Phenylalanine
Glutamine	Gln Q	G	Glycine
Glutamic acid	Glu E	H	Histidine
Glycine	Gly G	I	Isoleucine
Histidine	His H	K	Lysine
Isoleucine	Ile I	L	Leucine
Leucine	Leu L	M	Methionine
Lysine	Lys K	N	Asparagine
Methionine	Met M	P	Proline
Phenylalanine	Phe F	Q	Glutamine
Proline	Pro P	R	Arginine
Serine	Ser S	S	Serine
Threonine	Thr T	T	Threonine
Tryptophan	Trp W	V	Valine
Tyrosine	Tyr Y	W	Tryptophan
Valine	Val V	Y	Tyrosine

Appendix D

Plasmid vectors



pUC19 (2686 bp; Yanisch-Perron *et al.*, 1985). The nucleotide sequence of the multiple cloning site polylinker (in the *lacZ* gene) is shown below the circular plasmid map.

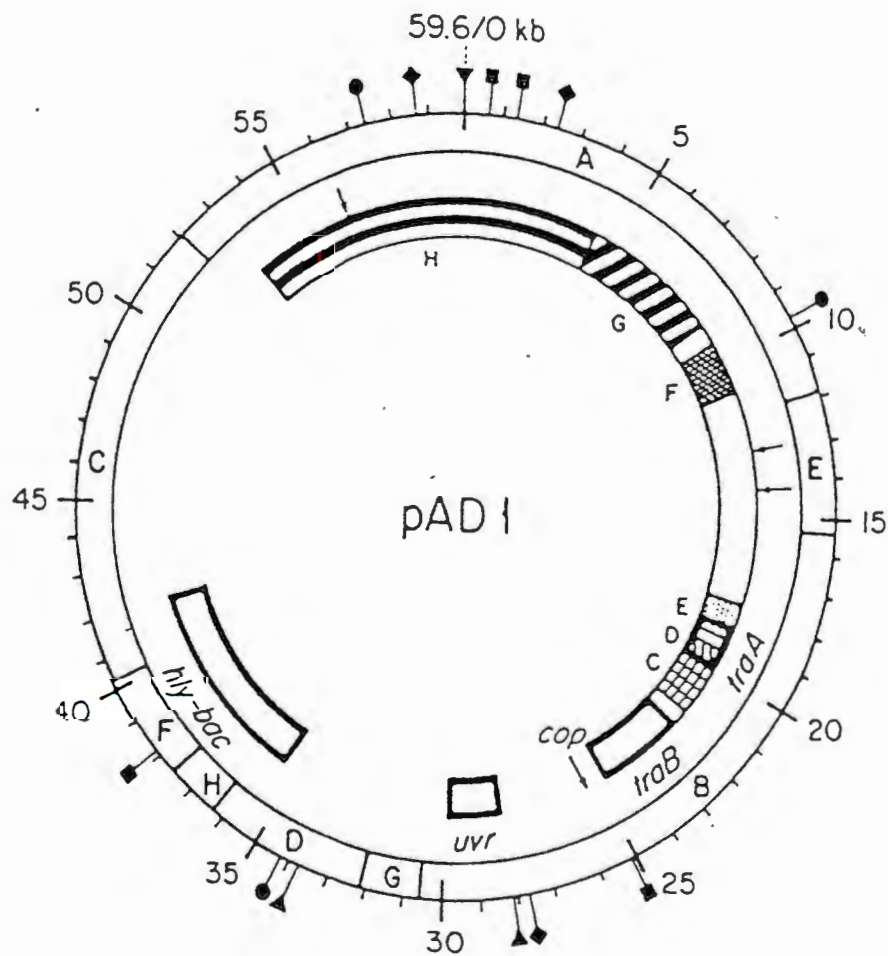


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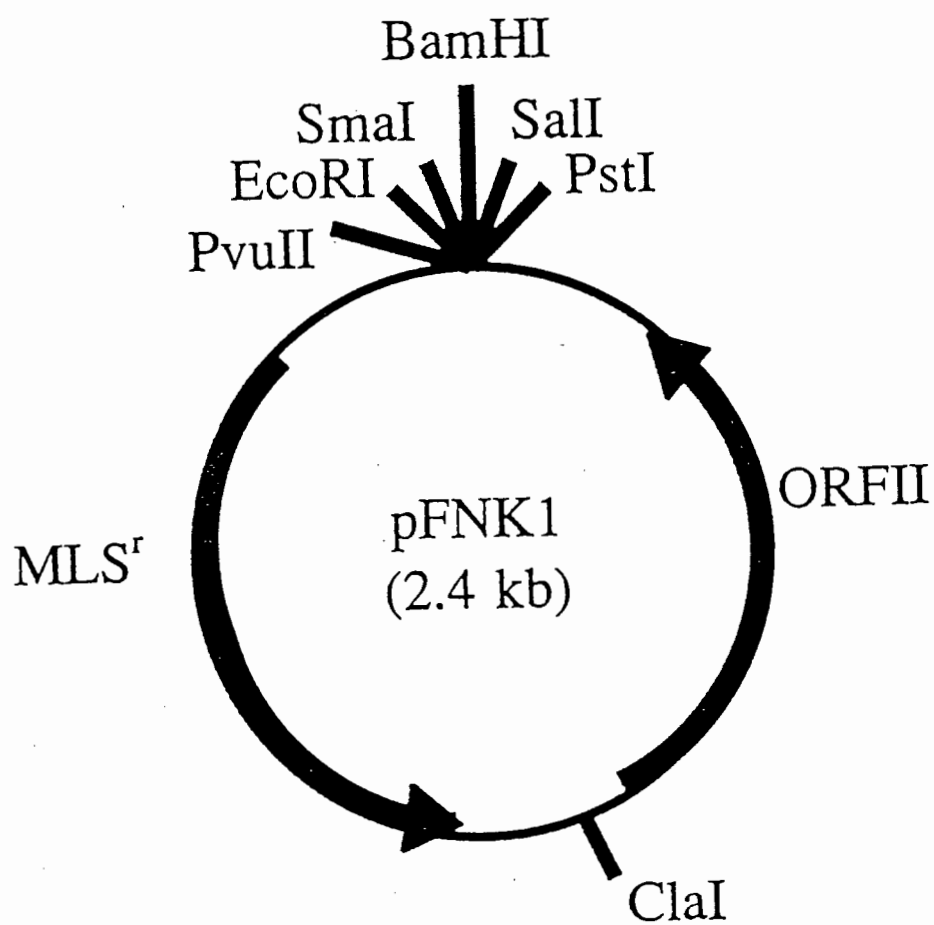
      KpnI   ApaI
GGTACCCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTC →
      DraII           SalI           HindIII           EcoRI
                   AccI
                   HincII

→ PstI   BamHI   XbaI   EagI   SacII   SacI
CTGCAGCCCGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTC
      SmaI           SpeI           NotI           BstXI
    
```

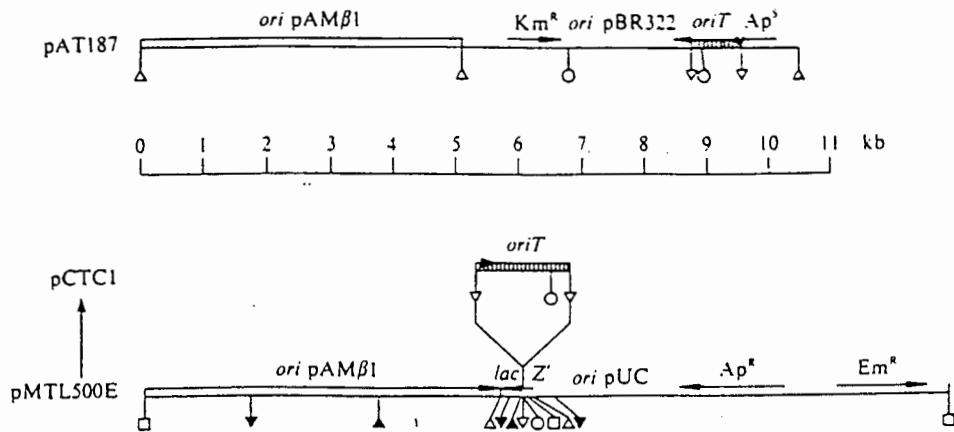
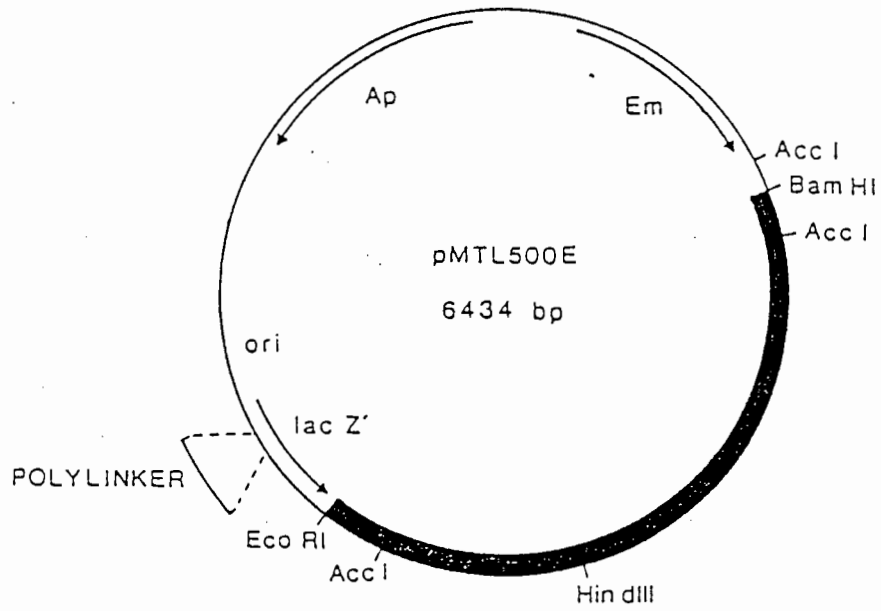
BlueScript SK (2959 bp; Stratagene, San Diego, CA). The nucleotide sequence of the multiple cloning site polylinker (in the *lacZ* gene) is shown below the circular plasmid map.



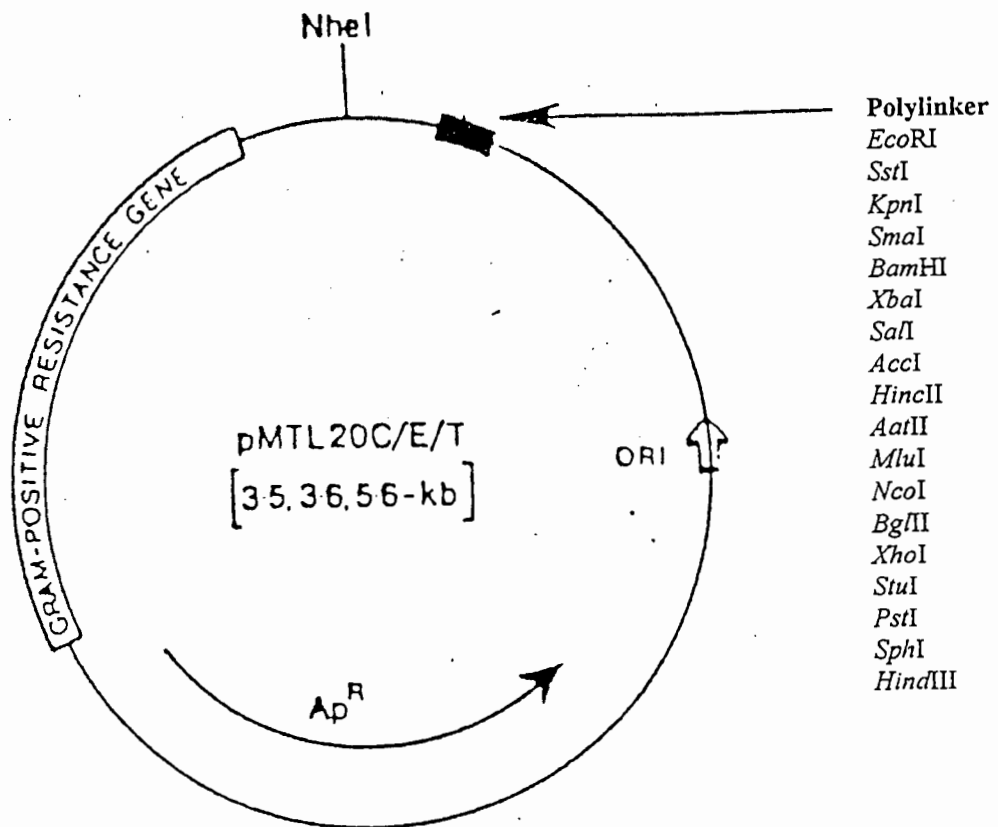
pAD1 (59.6 kb; Ehrenfeld and Clewell, 1987). Regions important for transfer are indicated as shaded or open boxes on the interior of the map and are labeled *traA*, *traB*, and C through H. Regions *hly-bac* and *uvr* represent hemolysin-bacteriocin and UV resistance determinants, respectively. *EcoRI* fragments are labeled within the outer circle. Other restriction sites are *SaII* (▼), *BamHI* (■), *KpnI* (◆), and *PstI* (●).



pFNK1 (2.4 kb; Mermelstein *et al.*, 1992), a *B. subtilis*/*C. acetobutylicum* shuttle vector. The MLS^r and putative replication (ORFII) genes are derived from the *B. subtilis* plasmid pIM13.



Origin of *C. acetobutylicum*/*E. coli* shuttle vector pCTC1 (Williams *et al.*, 1990). A 760 bp *PstI* fragment from pAT187 (Trieu-Cuot *et al.*, 1987), containing *oriT* of plasmid RK2, was inserted into the *PstI* site of pMTL500E (Oultram *et al.*, 1988) to generate pCTC1.



Origin of *C. acetobutylicum* integrative vector, pMTL30 (Minton *et al.*, 1988). The *oriT* fragment of RK2 was inserted into the *NheI* site of pMTL20E to generate pMTL30.

Literature cited

- Ahmed, M., Borsch, C.M., Taylor, S.S., Vázquez-Laslop, N. and Neyfakh, A.A. (1994). A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J Biol Chem* 269, 28506-28513.
- Aiba, H., Adhya, S. and de Crombrughe, B. (1981). Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J Biol Chem* 256, 11905-11910.
- Allcock, E.R., Reid, S.J., Jones, D.T. and Woods, D.R. (1981). Autolytic activity and an autolysis-deficient mutant of *Clostridium acetobutylicum*. *Appl Environ Microbiol* 42, 929-935.
- Allcock, E.R., Reid, S.J., Jones, D.T. and Woods, D.R. (1982). *Clostridium acetobutylicum* protoplast formation and regeneration. *Appl Environ Microbiol* 43, 719-721.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.M. and Lipman, D.J. (1990). Basic local alignment search tool. *J Mol Biol* 215, 403-410.
- Anderson, D.G. and McKay, L.L. (1983). Simple and rapid method for isolating large plasmid DNA from lactic streptococci. *Appl Environ Microbiol* 46, 549-552.
- Ayusawa, D., Yoneda, Y., Yamane, K. and Maruo, B. (1975). Pleiotropic phenomena in autolytic enzyme(s) content, flagellation and simultaneous hyperproduction of extracellular α -amylase and protease in a *Bacillus subtilis* mutant. *J Bacteriol* 124, 459-469.
- Azeddoug, H., Hubert, J. and Reysset, G. (1992). Stable inheritance of shuttle vectors based on plasmid pIM13 in a mutant strain of *Clostridium acetobutylicum*. *J Gen Microbiol* 138, 1371-1378.
- Babb, B.L. (1993). Studies on *Clostridium acetobutylicum* P262: sporulation induction and analysis of an oligosporogenous, solvent-deficient mutant. MSc Dissertation, University of Cape Town, South Africa.

- Babb, B.L., Collett, H.J., Reid, S.J. and Woods, D.R. (1993). Transposon mutagenesis of *Clostridium acetobutylicum* P262: isolation and characterization of solvent deficient and metronidazole resistant mutants. *FEMS Microbiol Lett* 114, 343-348.
- Bahl, H., Andersch, W., Braun, K. and Gottschalk, G. (1982). Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. *Eur J Appl Microbiol Biotechnol* 14, 17-20.
- Bahl, H., Gottwald, M., Kuhn, A., Rale, V., Andersch, W. and Gottschalk, G. (1986). Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*. *Appl Environ Microbiol* 52, 169-172.
- Bahl, H., Müller, H., Behrens, S., Joseph, H. and Narberhaus, F. (1995). Expression of heat shock genes in *Clostridium acetobutylicum*. *FEMS Microbiol Rev* 17, 341-348.
- Barber, J.M., Robb, F.T., Webster, J.R. and Woods, D.R. (1979). Bacteriocin production by *Clostridium acetobutylicum* in an industrial fermentation process. *Appl Environ Microbiol* 37, 433-437.
- Bartling, D., Heese, A. and Weiler, E.W. (1992). Nucleotide sequence of a cDNA encoding an *Arabidopsis* cyclophilin-like protein. *Plant Mol Biol* 19, 529-530.
- Béguin, P., Rocancourt, M., Chebrou, M.-C. and Aubert, J.-P. (1986). Mapping of mRNA encoding endoglucanase A from *Clostridium thermocellum*. *Mol Gen Genet* 202, 251-254.
- Bertram, J. and Dürre, P. (1989). Conjugal transfer and expression of streptococcal transposons in *Clostridium acetobutylicum*. *Arch Microbiol* 151, 551-557.
- Bertram, J., Kuhn, A. and Dürre, P. (1990). Tn916-induced mutants of *Clostridium acetobutylicum* defective in regulation of solvent formation. *Arch Microbiol* 153, 373-377.
- Bertram, J., Strätz, M. and Dürre, P. (1991). Natural transfer of conjugative transposon Tn916 between Gram-positive and Gram-negative bacteria. *J Bacteriol* 173, 443-448.

- Biswas, I., Gruss, A., Ehrlich, S.D. and Maguin, E. (1993). High-efficiency inactivation and replacement system for Gram-positive bacteria. *J Bacteriol* 175, 3628-3635.
- Blattner, F.R., Burland, V., Plunkett, G., III, Sofia, H.J. and Daniels, D.L. (1993). Analysis of *Escherichia coli* genome. 4. DNA sequence of the region from 89.2 to 92.8 minutes. *Nucleic Acids Res* 21, 5408-5417.
- Bowles, L.K. and Ellefson, W.L. (1985). Effects of butanol on *Clostridium acetobutylicum*. *Appl Environ Microbiol* 50, 1165-1170.
- Boynton, Z.L., Bennet, G.N. and Rudolph, F.B. (1994). Intracellular concentrations of coenzyme A and its derivatives from *Clostridium acetobutylicum* ATCC 824 and their roles in enzyme regulation. *Appl Environ Microbiol* 60, 39-44.
- Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72, 248-254.
- Brendel, V. and Trifonov, E.N. (1984). A computer algorithm for testing potential prokaryotic terminators. *Nucleic Acid Res* 12, 4411-4427.
- Brennan, R.G. and Matthews, B.W. (1989). The helix-turn-helix DNA binding motif. *J Biol Chem* 264, 1903-1906.
- Breuil, J., Dublanchet, A., Truffaut, N. and Sebald, M. (1989). Transferable 5-nitroimidazole resistance in *Bacteroides fragilis* group. *Plasmid* 21, 151-154.
- Bringel, F., van Alstine, G.L. and Scott, J.R. (1991). A host factor absent from *Lactococcus lactis* subspecies *lactis* MG1363 is required for conjugative transposition. *Mol Microbiol* 5, 2983-2993.
- Bringel, F., van Alstine, G.L. and Scott, J.R. (1992). Conjugative transposition of Tn916: the transposon *int* gene is required only in the donor. *J Bacteriol* 174, 4036-4041.
- Britz, M.L. and Wilkinson, R.G. (1979). Isolation and properties of metronidazole-resistant mutants of *Bacteroides fragilis*. *Antimicrob Agents Chemother* 16, 19-27.

- Brooker, J.D. and Lum, D.K. (1993). Use of transposon Tn916 as a genetic marker in the rumen. *Lett Appl Microbiol* 17, 224-227.
- Brooker, J.D., Lockington, R.A., Attwood, G.T. and Miller, S. (1990). The use of gene and antibody probes in identification and enumeration of rumen bacterial species. In *Gene Probes for Bacteria*, pp. 389-415. Edited by A.J.L. Macario and C de Macario. New York: Academic Press.
- Brown, D.P., Ganova-Raeva, L., Green, B.D., Wilkinson, S.R., Young, M. and Youngman, P. (1994a). Characterization of *spo0A* homologues in diverse *Bacillus* and *Clostridium* species identifies a probable DNA-binding domain. *Mol Microbiol* 14, 411-426.
- Brown, J.R., Masuchi, Y., Robb, F.T. and Doolittle, W.F. (1994b). Evolutionary relationships of bacterial and archaeal glutamine synthetase genes. *J Mol Evol* 38, 566-576.
- Brynstad, S., Iwanejko, L.A., Stewart, G.S.A.B. and Granum, P.E. (1994). A complex array of Hpr consensus DNA recognition sequences proximal to the enterotoxin gene in *Clostridium perfringens* type A. *Microbiol* 140, 97-104.
- Buchner, Y. and Edwards, D.I. (1975). The effect of mertonidazole and nitrofurans on the morphology of *Trichomonas vaginalis*. *J. Antimicrob. Chemother.* 1, 229-234.
- Burdett, V. (1990). Nucleotide sequence of the *tet(M)* gene of Tn916. *Nucleic Acid Res* 18, 6130.
- Burdett, V. (1993). tRNA modification activity is necessary for Tet(M)-mediated tetracycline resistance. *J Bacteriol* 175, 7209-7215.
- Burton, Z., Burgess, R.R., Lin, J., Moore, D., Holder, S. and Gross, C.A. (1981). The nucleotide sequence of the cloned *rpoD* gene for the RNA polymerase sigma subunit from *Escherichia coli* K-12. *Nucleic Acid Res* 9, 2889-2903.
- Buu-Hoï, A. and Horodniceanu, T. (1980). Conjugative transfer of multiple antibiotic resistance markers of *Streptococcus pneumoniae*. *J Bacteriol* 143, 313-320.

- Caillaud, F. and Courvalin, P. (1987). Nucleotide sequence of the ends of the conjugative shuttle transposon Tn1545. *Mol Gen Genet* 209:110-115.
- Calvacanti, S.M.B., Domingues, R.M.C.P., Ferreira, M.C.D. and Fihlo, F.C.E. (1991). Effect of metronidazole on the surface properties of *Bacteroides fragilis*. *J Antimicrob Chemother* 28, 819-826.
- Caparon, M.G. and Scott, J.R. (1989). Excision and insertion of the conjugative transposon Tn916 involves a novel recombination mechanism. *Cell* 59, 1027-1034.
- Carrier, C. and Courvalin, P. (1982). Resistance of streptococci to aminoglycoside-aminocyclitol antibiotics. In *Microbiology*, pp. 162-166. Edited by D. Schlessinger. Washington, DC: American Society for Microbiology.
- Cato, E.P. and Stakebrand, E. (1989). Taxonomy and phylogeny. In: *Clostridia*, pp. 1-26. Edited by N.P. Minton and D.J. Clarke. New York: Plenum Publishing Co.
- Cerkasovova, A., Novak, J., Cerkasov, J., Kulda, J. and Tachezy, J. (1988). Metabolic properties of *Trichomonas vaginalis* resistant to metronidazole under anaerobic conditions. *Acta Univ Carol Biol* 30, 505-512.
- Chen, C.C. and Cleary, P.P. (1990). Complete nucleotide sequence of the streptococcal C5a peptidase gene of *Streptococcus pyogenes*. *J Biol Chem* 265, 3161-3167.
- Chen, J.-S. and Blanchard, D.K. (1979). A simple hydrogenase-linked assay for ferredoxin and flavodoxin. *Anal Biochem* 93, 216-222.
- Chen, J.-S. (1993). Properties of acid-and solvent-forming enzymes of clostridia. In *The Clostridia and Biotechnology*, pp. 51-76. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Chen, J.-S. (1995). Alcohol dehydrogenase: multiplicity and relatedness in the solvent-producing clostridia. *FEMS Microbiol Rev* 17, 263-273.

- Chien, Y.W. (1976). Electrochemistry of drug action. I. Electroreduction of ferredoxins. *J Pharmaceut Sciences* 65, 1471-1476.
- Chou, P.Y. and Fasman, G.D. (1978). Prediction of secondary structure of proteins from their amino acid sequence. *Adv Enzymol* 47, 45-148.
- Christie, P.J. and Dunny, G.M. (1986). Identification of regions of the *Streptococcus faecalis* plasmid pCF-10 that encode antibiotic resistance and pheromone response functions. *Plasmid* 15, 230-241.
- Christie, P.J., Korman, R.Z., Zahler, S.A., Adsit, J.C. and Dunny, G.M. (1987). Two conjugation systems associated with *Streptococcus faecalis* plasmid pCF10: Identification of a conjugative transposon that transfers between *S. faecalis* and *Bacillus subtilis*. *J Bacteriol.* 169, 2529-2536.
- Chrystal, E.J.T., Koch, R.L., McLafferty, M.A. and Goldman, P. (1980). Relationship between metronidazole metabolism and bacteriacidal activity. *Antimicrob Agents Chemother* 18:566-573.
- Church, D.L. (1985). Selective toxicity of metronidazole against *Clostridium pasteurianum*. PhD Thesis, University of Calgary, Calgary, Alberta.
- Church, D.L. and Laishley, E.J. (1995). Reduction of metronidazole by hydrogenase from Clostridia. *Anaerobe* 1, 81-92.
- Church, D.L., Rabin, H.R. and Laishley, E.J. (1985). Role of the phosphoroclastic reaction of *Clostridium pasteurianum* in the reduction of metronidazole. *Antimicrob Agents Chemother* 27, 863-867.
- Church, D.L., Rabin, H.R. and Laishley, E.J. (1988). Role of hydrogenase 1 of *Clostridium pasteurianum* in the reduction of metronidazole. *Biochem Pharmacol* 37, 1525-1534.
- Church, D.L., Rabin, H.R. and Laishley, E.J. (1990). Reduction of 2-, 4- and 5-nitroimidazole drugs by hydrogenase in *Clostridium pasteurianum*. *J Antimicrob Chemother* 25, 15-23.

- Church, D.L., Rabin, H.R. and Laishley, E.J. (1991). Physiological effects of metronidazole on *Clostridium pasteurianum*. *Biochem Pharmacol* 37, 1525-1534.
- Clark, D.P. (1989). The fermentation pathways of *Escherichia coli*. *FEMS Microbiol Rev* 63, 223-234.
- Clark, S.W., Bennett, G.N. and Rudolph, R.B. (1989). Isolation and characterization of mutants of *Clostridium acetobutylicum* ATCC 824 deficient in acetoacetyl-coenzymeA:acetate/butyrate:coenzyme A transferase (EC 2.8.3.9) and in other solvent pathway enzymes. *Appl Environ Microbiol* 55, 970-976.
- Clewell, D.B., An, F.Y., White, B.A. and Gawron-Burke, C. (1985). *Streptococcus faecalis* sex pheromone (cAM373) also produced by *Staphylococcus aureus* and identification of a conjugative transposon (Tn918). *J Bacteriol* 162, 1212-1220.
- Clewell, D.B., Ehrenfeld, E.E., Kessler, R.E., Ike, Y., Franke, A.E., Madion, M., Shaw, J.H., Wirth, R., An, F., Mori, M., Kitada, C., Fujino, M. and Suzuki, A. (1986). Sex-pheromone systems in *Streptococcus faecalis*. In *Banbury Report 24: Antibiotic Resistance Genes: Ecology, Transfer and Expression* pp. 131-142. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.
- Clewell, D.B., Flannagan, S.E., Ike, Y., Jones, J.M. and Gawron-Burke, C. (1988). Sequence analysis of termini of conjugative transposon Tn916. *J Bacteriol* 170, 3046-3052.
- Collins, M.D., Lawson, P.A., Willems, A., Cordoba, J.J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. and Farrow, J.A.E. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44, 812-826.
- Conradt, H., Hohmann-Berger, M., Hohmann, H.P., Blaschkowski, H.P. and Knappe, J. (1984). Pyruvate formate-lyase (inactive form) and pyruvate formate-lyase activating enzyme of *Escherichia coli*: isolation and structural properties. *Arch Biochem Biophys* 122, 133-142.

- Contag, P.R., Williams, M.G. and Rogers, P. (1990). Cloning of lactate dehydrogenase gene from *Clostridium acetobutylicum* B643 and expression in *Escherichia coli*. *Appl Environ Microbiol* **56**, 3760-3765.
- Courvalin, P. and Carlier, C. (1986). Transposable multiple antibiotic resistance in *Streptococcus pneumoniae*. *Mol Gen Genet* **205**, 291-297.
- Croux, C. and Garcia, J.L. (1991). Sequence of the *lyc* gene encoding the autolytic lysozyme of *Clostridium acetobutylicum* ATCC 824: comparison with other lytic enzymes. *Gene* **104**, 25-31.
- Dachs, G.U., Abratt, V.R. and Woods, D.R. (1995). Mode of action of metronidazole and a *Bacteroides fragilis metaA* resistance gene in *Escherichia coli*. *J Antimicrob Chemother* **35**, 483-496.
- Datta, R. and Zeikus, J.G. (1985). Modulation of acetone-butanol-ethanol fermentation by carbon monoxide and organic acids. *Appl Environ Microbiol* **49**, 522-529.
- Davies, A., Oultram, J.D., Pennock, A., Williams, D.R., Richards, D.F., Minton, N.P. and Young, M. (1988). Conjugal gene transfer in *Clostridium acetobutylicum*. In *Genetics and Biotechnology of Bacilli*, pp. 391-395. Edited by A.T. Ganaesan and J.A. Hoch. Orlando: Academic Press.
- Deléage, G., Clerc, F.F., Roux, B. and Gautheron, D.C. (1988). Antheprot: a package for protein sequence analysis using a microcomputer. *Carbios* **4**, 351-356.
- Devereux, J., Haerberli, P. and Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucleic Acids Res* **12**, 387-395.
- Doetsch, R.N. (1981). Methods of light microscopy. In *Manual of Methods for General Bacteriology*, pp. 21-51. Edited by P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. New York: American Society for Microbiology.

- Doremus, M.G., Linden, J.C. and Moreira, A.R. (1985). Agitation and pressure effects on acetone-butanol fermentation. *Biotechnol Bioeng* 27, 852-860.
- Dubnau, D. (1993). Genetic exchange and homologous recombination. In *Bacillus subtilis and Other Gram-positive Bacteria. Biochemistry, Physiology, and Molecular genetics*, pp. 555-584. Edited by A.L. Sonenshine, J.A. Hoch and R. Losick. Washington, D.C: American Society for Microbiology.
- Dürre, P., Fischer, R.-J., Kuhn, A., Lorenz, K., Schreiber, W., Stürzenhofecker, B., Ullmann, S., Winzer, K. and Sauer, U. (1995). Solventogenic enzymes of *Clostridium acetobutylicum*: catalytic properties, genetic organization, and transcriptional regulation. *FEMS Microbiol Rev* 17, 251-262.
- Dürre, P., Kuhn, A., Gottwald, M. and Gottschalk, G. (1987). Enzymatic investigations on butanol dehydrogenase and butyraldehyde dehydrogenase in extracts of *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol* 26, 268-272.
- Edwards, D. I. (1980). Antimicrobial Drug Action, pp. 235-243. London: MacMillan Press Ltd.
- Edwards, D.I. (1986). Reduction of nitroimidazoles and DNA damage. *Biochem Pharmacol* 35, 53-58.
- Edwards, D.I. (1993a). Nitroimidazole drugs- action and resistance mechanisms I. Mechanisms of action. *J Antimicrob Chemother* 31, 9-20.
- Edwards, D.I. (1993b). Nitroimidazole drugs- action and resistance mechanisms II. Mechanisms of resistance. *J Antimicrob Chemother* 31, 201-210.
- Edwards, D.I. and Mathison, G.E. (1970). The mode of action of metronidazole against *Trichomonas vaginalis*. *J Gen Microbiol* 63, 297-302.
- Edwards, D.I., Dye, M. and Carne, H. (1973). The selective toxicity of antimicrobial nitroheterocyclic drugs. *J Gen Microbiol* 76, 135-145.

- Edwards, D.I., Rowley, D.A., Knox, R.J., Skolimowski, I.M. and Knight, R.C. (1980). Nature of DNA damage induced by electrolytically reduced nitroimidazole drugs. In *Current Chemotherapy and Infectious Disease*, pp. 561-575. Edited by J.D. Nelson and C. Grassi. Washington, DC: The American Society for Microbiology.
- Ehrenfeld, E.E. and Clewell, D.B. (1987). Transfer functions of the *Streptococcus faecalis* plasmid pAD1: organization of plasmid DNA encoding response to sex pheromone. *J Bacteriol* **169**, 3473-3481.
- Errington, J (1990). Gene cloning techniques. In *Molecular Biological Methods for Bacillus*, pp.175-220. Edited by C.R. Harwood and S.M. Cutting. Chichester: Wiley.
- Errington, J. (1993). *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. *Microbiol Rev* **57**, 1-33.
- Fan, S.P. (1970). Autolysin(s) of *Bacillus subtilis* as dechaining enzyme. *J Bacteriol* **103**, 494-499.
- Fein, J.E. (1979). Possible involvement of bacterial autolytic enzymes in flagellar morphogenesis. *J Bacteriol* **137**, 933-946.
- Fein, S.E. and Rogers, H.J. (1976). Autolytic enzyme-deficient mutants of *Bacillus subtilis*. *J Bacteriol* **127**, 1427-1442.
- Ferrari, F., Trach, K., LeCoq, D., Spence, J., Ferrari, E. and Hoch, J.A. (1985). Characterization of the *spo0A* locus and its deduced product. *Proc Natl Acad Sci USA* **82**, 2647-2651.
- Fischer, R.J., Helms, J. and Dürre, P. (1993). Cloning, sequencing, and molecular analysis of the *sol* operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. *J Bacteriol* **175**, 6959-6969.
- Fitzgerald, G.F. and Clewell, D.B. (1985). A conjugative transposon (Tn919) in *Streptococcus sanguis*. *Infect Immun* **47**, 415-420.

- Flannagan, S.E. and Clewell, D.B. (1991). Conjugative transfer of Tn916 in *Enterococcus faecalis*: *trans* activation of homologous transposons. *J Bacteriol.* 173, 7136-7141.
- Flannagan, S.E., Zitzow, L.A. and Clewell, D.B. (1994). Nucleotide sequence of the 18-kb conjugative transposon Tn916 from *Enterococcus faecalis*. *Plasmid* 32, 350-354.
- Foster, S.J. (1991). Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. *J Gen Microbiol* 137, 1987-1998.
- Foster, S.J. and Johnstone, K. (1990). Pulling the triggers: the mechanism of bacterial spore germination. *Mol Microbiol* 4, 137-141.
- Fourney, R.M., Miyskoshi, J., Day, R.S., III and Paterson, M.C. (1988). Northern blotting: efficient RNA staining and transfer. *Focus* 10, 5-7.
- Franke, A.E. and Clewell, D.B. (1981). Evidence for a chromosome-borne resistance transposon (Tn916) in *Streptococcus faecalis* that is capable of conjugal transfer in the absence of conjugative plasmid. *J Bacteriol* 145, 494-502.
- Gaur, N.K., Dubnau, E. and Smith, I. (1986). Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. *J Bacteriol* 168, 860-869.
- Gaur, N.K., Oppenheim, J. and Smith, I. (1991). The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. *J Bacteriol* 173, 678-686.
- Gerischer, U. and Dürre, P. (1990). Cloning, sequencing, and molecular analysis of the acetoacetate decarboxylase gene region from *Clostridium acetobutylicum*. *J Bacteriol* 172, 6907-6918.
- Gerischer, U. and Dürre, P. (1992). mRNA analysis of the *adc* gene region of *Clostridium acetobutylicum* during the shift to solventogenesis. *J Bacteriol* 174, 426-433.
- Gest, H. and Peck, H.D., Jr (1955). A study of the hydrogenlyase reaction with systems derived from normal and anaerogenic coli - aerogenes bacteria. *J Bact* 70, 326-334.

- Girbal, L. and Soucaille, P. (1994). Regulation of *Clostridium acetobutylicum* metabolism as revealed by mixed-substrate steady-state continuous cultures: role of NADH/NAD ratio and ATP pool. *J Bacteriol* 176, 6433-6438.
- Girbal, L., Croux, C., Vasconcelos, I. and Soucaille, P. (1995). Regulation of metabolic shifts in *Clostridium acetobutylicum* ATCC 824. *FEMS Microbiol Rev* 17, 287-297.
- Girbal, L., Vasconcelos, I. and Soucaille, P. (1994). Transmembrane pH of *Clostridium acetobutylicum* is inverted (more acidic) when the *in vivo* activity of hydrogenase is decreased. *J Bacteriol* 176, 6146-6147.
- Goldman, P. (1980). Metronidazole: proven benefits and potential risks. *The John Hopkins Med J* 147, 1-9.
- Goldstein, B.P., Nielsen, E., Berti, M., Bolzoni, G. and Silvestri, L.G. (1977). The mechanism of action of nitro-heterocyclic antimicrobial drugs. Primary target of 1-methyl-2-nitro-5-vinylimidazole is DNA. *J Gen Microbiol* 100, 271-281.
- Greenberg, J.T. and Demple, B. (1989). A global response induced in *Escherichia coli* by redox-cycling agents overlaps with that induced by peroxide stress. *J Bacteriol* 171, 3933-3939.
- Grupe, H. and Gottschalk, G. (1992). Physiological events in *Clostridium acetobutylicum* during the shift from acidogenesis to solventogenesis in continuous culture and presentation of a model for shift induction. *Appl Environ Microbiol* 58, 3896-3902.
- Guffanti, A.A., Quirk, P.G. and Krulwich, T.A. (1991). Transfer of Tn925 and plasmids between *Bacillus subtilis* and alkaliphilic *Bacillus firmus* OF4 during Tn925-mediated conjugation. *J Bacteriol* 173, 1686-1689.
- Guild, W.R., Smith, M.D. and Shoemaker, N.B. (1982). Conjugative transfer of chromosomal R determinants in *Streptococcus pneumoniae*. Resistance of streptococci to aminoglycoside-aminocyclitol antibiotics. In *Microbiology*, pp. 88-92. Edited by D. Schlessinger, Washington, DC: American Society for Microbiology.

- Hager, P.W. and Rabinowitz, J.C. (1985). Translational specificity in *Bacillus subtilis*. In *The Molecular Biology of the Bacilli*, pp.1-32. Edited by D. Dubnau. New York: Academic Press.
- Haggoud, A., Reysset, H. and Sebald, M. (1992). Cloning of a *Bacteroides fragilis* chromosomal determinant coding for 5-nitroimidazole resistance. *FEMS Microbiol Lett* **74**, 1-5.
- Haggoud, A., Reysset, H., Azeddoug, H. and Sebald, M. (1994). Nucleotide sequence analysis of two 5-nitroimidazole resistance determinants from *Bacteroides* strains and of a new insertion sequence upstream of the two genes. *Antimicrob Agents Chemother* **38**, 1047-1051.
- Haldenwang, W.G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59**, 1-30.
- Hartmanis, M.G.N. and Gatenbeck, S. (1984). Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate. *Appl Environ Microbiol* **47**, 1277-1283.
- Hayano, T., Takahashi, N., Kato, S., Maki, N. and Suzuki, M. (1991). Two distinct forms of peptidyl prolyl-cis-trans-isomerase are expressed separately in periplasmic and cytoplasmic compartments of *Escherichia coli* cells. *Biochemistry* **30**, 3041-3048.
- Hayashida, S. and Yoshino, S. (1990). Degeneration of solventogenic *Clostridium* caused by a defect in NADH generation. *Agric Biol Chem.* **54**, 427-435.
- Hendrick, C.A., Johnson, L.K., Tomes, N.J., Smiley, B.K. and Price, J.P. (1991). Insertion of Tn916 into *Bacillus pumilis* plasmid pMGD302 and evidence for plasmid transfer by conjugation. *Plasmid* **26**, 1-9.
- Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351-359.
- Hermann, M., Fayolle, F., Marchal, R., Podvin, L., Sebald, M. and Vandecasteele, J.P. (1985). Isolation and characterization of butanol-resistant mutants of *Clostridium acetobutylicum*. *Appl Environ Microbiol* **50**, 1238-1243.

- Herrero, A.A. (1983). End-product inhibition in anaerobic fermentations. *Trends Biotechnol* **1**, 49-53.
- Hippe, H., Andreesen, J.R. and Gottschalk, G. (1992). The genus *Clostridium* - nonmedical. In: *The Prokaryotes* Vol. II, pp. 1800-1866. Edited by A. Barlows, H.G. Trüper, M. Dworkin, W. Harder and K.H. Schleifer. New York: Springer Verlag.
- Hoch, J.A (1993). *spo0A* genes, the phosphorelay, and the initiation of sporulation. In *Bacillus subtilis and Other Gram-positive Bacteria. Biochemistry, Physiology and Molecular biology*, pp. 785-800. Edited by A.L. Sonenshein, J.A. Hoch and R. Losick. Washington, D.C: American Society for Microbiology.
- Hongo, M., Murata, A., Kono, K. and Kato, F. (1968). Lysogeny and bacteriocinogeny in strains of *Clostridium* species. *Agric Biol Chem* **32**, 27-33.
- Honjo, M., Nakayama, A., Fukazawa, K., Kawamura, K., Ando, K., Hori, M. and Furutani, Y. (1990). A novel *Bacillus subtilis* gene involved in negative control of sporulation and degradative-enzyme production. *J Bacteriol* **172**, 1783-1790.
- Huang, L., Gibbins, L.N. and Forsberg, C.W. (1985). Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions. *Appl Environ Microbiol* **50**, 1043-1047.
- Hüsemann, M.H.W. and Papoutsakis, E.T. (1989). Comparison between *in vivo* and *in vitro* enzyme activities in continuous and batch fermentations of *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol* **30**, 585-595.
- Hutkins, R.W. and Kashket, E.R. (1986). Phosphotransferase activity in *Clostridium acetobutylicum* from acidogenic and solventogenic phases of growth. *Appl Environ Microbiol* **51**, 1121-1123.
- Ingham, H.R., Hall, C.J., Sisson, P.R., Tharagonnet, D. and Selkon, J.B. (1980). The activity of metronidazole against facultatively anaerobic bacteria. *J Antimicrob Chemother* **6**, 343-347.

- Ings, R.M.J, McFadzean, J.A. and Ormead, W.E. (1974). The mode of action of metronidazole in *Trichomonas vaginalis* and other micro-organisms. *Biochem Pharmacol* **23**, 1421-1429.
- Ish-Horowicz, D. and Burke, J.F. (1981). Rapid and efficient cosmid cloning. *Nucleic Acid Res* **9**, 2989-2998.
- Jackson, D., Salem, A. and Coombs, G.H. (1984). The *in vitro* activity of metronidazole against strains of *Escherichia coli* with impaired DNA repair systems. *J Antimicrob Chemother* **13**, 227-236.
- Jaworski, D.D. and Clewell, D.B. (1994). Evidence that coupling sequences play a frequency-determining role in conjugative transposition of Tn916 in *Enterococcus faecalis*. *J Bacteriol* **176**, 3328-3335
- Johnson, J.L. and Chen, J.-S. (1995). Taxonomic relationships among strains of *Clostridium acetobutylicum* and other phenotypically similar organisms. *FEMS Microbiol Rev* **17**, 233-240.
- Johnson, P.J. (1993). Metronidazole and drug resistance. *Parasitol Today* **9**, 183-186.
- Jones, D.T. and Keis, S. (1995). Origins and relationships of industrial solvent-producing clostridial strains. *FEMS Microbiol Rev* **17**, 221-230.
- Jones, D.T. and Woods, D.R. (1986). Acetone-butanol fermentation revisited. *Microbiol Rev* **50**, 484-524.
- Jones, D.T., van der Westhuizen, A., Long, S., Allcock, E.R., Reid, S.J. and Woods, D.R. (1982). Solvent production and morphological changes in *Clostridium acetobutylicum*. *Appl Environ Microbiol* **43**, 1434-1439.
- Joris, B., van Beeumen, J., Casagrande, F., Gerday, C., Frere, J.M. and Ghysen, J.M. (1983). The complete amino acid sequence of the Zn²⁺-containing D-alanyl-D-alanine-cleaving carboxypeptidase of *Streptomyces albus*. *Eur J Biochem* **130**, 53-69.

- Junelles, A.M., Janati-Idrissi, R., Petitdemange, H. and Gay, R. (1988). Iron effect on acetone-butanol fermentation. *Curr Microbiol* **17**, 299-303.
- Kappen, L.S., Lee, T.R., Yang, C. and Goldberg, I.H. (1989). Oxygen transfer from the nitro group of a nitroaromatic radiosensitizer to a DNA sugar damage product. *Biochemistry* **28**, 4540-4542.
- Kappus, H. and Sies, H. (1981). Toxic drug effects associated with oxygen metabolism: redox cycling and lipid peroxidation. *Experientia* **37**, 1233-1241.
- Kashket, E.R. and Cao, Z-Y. (1993). Isolation of a degeneration-resistant mutant of *Clostridium acetobutylicum* NCIMB 8052. *Appl Environ Microbiol* **59**, 4198-4202.
- Kashket, E.R. and Cao, Z-Y. (1995). Clostridial strain degeneration. *FEMS Microbiol Rev* **17**, 307-315.
- Keilty, S. and Rosenberg, M. (1987). Constitutive function of a positively regulated promoter reveals new sequences essential for activity. *J Biol Chem* **262**, 6389-6395.
- Keis, S., Bennet, C.F., Ward, V.K. and Jones, D.T. (1995). Taxonomy and phylogeny of industrial solvent-producing clostridia. *Int J Sys Bacteriol* **45**, 693-705.
- Kessler, D., Herth, W. and Knappe, J. (1992). Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of *Escherichia coli*. *J Biol Chem* **267**, 18073-18079.
- Kessler, D., Leibrecht, I. and Knappe, J. (1991). Pyruvate-formate-lyase deactivase and acetyl-CoA reductase activities of *Escherichia coli* reside on a polymeric protein particle encoded by *adhE*. *FEBS Lett* **281**, 59-63.
- Kim, B. H. and Zeikus, J.K. (1985). Importance of hydrogen metabolism in regulation of solventogenesis by *Clostridium acetobutylicum*. *Dev Ind Microbiol* **26**, 1-14.

- Kim, B.H., Bellows, R., Datta, R. and Zeikus, J.G. (1984). Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields. *Appl Environ Microbiol* **48**, 764-770.
- Kim, T.S. and Kim, B.H. (1988). Electron flow shift in *Clostridium acetobutylicum* fermentation by electrochemically introduced reducing equivalent. *Biotechnol Lett* **10**, 123-128.
- Kiwaki, M., Ikemura, H., Shimizu-Kadota, M. and Hirashima, A. (1989). Molecular characterization of a cell wall-associated proteinase gene from *Streptococcus lactis* NCD0763.
- Kleier, A., Msadek, T. and Rapoport, G. (1992). Positive regulation in the Gram-positive bacterium: *Bacillus subtilis*. *Ann Rev Microbiol* **46**, 429-459.
- Kletzin, A., Mukund, S., Kelley-Crouse, T.L., Chan, M.K., Rees, D.C. and Adams, M. W.W. (1995). Molecular characterization of the genes encoding the tungsten-containing aldehyde ferredoxin oxidoreductase and formaldehyde ferredoxin oxidoreductase from *Thermococcus litoralis*. *J Bacteriol* **177**, 4817-4819.
- Knappe, J. (1987). Anaerobic dissimilation of pyruvate. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular biology*, Vol 1, pp. 151-156. Edited by F.C. Neidhardt. Washington, DC: American Society for Microbiology.
- Knappe, J. and Sawers, G. (1990). A radical-chemical route to acetyl-CoA: the anaerobically induced pyruvate formate-lyase system of *Escherichia coli*. *FEMS Microbiol Rev* **75**, 383-398.
- Knappe, J., Neugebauer, F.A., Blaschkowski, H.P. and Gänzler, M. (1984). Post-translational activation introduces a free radical into pyruvate formate-lyase. *Proc Natl Acad Sci USA* **81**, 1332-1335.
- Knight, R.C., Skolimowski, I.M. and Edwards, D.I. (1978). The interaction of reduced metronidazole with DNA. *Biochem Pharmacol* **27**, 2089-2093.

- Knox, R.J., Knight, R.C. and Edwards, D.I. (1981). Misonidazole-induced thymine release from DNA. *Biochem Pharmacol* 30, 1925-1929.
- Knox, R.J., Knight, R.C. and Edwards, D.I. (1983). Studies on the action of nitroimidazole reduction. *Biochem Pharmacol* 32, 2149-2156.
- Koide, Y., Nakamura, A., Uozumi, T. and Beppu, T. (1986). Cloning and sequencing of the major intracellular serine protease gene of *Bacillus subtilis*. *J Bacteriol* 167, 110-116.
- Kok, J., Leenhouts, K., Haandrikman, A.J., Ledebouer, A.M. and Venema, G. (1988). Nucleotide sequence of the cell wall proteinase gene of *Streptococcus cremoris* Wg2. *Appl Environ Microbiol* 54, 231-238.
- Kondo, Y., Shomura, T., Ogawa, Y., Tsuruoka, T., Watanabe, H., Totsukawa, K., Suzuki, T., Moriyama, C., Yoshida, J., Inouye, S. and Miida, T. (1973). Studies of a new antibiotic, SF-1293. I. Isolation and physicochemical and biological characterization of SF-1293 substances. *Sci Rep Meiji Seika Kaisha* 13, 34-41.
- Koo, H-S., Wu, H-M. and Crothers, D.M. (1986). DNA bending at adenine-thymine tracts. *Nature* 320, 501-506.
- Kraut, J. (1977). Serine proteases: structure and mechanism of catalysis. *Ann Rev Biochem* 46, 331-358.
- Krüger, E., Völker, U. and Hecker, M. (1994). Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J Bacteriol* 176, 3360-3367.
- Kulda, J., Tachezy, J. and Cerkasovova, A. (1993). *In vitro* induced anaerobic resistance to metronidazole in *Trichomonas vaginalis*. *J Euk Microbiol* 40, 262-269.
- Kulda, J.E., Nohýnková, E. and Ludvík, J. (1987). Basic structure and function of the trichomonad cell. *Acta Univ Carol-Biol* 30, 181-198.
- Kuroda, A. and Sekiguchi, J. (1990). Cloning, sequencing and genetic mapping of a *Bacillus subtilis* cell wall hydrolase. *J Gen Microbiol* 136, 2209-2216.

- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- LaRusso, N.F., Tomasz, F., Müller, M. and Lipman, R. (1977). *In vitro* interaction of metronidazole with nucleic acids. *Mol Pharmacol* **13**, 872-882.
- Lawson, P.A., Llop-Perez, P., Hutson, R.A., Hippe, H. and Collins, M.D. (1993). Towards a phylogeny of the clostridia based on 16S rRNA sequences. *FEMS Microbiol Lett* **113**, 87-92.
- Lee, S.Y., Mermelstein, L.D. and Papoutsakis, E.T. (1993). Determination of plasmid copy number and stability in *Clostridium acetobutylicum* ATCC 824. *FEMS Microbiol Lett* **108**, 319-324.
- Lin, W-J. and Johnson, E.A. (1991). Transposon Tn916 mutagenesis in *Clostridium botulinum*. *Appl Environ Microbiol* **57**, 2946-2950.
- Lindmark, D.G. and Müller, M. (1973). Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Tritrichomonas foetus*, and its role in pyruvate metabolism. *J Biol Chem* **248**, 7724-7728.
- Lindmark, D.G. and Müller, M. (1976). Antitrichomonad action, mutagenicity, and reduction of metronidazole and other nitroimidazoles. *Antimicrob Agents Chemother* **10**, 476-482.
- Ljungdahl, L.G. and Andreesen, J.R. (1978). Formate dehydrogenase, a selenium-tungsten enzyme from *Clostridium thermoaceticum*. *Methods Enzymol* **53**, 360-372.
- Lloyd, D. and Kristensen, B. (1985). Metronidazole inhibition of hydrogen production *in vivo* in drug sensitive and resistant strains of *Trichomonas vaginalis*. *J Gen Microbiol* **131**, 849-853.
- Lloyd, D. and Pedersen, J.Z. (1985). Metronidazole radical anion generation *in vivo* in *Trichomonas vaginalis*: oxygen quenching is enhanced in a drug resistant strain. *J Gen Microbiol* **131**, 87-92.

- Lockerby, D.L., Rabin, H.R., Bryan, L.E. and Laishley, E.J. (1984). Ferredoxin-linked reduction of metronidazole in *Clostridium pasteurianum*. *Antimicrob Agents Chemother* **26**, 665-669.
- Long, S. (1984). Studies on solvent production and endospore formation in *Clostridium acetobutylicum* P262. PhD thesis, University of Cape Town, South Africa.
- Long, S., Jones, D.T. and Woods, D.R. (1983). Sporulation of *Clostridium acetobutylicum* P262 in a defined medium. *Appl Environ Microbiol* **45**, 1389-1393.
- Long, S., Jones, D.T., Woods, D.R. (1984a). Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262. *Appl Microbiol Biotechnol* **20**, 256-261.
- Long, S., Jones, D.T. and Woods, D.R. (1984b). The relationship between sporulation and solvent production in *Clostridium acetobutylicum* P262. *Biotechnol Lett* **6**, 529-534.
- Lu, F. and Churchward, G. (1994). Conjugative transposition: Tn916 integrase contains two independent DNA binding domains that recognize different DNA sequences. *EMBO J* **13**, 1541-1548.
- Lu, F. and Churchward, G. (1995). Tn916 target DNA sequences bind the C-terminal domain of integrase protein with different affinities that correlate with transposon insertion frequency. *J Bacteriol* **177**, 1938-1946.
- Ludlum, D.B., Colinas, R.J., Kirk, M.C. and Mehta, J.R. (1988). Reaction of reduced metronidazole with guanosine to form unstable adduct. *Carcinogen* **9**, 593-596.
- Maguin, E., Duwat, P., Hege, T., Ehrlich, D. and Gruss, A. (1992). New thermosensitive plasmid for Gram-positive bacteria. *J Bacteriol* **174**, 5633-5638.
- Marczak, R., Ballongue, H., Petitdemange, H. Alimi, F. and Gay, R. (1983a). Biochimie cellulaire - influence de la phase de croissance et de la composition du milieu sur le taux de biosynthese de la NADH: rubredoxin oxydoreductase chez *Clostridium acetobutylicum*. *C R Acad Sci* **3**, 469-474.

- Marczak, R., Ballongue, H., Petitdemange, H. and Gay, R. (1985). Differential levels of ferredoxin and rubredoxin in *Clostridium acetobutylicum*. *Biochimie* 67, 241-248.
- Marczak, T., Gorell, T.E. and Müller, M. (1983b). Hydrogenosomal ferredoxin of the anaerobic protozoan, *Tritrichomonas foetus*. *J Biol Chem* 258, 12427-12433.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* 3, 208-218.
- Mathews, F.S. (1985). The structure, function and evolution of cytochromes. *Prog Biophys Mol Biol* 45, 1-56.
- McFazdean, J.A. (1986). Flagyl. The story of a pharmaceutical discovery. London: The Parthenon Publishing Group Ltd.
- Meinecke, B., Bahl, H. and Gottschalk, G. (1984). Selection of an asporogenous strain of *Clostridium acetobutylicum* in continuous culture under phosphate limitation. *Appl Environ Microbiol* 48, 1064-1065.
- Meinecke, B., Bertram, J. and Gottschalk, G. (1989). Purification and characterization of the pyruvate-ferredoxin oxidoreductase from *Clostridium acetobutylicum*. *Arch Microbiol* 152, 244-250.
- Mermelstein, L.D. and Papoutsakis, E.T. (1993). *In vivo* methylation in *Escherichia coli* by the *Bacillus subtilis* phage ϕ 3T I methyltransferase to protect plasmids from restriction upon transformation of *Clostridium acetobutylicum* ATCC 824. *Appl Environ Microbiol* 59, 1077-1081.
- Mermelstein, L.D., Welker, N.E., Bennett, G.N. and Papoutsakis, E.T. (1992). Expression of cloned homologous fermentative genes in *Clostridium acetobutylicum* ATCC 824. *Biotechnol* 10, 190-195.
- Messing, J. (1983). New M13 vectors for cloning. *Methods Enzymol* 101, 20-78.

- Meyer, C.L. and Papoutsakis, E.T (1989a). Increased levels of ATP and NADH are associated with increased solvent production in continuous cultures of *Clostridium acetobutylicum*. *Biotechnol Lett* 7, 37-42.
- Meyer, C.L. and Papoutsakis, E.T (1989b). Continuous and biomass recycle fermentations of *Clostridium acetobutylicum* in a two-stage phosphate limited chemostat. *Eur J Appl Microbiol Biotechnol* 15, 201-205.
- Meyer, C.L., Roos, J.W. and Papoutsakis, E.T. (1986). Carbon monoxide gassing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol* 24, 159-167.
- Minton, N.P., Brehm, J.K., Oultram, J.D., Swinfield, T.J. and Thompson, D.E. (1988). Construction of plasmid vector systems for gene transfer in *Clostridium acetobutylicum*. In *Anaerobes Today*, pp. 125-140. Edited by J.M. Hardie and S.P. Borriello. Chichester: Wiley.
- Mishra, S., Béguin, P. and Aubert, J.-P. (1991). Transcription of *Clostridium thermocellum* endoglucanase genes *celF* and *celD*. *J Bacteriol* 173, 80-85.
- Miyata, S., Moriyama, R., Miyahara, N. and Makino, S. (1995a). A gene (*sleC*) encoding a spore-cortex-lytic enzyme from *Clostridium perfringens* S40 spores; cloning, sequence analysis and molecular characterization. *Microbiol* 141, 2643-2650.
- Miyata, S., Moriyama, R., Sugimoto, K. and Makino, S. (1995b). Purification and partial characterization of a spore-cortex-lytic enzyme of *Clostridium perfringens* S40 spores. *Biosci Biotech Biochem* 59, 514-515.
- Modrich, P. (1994). Mismatch repair, genetic stability, and cancer. *Nature* 266, 1959-1960.
- Moreno, S.N.J, Mason, R.P. and Docampo, R. (1984). Distinct reduction of nitrofurans and metronidazole to free radical metabolites by *Trichomonas foetus* hydrogenosomal and cytosolic enzymes. *J Biol Chem* 259, 8252-8259.

- Moreno, S.N.J, Mason, R.P., Muniz, R.P.A., Cruz, S.F. and Docampo, R. (1983). Generation of free radicals from metronidazole and other nitroimidazoles by *Trichomonas foetus*. *J Biol Chem* 258, 4051-4054.
- Msadek, T., Kunst, F. and Rapoport, G. (1994). MecB of *Bacillus subtilis*, a member of the ClpC ATPase family, is a pleiotropic regulator controlling competence gene expression and growth at high temperature. *Proc Natl Acad Sci USA* 91, 5788-5792.
- Mullany, P., Wilks, M. and Tabaqchali, S. (1991). Transfer of Tn916 and Tn916 Δ E into *Clostridium difficile*: demonstration of a hot-spot for these elements in the *C. difficile* genome. *FEMS Microbiol Lett* 79, 191-194.
- Müller, M. (1983). Mode of action of metronidazole on bacteria and protozoa. *Surgery* 93, 165-171.
- Müller, M. and Lindmark, D.G. (1976). Uptake of metronidazole and its effect on viability in trichomonads and *Entamoeba invadens* under anaerobic and aerobic conditions. *Antimicrob Agents Chemother* 9, 696-700.
- Müller, M., Lossick, J. G. and Gorrell, T.E. (1988). *In vitro* susceptibility of *Trichomonas vaginalis* to metronidazole and treatment outcome in vaginal trichomoniasis. *Sex Transmit Dis* 15, 17-24.
- Nair, R.V. and Papoutsakis, E.T (1994). Expression of plasmid-encoded *aad* in *Clostridium acetobutylicum* M5 restores vigorous butanol production. *J Bacteriol* 176, 5843-5846.
- Nair, R.V., Bennett G.N. and Papoutsakis, E. T. (1994). Molecular characterization of an aldehyde/alcohol dehydrogenase gene from *Clostridium acetobutylicum* ATCC 824. *J Bacteriol* 176, 871-885.
- Narikawa, S. (1986). Distribution of metronidazole susceptibility factors in obligate anaerobes. *J Antimicrob Chemother* 18, 565-574.
- Narikawa, S., Suzuki, T., Yamamoto, M. and Nakamura, M. (1991). Lactate dehydrogenase activity as a cause of metronidazole resistance in *Bacteroides fragilis* NCTC 11295. *J Antimicrob Chemother* 28, 47-53.

- Navarre, W.W. and Schneewind, O. (1994). Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram-positive bacteria. *Mol Microbiol* **14**, 115-121.
- Nedkov, P., Oberthur, W. and Braunitzer, G. (1983). Primary structure of subtilisin DY. *Hoppe Seyler's Z Physiol Chem* **364**, 1537-1540.
- Neyfakh, A.A. (1992). The multidrug efflux transporter of *Bacillus subtilis* is a structural and functional homolog of the *Staphylococcus* NorA protein. *Antimicrob Agents Chemother* **36**, 484-485.
- Neyfakh, A.A., Borsch, C.M. and Kaatz, G.W. (1993). Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrob Agents Chemother* **37**, 128-129.
- Nomura, N., Miyajima, N., Sazuka, T., Tanaka, A., Kawarabayashi, Y., Sato, S., Nagase, T., Seki, T., Ishikawa, K. and Tabata, S. (1994). Prediction of the coding sequences of unidentified human genes. II. The coding sequences of 40 new genes (KIAA 0041-KIAA 0080) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG1 (unpublished).
- Norgren, M. and Scott, J.R. (1991). The presence of conjugative transposon Tn916 in the recipient strain does not impede transfer of a second copy of the element. *J Bacteriol* **173**, 319-324.
- Norgren, M., Caparon, M.G. and Scott, J.R. (1989). A method for allelic replacement that uses the conjugative transposon Tn916: deletion of the *emm6.1* allele in *Streptococcus pyogenes* JRS4. *Infect Immun* **57**, 3846-3850.
- Novaes, A.B., DeUzeda, M., Fonesca, M.E.F. and Feitosa, A.C.R. (1991). The effect of subinhibitory concentrations of metronidazole and tetracycline on the ultrastructure of periodontopathic bacteria. *J Antimicrob Chemother* **28**, 151-154.
- O'Brien, R.W. and Morris, J.G. (1972). Effect of metronidazole on hydrogen production by *Clostridium acetobutylicum*. *Arch Microbiol* **84**, 225-233.

- Ochman, H. and Wilson, A.C. (1987). Evolution in bacteria: evidence for a universal substitution rate in cellular genomes. *J Mol Evol* 26, 74-88.
- Oda, Y., Nakayama, R., Kuroda, A. and Sekiguchi, J. (1993). Molecular cloning, sequence analysis, and characterization of a new cell wall hydrolase, CwlL, of *Bacillus licheniformis*. *Mol Gen Genet* 241, 380-388.
- Onderdonk, A.B., Louie, T.J., Tally, F.P. and Bartlett, J.G. (1979). Activity of metronidazole against *Escherichia coli* in experimental intra-abdominal sepsis. *J Antimicrob Chemother* 5, 201-210.
- Oultram, J.D and Young, M. (1985). Conjugal transfer of plasmid pAM β 1 from *Streptococcus lactis* and *Bacillus subtilis* to *Clostridium acetobutylicum*. *FEMS Microbiol Lett* 27, 129-134.
- Oultram, J.D., Burr, I.D., Elmore, M.J. and Minton, N.P. (1993). Cloning and sequence analysis of the genes encoding phosphotransbutyrylase and butyrate kinase from *Clostridium acetobutylicum* NCIMB 8052. *Gene* 131, 107-112.
- Oultram, J.D., Loughlin, M., Swinfield, T-J., Brehm, J.K., Thompson, D.E. and Minton, N.P. (1988). Introduction of plasmids into whole cells of *Clostridium acetobutylicum* by electroporation. *FEMS Microbiol Lett* 56, 83-88.
- Pabo, C.O. and Sauer, R.T. (1984). Protein-DNA recognition. *Ann Rev Biochem* 53, 293-321.
- Papoutsakis, E.T. (1983). A useful equation for fermentations of butyric acid bacteria. The acetone-butanol fermentation and related topics. In *Science and Technology Letters*, pp. 121-126. Edited by J.D. Bu'Lock and A.J. Bu'Lock. Kew, Surrey, United Kingdom.
- Papoutsakis, E.T. and Bennet, G.N. (1993). Cloning, structure, and expression of acid and solvent pathway genes of *Clostridium acetobutylicum*. In *The Clostridia and Biotechnology*, pp. 157-199. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Peguín, S. and Soucaille, P. (1995). Modulation of carbon and electron flow in *Clostridium acetobutylicum* by iron limitation and methyl viologen addition. *Appl Environ Microbiol* 61, 403-405.

- Peguin, S., Delmore, P., Goma, G. and Soucaille, P. (1995). Metabolic flexibility of *Clostridium acetobutylicum* in response to methyl viologen. *Appl Microbiol Biotechnol* (in press).
- Peguin, S., Delmore, P., Goma, G. and Soucaille, P. (1994). Enhanced alcohol yields in batch cultures of *Clostridium acetobutylicum* using three-electrode potentiometric system with methyl viologen as electron carrier. *Biotechnol Lett* 16, 269-274.
- Perego, M., Higgins, C.F., Pearce, S.R., Gallagher, M.P. and Hoch, J.A. (1991). The oligopeptide transport system of *Bacillus subtilis* plays a role in the initiation of sporulation. *Mol Microbiol* 5, 173-185.
- Perego, M., Spiegelman, G.B. and Hoch, J.A. (1988). Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. *Mol Microbiol* 2, 689-699.
- Perez-Reyes, E., Kalyranaman, B. and Mason, R.P. (1980). The reduction metabolism of metronidazole and ornidazole by aerobic liver microsomes. *Mol Pharmacol* 17, 239-244.
- Petersen, D.J., Welch, R.W., Rudolph, F.B. and Bennett, G.N. (1991). Molecular cloning of an alcohol dehydrogenase gene cluster from *Clostridium acetobutylicum* ATCC 824. *J Bacteriol* 173, 1831-1834.
- Petitdemange, E., Fond, O., Caillet, H., Petitdemange, H. and Gay, R. (1983). A novel one step process for cellulose fermentation using mesophilic cellulolytic and glycolytic Clostridia. *Biotechnol Lett* 5, 119-124.
- Petitdemange, H., Blusson, H. and Gay, R. (1981). Detection of NAD(P)H-rubredoxin oxidoreductases in Clostridia. *Anal Biochem* 116, 564-570.
- Pich, A., Narberhaus, F. and Bahl, H. (1990). Induction of heat shock proteins during initiation of solvent formation in *Clostridium acetobutylicum*. *Appl Microbiol Biotechnol* 33, 697-704.
- Ponnambalam, S., Webster, C., Bringham, A. and Busby, S. (1986). Transcription initiation in the *Escherichia coli* galactose operon promoters in the absence of normal -35 region sequences. *J Biol Chem* 261, 16043-16048.

- Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C. and Courvalin, P. (1990). The integration-excision system of the conjugative transposon Tn1545 is structurally and functionally related to those of lambdoid phages. *Mol Microbiol* 4, 1513-1521.
- Poyart-Salmeron, C., Trieu-Cuot, P., Carlier, C., and Courvalin, P. (1989). Molecular characterization of two proteins involved in the excision of the pneumococcal transposon Tn1545: homologies with other site-specific recombinases. *EMBO J* 8, 2425-2433.
- Pratviel, G., Bernadou, J. and Meunier, B. (1986). DNA breaks generated by the bleomycin-iron III complex in the presence of KHSO₅, a single oxygen donor. *Biochem Biophys Res Commun* 136, 1013-1020.
- Quon, D.V.K., D'oliveira, C.E. and Johnson, P.J. (1992). Reduced transcription of the ferredoxin gene in metronidazole-resistant *Trichomonas vaginalis*. *Proc Natl Acad Sci USA* 89, 4402-4406.
- Rao, G. and Mutharasan, R. (1986). Alcohol production by *Clostridium acetobutylicum* induced by methyl viologen. *Biotechnol Lett* 8, 893-896.
- Rao, G. and Mutharasan, R. (1987). Altered electron flow in continuous cultures of *Clostridium acetobutylicum* induced by viologen dyes. *Appl Environ Microbiol* 53, 1232-1235.
- Rather, P.N., Hay, R.E., Ray, G.L., Haldenwang, W.G. and Moran, C.P., Jr (1986). Nucleotide sequences that define promoters that are used by *Bacillus subtilis* sigma-29 RNA polymerase. *J Mol Biol* 192, 557-565.
- Reizer, J., Reizer, A. and Saier, M.H., Jr (1995). Novel phosphotransferase system genes revealed by bacterial genome analysis - a gene cluster encoding a unique Enzyme I and the proteins of a fructose-like permease system. *Microbiol* 141, 961-971.
- Reynolds, A.V. (1981). The activity of nitro-compounds against *Bacteroides fragilis* is related to their electron affinity. *J Antimicrob Chemother* 8, 91-99.

- Reysset, G. and Sebald, M. (1985). Conjugal transfer of plasmid antibiotic resistance from streptococci to *Clostridium acetobutylicum*. *Ann Inst Pasteur Microbiol* 136, 275-282.
- Reysset, G. and Sebald, M. (1993). Transformation/electrotransformation of Clostridia. In *The Clostridia and Biotechnology*, pp. 151-156. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Reysset, G., Haggoud, A. and Sebald, M. (1992). Genetics of resistance of *Bacteroides* species to 5-nitroimidazole. *Clin Infect Dis* 16 Suppl 4, S401-S403.
- Reysset, G., Haggoud, A., Su, W.J. and Sebald, M. (1993). Genetic and molecular analysis of pIP417 and pIP419: *Bacteroides* plasmids encoding 5-nitroimidazole resistance. *Plasmid* 27, 181-190.
- Reysset, G., Hubert, J., Podvin, L. and Sebald, M. (1988). Transfection and transformation of *Clostridium acetobutylicum* strain N1-4081 protoplasts. *Biotechnol Tech* 2, 199-204.
- Riebeling, V., Thauer, R.K. and Jungermann, K. (1975). The internal-alkaline pH gradient, sensitive to uncoupler and ATPase inhibitor, in growing *Clostridium pasteurianum*. *Eur J Biochem* 55, 445-453.
- Rödel, W., Plaga, W., Frank, R. and Knappe, J. (1988). Primary structures of *Escherichia coli* pyruvate formate-lyase and pyruvate-formate-lyase-activating enzyme deduced from the DNA nucleotide sequences. *Eur J Biochem* 177, 153-158.
- Rogers, P. and Gottschalk, G. (1993). Biochemistry and regulation of acid and solvent production in clostridia. In *The Clostridia and Biotechnology*, pp. 201-225. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Rood, J.I. and Cole, S.T. (1991). Molecular genetics and pathogenesis of *Clostridium perfringens*. *Microbiol Rev* 55, 621-648.
- Roos, J.W., McLaughlin, J.K. and Papoutsakis, E.T. (1985). The effect of pH on nitrogen supply, cell lysis and solvent production in fermentations of *Clostridium acetobutylicum*. *Biotechnol. Bioeng.* 27, 681-694.

- Rotimi, V.O., Duerden, B.I. and Hafix, A. (1981). Transferable plasmid-mediated antibiotic resistance in *Bacteroides*. *J Med Microbiol* **14**, 359-370.
- Rowley, D.A. and Edwards, D.I. (1982). Possible mechanisms of resistance of anaerobes to nitroimidazoles. In *Current Chemotherapy and Immunotherapy*, Vol. 1, pp. 183-184. Edited by P. Periti and G.C. Grassi. Washington, DC: American Society for Microbiology.
- Rowley, D.A., Knight, R.C., Skolimowski, I.M. and Edwards, D.I. (1979). The effect of nitroheterocyclic drugs on DNA: *in vitro* model of cytotoxicity. *Biochem Pharmacol* **28**, 3009-3013.
- Rowley, D.A., Knight, R.C., Skolimowski, I.M. and Edwards, D.I. (1980). The relationship between misonidazole cytotoxicity and base composition of DNA. *Biochem Pharmacol* **29**, 2095-2098.
- Rudner, D.Z., LeDeaux, J.R., Ireton, K. and Grossman, A.D. (1991). The *spo0K* locus of *Bacillus subtilis* is homologous to the oligopeptide permease locus and is required for sporulation and competence. *J Bacteriol* **173**, 1388-1398.
- Rudy, C.K. and Scott, J.R. (1994). Length of the coupling sequence of Tn916. *J Bacteriol* **176**, 3386-3388.
- Rutherford, S.L. and Zuker, D.S. (1994). Protein folding and the regulation of signalling pathways. *Cell* **79**, 1129-1132.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbour, New York: Cold Spring Harbor Laboratory.
- Sanchez-Pescador, R., Brown, J.T., Roberts, M. and Ureda, M.S. (1988). Homology of the TetM with translational elongation factors: implications for potential modes of tetM conferred tetracycline resistance. *Nucleic Acid Res* **3**, 1218.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463-5467.

- Santangelo, J.D. (1991). Studies on nucleotide levels and electron transport genes of *Clostridium acetobutylicum* P262. PhD Thesis, University of Cape Town, South Africa.
- Santangelo, J.D., Dürre, P., and Woods, D.R. (1995). Characterization and expression of the hydrogenase-encoding gene from *Clostridium acetobutylicum* P262. *Microbiol* **141**, 171-180.
- Santangelo, J.D., Jones, D.T. and Woods, D.R. (1991). Metronidazole activation and isolation of *Clostridium acetobutylicum* electron transport genes. *J Bacteriol* **173**, 1088-1095.
- Sass, C., Walter, J. and Benett, G.N. (1993). Isolation of mutants of *Clostridium acetobutylicum* ATCC 824 deficient in protease activity. *Curr Microbiol* **26**, 151-154.
- Sauer, U. and Dürre, P. (1992). Possible function of tRNA^{Thr}_{ACG} in regulation of solvent formation in *Clostridium acetobutylicum*. *FEMS Microbiol Lett* **100**, 147-154.
- Sauer, U. and Dürre, P. (1995). Differential induction of genes related to solvent formation during the shift from acidogenesis to solventogenesis in continuous culture of *Clostridium acetobutylicum*. *FEMS Microbiol Lett* **125**, 115-120.
- Sauer, U., Santangelo, J.D., Treuner, A., Buchholz, M. and Dürre, P. (1995). Sigma factor and sporulation genes in *Clostridium*. *FEMS Microbiol Rev* **17**, 331-340.
- Sauer, U., Treuner, A., Buchholz, M., Santangelo, J.D. and Dürre, P. (1994). Sporulation and primary sigma factor homologous genes in *Clostridium acetobutylicum*. *J Bacteriol* **176**, 6572-6582.
- Sauter, M. and Sawers, R.G. (1990). Transcriptional analysis of the gene encoding pyruvate formate-lyase-activating enzyme of *Escherichia coli*. *Mol Microbiol* **4**, 355-363.
- Sawers, G. and Böck, A. (1989). Novel transcriptional control of the pyruvate formate-lyase gene: upstream regulatory sequences and multiple promoters regulate anaerobic expression. *J Bacteriol* **171**, 2485-2498.

- Schneewind, O., Mihaylova-Petkov, D. and Model, P. (1993). Cell wall sorting signals in surface proteins of Gram-positive bacteria. *EMBO J* **12**, 4803-4811.
- Schneewind, O., Model, P. and Fischetti, V.A. (1992). Sorting of protein A to the staphylococcal cell wall. *Cell* **70**, 267-281.
- Scott, J.R. (1992). Sex and the single circle: conjugative transposition. *J Bacteriol* **174**, 6005-6010.
- Scott, J.R. (1993). Cojugative transposons. In *Bacillus subtilis and Other Gram-positive Bacteria. Biochemistry, Physiology and Molecular biology*, pp. 597-614. Edited by A.L. Sonenshein, J.A. Hoch and R. Losick, R. Washington, D.C: American Society for Microbiology.
- Scott, J.R. and Churchward, G.G. (1995). Conjugative transposition. *Ann Rev Microbiol* **49**, 367-397.
- Scott, J.R., Bringel, F., Marra, D., van Alstine, G. and Rudy, C.K. (1994). Conjugative transposition of Tn916: preferred targets and evidence for conjugative transfer of a single strand and for a double-stranded intermediate. *Mol Microbiol* **11**, 1099-1108.
- Scott, J.R., Kirchman, P.A. and Caparon, M.G. (1988). An intermediate in transposition of the conjugative transposon Tn916. *Proc Natl Acad Sci USA* **85**, 4809-4813.
- Sebban, C., Blanchard, L., Bruschi, M. and Guerlesquin, F. (1995). Purification and characterization of the formate dehydrogenase from *Desulfovibrio vulgaris* Hildenborough. *FEMS Microbiol Lett* **133**, 143-149.
- Sekiguchi, J., Ohsu, H., Kuroda, A., Moriyama, H. and Akamatsu, T. (1990). Nucleotide sequences of the *Bacillus subtilis* *flaD* locus and a *B. licheniformis* homologue affecting the autolysin level and flagellation. *J Gen Microbiol* **136**, 1223-1230.
- Sen, S. and Oriel, P. (1990). Transfer of transposon Tn916 from *Bacillus subtilis* to *Thermus aquaticus*. *FEMS Microbiol Lett* **67**, 131-134.
- Senghas, E., Jones, J.M., Yamamoto, M., Gawron-Burke, C. and Clewell, D.B. (1988). Genetic organization of the bacterial conjugative transposon Tn916. *J Bacteriol* **170**, 245-249.

- Shine, J. and Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome-binding sites. *Proc Natl Acad Sci USA* 71, 1342-1346.
- Shiroza, T. and Kuramitsu, H.K. (1988). Sequence analysis of the *Streptococcus mutans* fructosyltransferase gene and flanking regions. *J Bacteriol* 170, 810-816.
- Shoemaker, N.B, Smith, M.D. and Guild, W.R. (1980). DNase-resistant transfer of chromosomal *cat* and *tet* insertions by filter mating in pneumococcus. *Plasmid* 3, 80-87.
- Showsh, S.A. and Andrews, R.E., Jr (1992). Tetracycline enhances Tn916-mediated conjugal transfer. *Plasmid* 28, 213-224.
- Silva-Filho, F.C. and de Souza, W. (1988). Effect of metronidazole on the cell surface charge of *Trichomonas vaginalis* and *Tritrichomonas foetus*. *Braz J Med Biologic Res* 21, 1037-1045.
- Simic, D., Vukovic-Gacic, B., Ajanovic, A. and Knezevic-Vukcevic, J. (1991). Activation of RecA protein in recombination-deficient strains of *Escherichia coli* following DNA-damaging treatments. *Mutation Res* 254, 255-262.
- Sindar, P., Britz, M.L. and Wilkinson, R.G. (1982). Isolation and properties of metronidazole resistant mutants of *Clostridium perfringens*. *J Med Microbiol* 15, 503-509.
- Skarin, A. and Mardh, P.A. (1981). Scanning electron microscopic examination of *Bacteroides fragilis* and *Gardnerella vaginalis* after exposure to concentration gradients of metronidazole and tinidazole. *Scandinavian J of Infectious Diseases, Suppl* 26, 54-59.
- Smibert, R.M. and Krieg, N.R. (1981). General characterization. In *Manual of Methods for General Bacteriology*, pp. 40-443. Edited by P. Gerhardt, R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips. New York: American Society for Microbiology.

- Smith, I. (1993). Regulatory proteins that control late-growth development. In *Bacillus subtilis and Other Gram-positive Bacteria. Biochemistry, Physiology and Molecular Biology*, pp. 785-800. Edited by A.L. Sonenshein, J.A. Hoch and R. Losick, R.. Washington, D.C: American Society for Microbiology.
- Sorokin, A., Zumstein, E., Azevedo, V., Ehrlich, S.D. and Serror, P. (1993). The organization of the *Bacillus subtilis* 168 chromosome region between the *spoVA* and *serA* genetic loci, based on sequence data. *Mol Microbiol* 10, 385-395.
- Soucaille, P., and Goma, G. (1986). Acetonobutylic fermentation by *Clostridium acetobutylicum* ATCC 824; autobacteriocin production, properties, and effects. *Curr Microbiol* 13, 163-169.
- Soucaille, P., Joliff, G., Izard, A. and Goma, G. (1987). Butanol tolerance and autobacteriocin production by *Clostridium acetobutylicum*. *Curr Microbiol* 14, 295-299.
- Stewart, V. (1988). Nitrate respiration in relation to facultative metabolism in Enterobacteria. *Microbiol Rev* 52, 190-232.
- Störmer, F. (1975). 2,3-butanediol biosynthetic system in *Acetobacter aerogenes*. *Methods Enzymol* 41, 518-533.
- Storrs, M.J., Poyart-Salmeron, C., Trieu-Cuot, P. and Courvalin, P. (1991). Conjugative transposition of Tn916 requires the excisive and integrative activities of the transposon-encoded integrase. *J Bacteriol* 173, 4347-4352.
- Strätz, M., Gottschalk, G. and Dürre, P. (1990). Transfer and expression of the tetracycline resistance transposon Tn925 in *Acetobacterium woodii*. *FEMS Microbiol Lett* 68, 171-176.
- Strauch, M.A. and Hoch, J.A. (1993). Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Mol Microbiol* 7, 337-342.
- Su, Y.A. and Clewell, D.B. (1993). Characterization of the left 4 kb of conjugative transposon Tn916: determinants involved in excision. *Plasmid* 30, 234-250.

- Su, Y.A., He, P. and Clewell, D.B. (1992). Characterization of the *tet(M)* determinant of Tn916: evidence for regulation by transcription attenuation. *Antimicrob Agents Chemother* **36**, 769-778.
- Supelco Inc. (1975). Analysis of VFAs from anaerobic fermentation. Bulletin 748F.
- Svendsen, I., Genov, M. and Idakieva, K. (1986). Complete amino acid sequence of alkaline mesenteropeptidase. *FEBS Lett* **196**, 228-232.
- Swinfield, T.-J., Oultram, J.D., Thompson, D.E., Brehm, J.K. and Minton, N.P. (1990). Physical characterization of the replication of the *Streptococcus faecalis* plasmid pAMβ1. *Gene* **87**, 79-90.
- Takemaru, K., Mizuno, M., Sato, T., Takeuchi, M. and Kobayashi, Y. (1995). Complete nucleotide sequence of a *skin* element excised by DNA rearrangement during sporulation in *Bacillus subtilis*. *Microbiol* **141**, 323-327.
- Takeshita, M., Grollman, A.P., Ohtsubo, E., Ohtsubo, H. (1978). Interaction of bleomycin with DNA. *Proc Natl Acad Sci USA* **75**, 5983-5987.
- Tally, F.P., Goldin, B.R., Sullivan, N., Johnston, J. and Gorbach, S.L. (1978). Antimicrobial activity of metronidazole in anaerobic bacteria. *Antimicrob Agents Chemother* **13**, 460-465.
- Tally, F.P., Goldman, B.R., Jacobus, N.V. and Gorbach, S.L. (1977). Superoxide dismutase in anaerobic bacteria of clinical significance. *Infect Immun* **16**, 20-25.
- Tally, F.P., Snyderman, D.R., Shimell, M.J., Goldin, B.R. (1979). Mechanisms of antimicrobial resistance of *Bacteroides fragilis*. In *Metronidazole Proceedings of the Second International Symposium on Anaerobic Infections*. Edited by I. Phillips and J. Collier. London: The Royal Society of Medicine, New York: Academic Press.
- Tao, T., Bourne, J.C. and Blumenthal, R.M. (1991). A family of regulatory genes associated with type II restriction-modification systems. *J Bacteriol* **173**, 1367-1375.

- Terracciano, J.S. and Kashket, E.R. (1986). Intracellular concentrations required for initiation of solvent production by *Clostridium acetobutylicum*. *Appl Environ Microbiol* **52**, 86-91.
- Terracciano, J.S., Rapaport, E. and Kashket, E.R. (1988). Stress and growth-phase associated proteins of *Clostridium acetobutylicum*. *Appl Environ Microbiol* **54**, 1989-1995.
- Thauer, R.K., Jungermann, K.A. and Decker, K. (1977). Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* **41**, 100-180.
- Thauer, R.K., Kaufer, B., Zahringer, M. and Jungermann, K. (1974). The reaction of the iron-sulfur protein with carbon monoxide. *Eur. J. Biochem.* **42**, 447-452.
- Thauer, R.K., Kirchniawy, F.H. and Jungermann, K.A. (1972). Properties and function of the pyruvate-formate-lyase reaction in Clostridia. *Eur J Biochem* **27**, 282-290.
- Tocher, J.H. and Edwards, D.I (1992). The interaction of reduced metronidazole with DNA bases and nucleosides. *Int J Radiation Onc Biol Phys* **22**, 661-663.
- Torres, O.R., Korman, R.Z., Zahler, S.A. and Dunny, G.M. (1991). The conjugative transposon Tn925: enhancement of conjugal transfer by tetracycline in *Enterococcus faecalis* and mobilization of chromosomal genes in *Bacillus subtilis* and *E. faecalis*. *Mol Gen Genet* **225**: 395-400.
- Townson, S.M., Hanson, G.R., Upcroft, J.A. and Upcroft, P. (1994). A purified ferredoxin from *Giardia duodenalis*. *Eur J Biochem* **220**, 439-446.
- Tran, P.V., Bannor, T.A., Doktor, S.Z. and Nichols, B.P. (1990). Chromosomal organization and expression of *Escherichia coli pabA*. *J Bacteriol* **172**, 397-410.
- Trieu-Cuot, P., Carlier, C., Poyart-Salmeron, C. and Courvalin, P. (1991). An integrative vector exploiting the transposition properties of Tn1545 for insertional mutagenesis and cloning of genes from Gram-positive bacteria. *Gene* **106**, 21-27.
- Trieu-Cuot, P., Carlier, C., Martin, P. and Courvalin, P. (1987). Plasmid transfer by conjugation from *Escherichia coli* to Gram-positive bacteria. *FEMS Microbiol Lett* **48**, 289-294.

- Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. and Courvalin, P. (1990). Conjugative transposons of gram-positive cocci. In *Proceedings of the Sixth International Symposium on Genetics of Industrial Microorganisms*, GIM '90, pp. 195-205. Strasbourg: Société Française de Microbiologie.
- Trieu-Cuot, P., Poyart-Salmeron, C., Carlier, C. and Courvalin, P. (1993). Sequence requirements for target activity in site-specific recombination mediated by the Int protein of transposon Tn1545. *Mol Microbiol* 8, 179-185.
- Trinh, S., Haggoud, A., Reysset, G. and Sebald, M. (1995). Plasmids pIP419 and pIP421 from *Bacteroides*: 5-nitroimidazole resistance genes and their upstream insertion sequence elements. *Microbiol* 141, 927-935.
- Truffaut, N., Hubert, J. and Reysset, G. (1989). Construction of shuttle vectors useful for transforming *Clostridium acetobutylicum*. *FEMS Microbiol Lett* 58, 15-20.
- Van de Guchte, M., Daly, C., Fitzgerald, G. and Arendt, E.K. (1994). Identification of the putative repressor-encoding gene *cl* of the temperate lactococcal bacteriophage Tuc2009. *Gene* 144, 93-95.
- Van der Westhuizen, A., Jones, D.T. and Woods, D.R. (1982). Autolytic activity and butanol tolerance of *Clostridium acetobutylicum*. *Appl Environ Microbiol* 44, 1277-1281.
- Van Hoy, B.E. and Hoch, J.A. (1990). Characterization of the *spoIVB* and *recN* loci of *Bacillus subtilis*. *J Bacteriol* 172, 1306-1311.
- Van Kaer, L., Van Motagu, M. and Dhaese, P. (1987). Transcriptional control in the *EcoRI*-F immunity region of *Bacillus subtilis* phage ϕ 105. *J Mol Biol* 197, 55-67.
- Vasconcelos, I., Girbal, L. and Soucaille, P. (1994). Regulation of carbon and electron flow in *Clostridium acetobutylicum* grown in chemostat culture at neutral pH on mixtures of glucose and glycerol. *J Bacteriol* 176, 1443-1450.

- Verhasselt, P. and Vanderleyden, J. (1993). Cloning and expression of *Clostridium acetobutylicum* genes involved in carbohydrate utilization. In *Genetics and Molecular Biology of Anaerobic Bacteria*, pp. 301-316. Edited by M. Sebald. New York: Springer-Verlag.
- Volk, W.A., Bizzini, B., Jones, K.R. and Macrina, F.L. (1988). Inter- and intragenic transfer of Tn916 between *Streptococcus faecalis* and *Clostridium tetani*. *Plasmid* 19, 255-259.
- Wagner, A.F.V., Frey, M., Neugebauer, F.A., Schäfer, W. and Knappe, J. (1992). The free radical in pyruvate-formate lyase is located on glycine-734. *Proc Natl Acad Sci USA* 89, 996-1000.
- Walter, K.A., Bennet, G.N. and Papoutsakis, E.T. (1992). Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isozyme genes. *J Bacteriol* 174, 7149-7158.
- Walter, K.A., Nair, R.V., Cary, J.W., Bennett, G.N. and Papoutsakis, E.T. (1993). Sequence arrangement of two genes of the butyrate-synthesis pathway of *Clostridium acetobutylicum* ATCC 824. *Gene* 134, 107-111.
- Watson, D.A., Musher, D.M. and Hamill, R.J. (1993). Promotion of transposon Tn916 excision via exposure to streptomycin. *J Infect Dis* 168, 1334-1335.
- Wehnert, G.U., Abratt, V.R. and Woods, D.R. (1992). Molecular analysis of a gene from *Bacteroides fragilis* involved in metronidazole resistance in *Escherichia coli*. *Plasmid* 27, 242-245.
- Wehnert, G.U., Abratt, V.R., Goodman, H.J.K. and Woods, D.R. (1990). Cloning of *Bacteroides fragilis* plasmid genes affecting metronidazole resistance and ultraviolet survival in *Escherichia coli*. *Plasmid* 23, 155-158.
- Widdick, D.A. and Edwards, D.I. (1991). A comparison of the relative activities of 8 radiosensitizers in the SOS chromotest. *Mutation Res* 259, 89-93.
- Wilkinson, S.R. and Young, M. (1994). Targeted integration of genes into the *Clostridium acetobutylicum* chromosome. *Microbiol* 140, 89-95.

- Wilkinson, S.R. and Young, M. (1995). Physical map of the *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052 chromosome. *J Bacteriol* 177, 439-448.
- Wilkinson, S.R., and Young, M. (1993). Wide diversity of genome size among different strains of *Clostridium acetobutylicum*. *J Gen Microbiol* 139, 1069-1076.
- Wilkinson, S.R., Young, D.I., Morris, J.G. and Young, M. (1995a). Molecular genetics and initiation of solventogenesis in *Clostridium beijerinckii* (formerly *Clostridium acetobutylicum*) NCIMB 8052. *FEMS Microbiol Rev* 17, 275-285.
- Wilkinson, S.R., Young, M., Goodacre, R., Morris, J.G., Farrow, J.A.E. and Collins, M.D. (1995b). Phenotypic and genotypic differences between certain strains of *Clostridium acetobutylicum*. *FEMS Microbiol Lett* 125, 199-204.
- Williams, D.R., Young, D.I. and Young, M. (1990). Conjugative plasmid transfer from *Escherichia coli* to *Clostridium acetobutylicum*. *J Gen Microbiol* 36, 819-826.
- Winzer, K. and Dürre, P. (1994). Cloning and sequencing of two thiolase genes from *Clostridium acetobutylicum* DSM 792. *Bioengineering (Spec Iss 2)* 48, 191.
- Wong, J., Saso, C. and Bennett, G.N. (1995). Sequence and arrangement of genes encoding sigma factors in *Clostridium acetobutylicum*. *Gene* 153, 89-92.
- Wong, K.K., Murray, B.W., Lewis, S.A., Baxter, M.K., Ridky, T.W., Ulissi-DeMario, L. and Kozarich, J.W. (1993). Molecular properties of pyruvate formate-lyase activating enzyme. *Biochemistry* 32, 14102-14110.
- Wong, S.L., Price, C.W., Goldfarb, D.S. and Doi, R.H. (1984). The subtilisin E gene of *Bacillus subtilis* is transcribed from a σ^{37} promoter in vivo. *Proc Natl Acad Sci USA* 81, 1184-1188.
- Wood, N.P. and Jungermann, K. (1972). Inactivation of the pyruvate formate lyase in *Clostridium butyricum*. *FEBS Lett* 27, 49-52.

- Woods, D.R. and Santangelo, J. (1993). Molecular analysis and expression of nitrogen metabolism and electron transport genes of *Clostridium*. In *The Clostridia and Biotechnology*, pp 201-225. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Woolley, R.C. and Morris, J.G. (1990). Stability of solvent production by *C. acetobutylicum* in continuous culture: strain differences. *J Appl Bacteriol* **69**, 718-728.
- Woolley, R.C., Pennock, A., Ashton, R.J., Davies, A. and Young, M. (1989). Transfer of Tn1545 and Tn916 to *Clostridium acetobutylicum*. *Plasmid* **22**, 169-174.
- Yamamoto, M., Jones, J.M., Senghas, E., Gawron-Burke, C. and Clewell, D.B. (1987). Generation of Tn5 insertions in streptococcal conjugative transposon Tn916. *Appl Environ Microbiol* **53**, 1069-1072.
- Yanisch-Perron, C., Vieira, J. and Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103-119.
- Yanke, L.J., Bryant, R.D. and Laishley E.J. (1995). Hydrogenase 1 of *Clostridium pasteurianum* functions as a novel selenite reductase. *Anaerobe* **1**, 61-67.
- Yarlett, N., Gorrell, T.E., Marczak, R. and Müller, M. (1985). Reduction of nitroimidazole derivatives by hydrogenosomal extracts of *Trichomonas vaginalis*. *Mol Biochem Parasitol* **14**, 29-40.
- Yerushalmi, L and Volesky, B. (1985). Importance of agitation in acetone-butanol fermentation. *Biotechnol Bioeng* **27**, 1297-1305.
- Yeung, T.C., Beaulieu, B.B., McLafferty, M.A. and Goldman, P. (1984). Interaction of metronidazole with repair mutants of *Escherichia coli*. *Antimicrob Agents Chemother* **25**, 65-70.
- Yoneda, Y. and Maruo, B. (1975). Mutation of *Bacillus subtilis* causing hyperproduction of α -amylase and protease and its synergistic effect. *J. Bacteriol.* **124**, 48-54.

- Young, M. (1993). Development and exploitation of conjugative gene transfer in clostridia. In *The Clostridia and Biotechnology*, pp. 99-117. Edited by D.R. Woods. Stoneham, MA: Butterworth-Heinemann.
- Youngleson, J.S., Jones, W.A., Jones, D.T. and Woods, D.R. (1989). Molecular analysis and nucleotide sequence of the *adhI* gene encoding a NADPH-dependent butanol dehydrogenase in the Gram positive anaerobe *Clostridium acetobutylicum*. *Gene* **78**, 355-364.
- Youngleson, J.S., Santangelo, J.D., Jones, D.T. and Woods, D.R. (1988). Cloning and expression of a *Clostridium acetobutylicum* alcohol dehydrogenase gene in *Escherichia coli*. *Appl Environ Microbiol* **54**, 676-682.
- Yu, P.-L. and Pearce, L.E. (1986). Conjugal transfer of streptococcal antibiotic resistance plasmids into *Clostridium acetobutylicum*. *Biotechnol Lett* **8**, 469-474.
- Zabeau, M. and Stanley, K.K. (1982). Enhanced expression of cro- β -galactosidase fusion proteins under the control of P_R promoter of bacteriophage λ . *EMBO J* **1**, 1217-1224.
- Zappe, H., Jones, D.T. and Woods, D.R. (1986). Cloning and expression of *Clostridium acetobutylicum* endoglucanase, cellobiase and amino acid biosynthesis genes in *Escherichia coli*. *J Gen Microbiol* **132**, 1367-1372.
- Zappe, H., Jones, W.A. and Woods, D.R. (1988). Structure of an endo- β -1,4-glucanase gene from *Clostridium acetobutylicum* P262 showing homology with endoglucanase genes from *Bacillus* spp. *Appl Environ Microbiol* **B54**, 1289-1292.
- Zappe, H., Jones, W.A. and Woods, D.R. (1990). Nucleotide sequence of a *Clostridium acetobutylicum* P262 xylanase gene (*xynB*). *Nucleic Acid Res* **18**, 2179.
- Zhou, X. and Traxler, R.W. (1992). Enhanced butanol production and reduced autolysin activity after chloramphenicol treatment of *Clostridium acetobutylicum* ATCC 824. *Appl Microbiol Biotechnol* **37**, 293-297.