Studies on *Clostridium acetobutylicum* P262: sporulation induction and analysis of an oligosporogenous, solvent-deficient mutant

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"Hindsight is an exact science"

Abbreviations

A	deoxyadenosine
AADC	acetoacetate decarboxylase
ABE	acetone-butanol-ethanol
ADP	adenosine diphosphate
Ap	ampicillin
ATP	adenosine triphosphate
BAD	butyraldehyde dehydrogenase
BC	BASOL medium supplemented with xylose and casamino acids
BDH	butanol dehydrogenase
BK	butyrate kinase
bp	base pairs
C	deoxycytidine
CAMM	*C. acetobutylicum* minimal medium
CBM	clostridial basal medium
CBX	CBM with xylose as the carbon source
Cm	chloramphenicol
CO_2	carbon dioxide
CoAT	acetoacetate-CoA:acetate/butyrate:CoA transferase
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
Em	erythromycin
Em^R	erythromycin resistance
G	deoxyguanosine
GTP	guanosine triphosphate
H_2	hydrogen
HSP	heat shock protein
kb	kilobase pairs
λ	bacteriophage lambda
NADP	oxidized form of nicotinamide adenine dinucleotide phophate
NADPH	reduced form of nicotinamide adenine dinucleoide phophate
NCBI	nastional centre for biotechnology information
NTP	nucleoside triphosphate
OD	optical density at 600nm
PCR	polymerase chain reaction
pers. comm.	personal communication
PTB	phospho-transbutyrylase
RNA	ribonucleic acid
σ	sigma
SDS	sodium dodecyl sulphate
SSP	single specific primer
T	thymidine
Tc	tetracycline
Tc^R	tetracycline resistance
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer ribonucleic acid
v/v	volume for volume
VS	viable spores
w/v	weight by volume
WIBR	Whitehead institute for biomedical research
WT	wild type
YT	yeast tryptone medium

Abstract

The ability of various fermentation products to induce sporulation was tested in order to design a sporulation induction medium for *Clostridium acetobutylicum* P262. Of acetic acid, butyric acid, acetone and butanol, acetic acid was found to be most effective at induction. Induction was more efficient at low pH values under certain conditions. The heat resistance of mature spores was determined and the optimal temperature for spore quantification was shown to be 75°C.

C. acetobutylicum mutants m5 and o6 were generated by transposon mutagenesis using the conjugative transposon Tn*925*::Tn*917*, and not transposon Tn*925* as previously thought [Babb, B.L. 1990. B.Sc. (Honours) thesis, University of Cape Town, South Africa]. The spore development and the fermentation profiles of these mutants were analyzed in batch culture over a time period of 60h. Mutant m5 was shown to be oligosporogenous with the majority of cells blocked at sporulation stage *II*. It was deficient in acetone and butanol production. Mutant o6 proceeded to sporulation stage VII and produced acetone and butanol at levels similar to that of the wild type strain.

Mutants m5 and o6 appeared to contain three and two transposon insertions respectively from Southern hybridization experiments using a probe for the left transposon junction. However, when a probe to the right transposon junction was used, the chromosomal deoxyribonucleic acid (DNA) of mutant m5 was shown to contain approximately eight junction sites. The cause for the anomalous hybridization pattern was neither non-specific restriction enzyme activity nor a result of independent transposition of transposon Tn*917*.

From a genomic library of mutant m5 chromosomal DNA transformed into *Escherichia coli*, eight different plasmids containing DNA homologous to the right end of transposon Tn*925*::Tn*917* were identified in colony hybridization experiments. The plasmids were either 7.6, 9.9 or 7.7 kilobase pairs in size, but the pattern of restriction enzyme sites changed for new extractive preparations of two plasmids. Nucleotide sequence of the transposon junction sites in seven of the plasmid clones revealed three different DNA sequences. There was a stretch of five adenine residues near each transposon junction site, which may form part of a target insertion site for transposon Tn*925*::Tn*917*. The categorization of the plasmid clones by size and by transposon junction sequence did not correlate.

Computer analysis of the three chromosomal DNA sequences of the transposon junction sites revealed that one contained homology to a DNA sequence containing a β-galactosidase from *C. acetobutylicum* NCIB 2951. No significant homology was found for the other transposon junction site DNA sequences. The DNA sequences of the transposon junction sites were used to design chromosome-specific oligonucleotide probes. These probes did not hybridize to either the mutant or the wild type *C. acetobutylicum* chromosome, but one oligonucleotide was shown to hybridize to plasmid DNA representing a different transposon junction sequence. Polymerase chain reaction (PCR) of three plasmids representing the different transposon junction sequences yielded product for one plasmid. This PCR product hybridized to different sized chromosomal DNA fragments of the wild type and mutant m5 *C. acetobutylicum*, which indicated that the chromosomal DNA flanking a transposon insertion site had been amplified in the PCR reaction.

The chromosome of mutant m5 appeared to have rearranged, and the plasmid clones of the right transposon junctions of mutant m5, appeared to be unstable in *E. coli*. The instability of the chromosome and the plasmids was possibly caused by a gene of the conjugative transposon Tn*925*::Tn*917*.

Chapter 1

Introduction

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1.1. Introduction

1.1.1. Importance of Clostridia

The genus *Clostridium* includes spore-forming, Gram-positive, rod-shaped bacteria that are strictly anaerobic [Cato and Stackebrandt, 1989]. Included by this definition, are bacteria representative of a diverse range of metabolic activities. The metabolic diversity of this group of bacteria makes them important both industrially and medically. Clostridia cause disease in man and animals, that can be severe (botulism, tetanus and gas gangrene) or debilitating (food poisoning, diarrhea and colitis). The botulinum toxins are medically useful and are being exploited as therapeutic agents [Shone and Hambleton, 1989].

The industrial usefulness of clostridia is derived largely from their ability to utilize a wide variety of substrates, including the waste products of other industries, and from their production of potentially useful substances through fermentation [Morris, 1993]. Aeration, a major cost in industrial fermentation, is not necessary with clostridial fermentations, which are anaerobic. Furthermore, there is a market for "natural" products, compared to synthetic products, in our environmentally aware society, and bacteria can be utilized for the production of specific stereoscopic isomers of chemical compounds that are difficult to synthesize [Minton *et al.*, 1993].

1.1.2. Industrial application of *Clostridium acetobutylicum*

Clostridia are well known industrially, largely for the production of acetone and butanol by *C. acetobutylicum*. Acetone was an important solvent in the munitions industry during World War I and II [Jones and Woods, 1986]. It was at this time that the Weizmann process [the acetone-butanol-ethanol (ABE) fermentation by *C. acetobutylicum*] was developed. Initially, butanol was an unwanted by-product, but it has subsequently been utilized as a solvent, for example in quick-drying lacquers in car-body paints [Jones and Woods, 1986]. In 1987, 364×10^6 kg of butanol were used in North America alone [Ladisch, 1991]. Butanol can also be used as a fuel extender and the potential market for butanol in this regard is more than 1×10^9 kg per year, provided that production costs can be lowered [Ladisch, 1991]. Micro emulsions of butanol in petrol reduce fire hazard and improve water tolerance of the fuel. Butanol has a higher heating value than ethanol or methanol, and oxygenates of butanol serve as octane boosters, reduce carbon monoxide emissions and reduce ozone damage [Ladisch, 1991].

The yield of acetone and butanol from fermentation is not high (less than $22g.l^{-1}$) as these solvents are inhibitory to *C. acetobutylicum* [Jones and Woods, 1986]. In addition, this fermentation occurs via branched pathways so that not all the substrate is necessarily converted to the desired products [Rogers and Gottschalk, 1993]. As the price of raw materials increased, attempts were made to operate integrated plants by selling by-products: carbon dioxide (CO_2) was marketed as dry ice, hydrogen (H_2) was sold for use in the manufacture of synthetic methanol or hydrogenation of edible oils, and the residual solids, rich in riboflavin, were sold as animal feed supplements [Morris, 1993]. The cracking of petrochemicals to produce acetone and butanol eventually proved to be more cost effective and the ABE fermentation process was discontinued in the early 1980's. However, the use of *C. acetobutylicum* is less damaging to the environment than the exploitation of fossil fuel reserves [Brehm *et al.*, 1992], and the possibility of fuel shortages and of price increases has revived interest in the ABE fermentation. The construction of a new ABE fermentation plant has been planned in Brazil, where the cost of the molasses substrate is low [Afschar *et al.*, 1990].

Of the different strains of *C. acetobutylicum*, *C. acetobutylicum* P262 has been shown to produce the most solvents naturally [Jones and Woods, 1986]. The various strains differ with respect to total solvent yield as well as in general physiology and genome structure [Young *et al.*, 1989b]. The extent of these differences is so great that *C. acetobutylicum* is perhaps incorrectly viewed as being one species. Genome mapping led to the division of *C. acetobutylicum* into three groups [Wilkinson and Young, 1993], which are represented by strains NCIB 8052, ATCC 824 and DSM 1731, and P262. *C. acetobutylicum* P262 has a genome size of $2.85x10^6$ base pairs (bp), which is significantly smaller than the $6.5x10^6$bp of *C. acetobutylicum* NCIB 8052 and the $4.0x10^6$bp of *C. acetobutylicum* ATCC 824. Only one of four solvent gene-specific probes from *C. acetobutylicum* ATCC 824 hybridized to the P262 genome, but all the probes showed homology to the chromosome of strain NCIB 8052 [Wilkinson and Young, 1993].

C. acetobutylicum P262, as well as producing high levels of solvents, has the additional advantage of being physically large, which facilitates microscopic analyses and the study of differentiation [Awang *et al.*, 1992]. Differentiation in *C. acetobutylicum* is the growth of the organism through different developmental stages, culminating in the production of aerotolerant spores. An understanding of the differentiation process in the clostridia is important both medically and industrially, as there are links between sporulation and toxin production [for example, Kaniya *et al.*, 1992], and sporulation and solvent production [Jones and Woods, 1986]. These links need to be elucidated if the control of basic gene regulation is to have practical application in clostridia.

Studies on the differentiation links and switches in a particular mutant of *C. acetobutylicum* P262 are presented in this thesis. This sporulation-deficient mutant was generated with conjugative transposons and was blocked in acetone and butanol production [Babb, 1990]. Conjugative transposons and topics relating to *C. acetobutylicum* have been reviewed recently [Woods, 1993], and sporulation in *Bacillus subtilis* has been reviewed recently [Errington, 1993]. Rather than try to rewrite these excellent reviews, I have emphasized relevant points concerning *C. acetobutylicum*, sporulation and conjugative transposons.

1.2. Physiology of *C. acetobutylicum*

As the name of the organism suggests, *C. acetobutylicum* is able to produce acetone and butanol. These solvents are produced through an anaerobic fermentation. The industrial attractiveness of *C. acetobutylicum* is its ability to ferment cheap substrates including maize mash and molasses. In addition, there are cheaper potential fermentation substrates, such as algal biomass, whey, wood wastes and Jerusalem artichokes [Jones and Woods, 1986]. The fermentation end products, acetone and butanol, are not produced constitutively, but rather as part of a detoxification system [Jones and Woods, 1986]. *C. acetobutylicum* has a biphasic metabolism, the phases of which are known as acidogenesis and solventogenesis. Acetic and butyric acid are produced by vegetative cells of *C. acetobutylicum* in the acidogenic phase of development. This lowers the pH in the immediate vicinity of the organism until a critical breakpoint pH is reached, when the energy gradient across the cell membrane can no longer be maintained. Acids are then re-assimilated and reduced to produce the solvents acetone and butanol in the solventogenic phase of development. The pH is increased in this process and the energy gradient is restored. Butanol is produced at inhibitory concentrations [Jones and Woods, 1986], but by producing solvents, the organism is able to complete the process of spore formation, resulting in spores that are resistant to both solvents and oxygen. The genetic switches that are involved in solvent production may therefore be responsible for developmental changes involved with sporulation.

1.2.1. Acidogenesis and solventogenesis

Most of the enzymes involved in both acidogenesis and solventogenesis in *C. acetobutylicum* have been identified and purified [Chen, 1993]. Common to both metabolic pathways is the breakdown of glucose to acetyl-CoA with the concurrent release of hydrogen and carbon dioxide and the production of adenosine triphosphate (ATP). Acetyl-CoA is a key intermediate compound as all the fermentation products can be derived from it. In acidogenesis, acetate and butyrate are produced with the formation of ATP. In solventogenesis, *de novo* synthesis of solvents takes place as well as the production of solvents through the reduction of acids produced in acidogenesis. Using radio-isotopes, carbon in acetone, butanol and ethanol was traced to carbon from acetate, butyrate and sodium carbonate [Zhou and Traxler, 1992]. The re-entry of acetic and butyric acids into metabolism may involve different mechanisms under different growth conditions [Chen, 1993]. This is corroborated by the fact that fermentation in media containing different carbon sources results in different fermentation products [Awang *et al.*, 1992]. Of the tested substrates, cellobiose allowed the complete re-utilization of acids and the highest production of butanol. When pyruvate was the sole carbon source, only acids were produced [Verhasselt and Vanderleyden, 1993]. Glucose utilization decreased in acidogenic cultures to which pyruvate had been added and no solvent production was demonstrated. When pyruvate was added to cultures in the solventogenic phase, solventogenesis ceased and acid production commenced [Janati-Idrissi *et al.*, 1989].

Minor metabolic pathways exist in *C. acetobutylicum*, that lower the yield of the major products, acetone and butanol, by competing with the production of acetyl-CoA from pyruvate [Papoutsakis and Bennett, 1993]. This can, for example, result in the formation of acetoin and lactate. Inhibition of protein synthesis can reduce the flow of metabolites to these side pathways. The addition of chloramphenicol (Cm) to cultures with radioactive labeled carbon sources, increased the recovery of labeled carbon from acetone, butanol and ethanol as well as increasing the total yield of solvents [Zhou and Traxler, 1992]. The production of ethanol was thought to take place in solventogenesis [Jones and Woods, 1986], but a class of mutant has been identified that is able to produce ethanol and is blocked in solventogenesis [Bertram *et al.*, 1990, Babb *et al.*, in press]. Ethanol production is therefore not directly associated with the production of acetone and butanol.

In *C. acetobutylicum*, no ATP is formed when the solvents acetone and butanol are formed from acetyl-CoA, but the redox balance is restored by this metabolic pathway [Jones and Woods, 1986]. This helps to maintain the energy gradient across the membrane. *C. acetobutylicum* strains ATCC 824 and P262 both produce extracellular polysaccharide material associated with the solvent producing clostridial form. This may also act as a sink for non-reduced compounds [Jones and Woods, 1986].

The physiological switch from acidogenesis to solventogenesis is linked to culture conditions, but the critical pH value at the pH breakpoint ranges from pH 5.0 to pH 6.5 for different strains of *C. acetobutylicum* [Jones and Woods, 1986]. The pH is not the only critical factor. By raising the levels of acids and maintaining a constant pH value of 7.0, the switch in metabolism can be induced [Jones and Woods, 1986]. High acetate and butyrate concentrations at low pH mimic conditions at the end of acidogenesis. It is the products of acidogenesis that induce solventogenesis. Acetate alone was insufficient to initiate solventogenesis in *C. acetobutylicum* ATCC 824 [Hüsemann and Papoutsakis, 1990]. *Clostridium butylicum*, which also undergoes a biphasic metabolic cycle, needed both acetate and butyrate for effective solventogenesis induction [George and Chen, 1983]. Reproducible induction of solvent production in continuous culture of *C. acetobutylicum* was obtained by addition of butyrate at low pH [Bahl and Gottschalk, 1982].

The concentration of fermentation products, H_2 and CO_2, also affected the ratio of solvents produced in *C. acetobutylicum* [Mollah and Stuckey, 1992]. When continuous cultures of *C. acetobutylicum* NCIB 8052 were sparged with nitrogen and denied H_2 and CO_2, acids were the only fermentation products. However, conflicting results have been obtained from batch cultures [Mollah and Stuckey, 1992]. The switch to solventogenesis in *C. acetobutylicum* can be artificially induced by the inhibition of H_2 evolution, for example, by the addition of substances such as methyl viologen [Rao and Mutharasan, 1986]. Phosphate limitation has also been reported to result in production of solvents [Bahl and Gottschalk, 1982].

The shift to solventogenesis was induced by removing the pH control in continuous cultures of *C. acetobutylicum* DSM 1731 [Grupe and Gottschalk, 1992]. This resulted in a drop in the ratio of ATP to adenosine diphosphate (ADP) and a drop in reduced nicotinamide adenine dinucleotide phosphate (NADPH) levels. The addition of acetate or butyrate resulted in similar physiological changes, but the addition of methyl viologen affected neither. In methyl viologen-induced solventogenesis, butanol was the predominant product. This led to a model for initiation of solventogenesis, with the level of ATP/ADP and NADPH each signaling different regulatory controls [Grupe and Gottschalk, 1992]. A mathematical model for solvent production has been proposed for batch cultures, based on data from *C. acetobutylicum* ATCC 824 [Jarzebski *et al.*, 1992]. In this model, a critical level of intracellular butyrate triggered the metabolic shift. These models are not necessarily contradictory, but the implied differences could be due to strain differences or differences in growth conditions.

The possible methods by which signals controlling the switch in metabolism are processed to bring about differential gene expression include: 1) signal transduction via protein phosphorylation; 2) small molecule effectors such as guanosine tetraphosphate (ppGpp); 3) sigma factor control of promoter specificity [Rogers and Gottschalk, 1993]. These signal processing mechanisms are known to operate in regulatory pathways of other bacteria [Rogers and Gottschalk, 1993], but their importance and relevance remains to be elucidated in *C. acetobutylicum*.

1.2.2. Developmental morphology of *C. acetobutylicum*

The switch between acidogenesis and solventogenesis is accompanied by changes in cellular structure. *C. acetobutylicum* P262 exhibits a clear progression of well defined morphological stages [Long *et al.*, 1983] (Fig. 1-1.). During acidogenesis, cells are phase dark and motile [stage *O*]. A decrease in motility is accompanied by granulose accumulation in the cells. This is not true for all *C. acetobutylicum* [Reysenbach *et al*, 1986]; of 15 strains tested, 14 could produce granulose and only 5 strains could produce granulose in all the media tested. Classically, solvent production is typified by cigar-shaped clostridial forms [stage *I*]. Subsequent morphological changes are involved with the production of spores, a process requiring the ordered progression of several biochemical steps. The forespore is formed [stage *II*] and engulfed by the mother-cell [stage *III*]. Coat formation of the spore [stage *IV*] is followed by cortex formation [stage *V*], spore maturation [stage *VI*] and, finally, by the release of the spore through cell lysis of the mother-cell [stage *VII*]. The mother cell is sacrificed to produce the resistant spore.

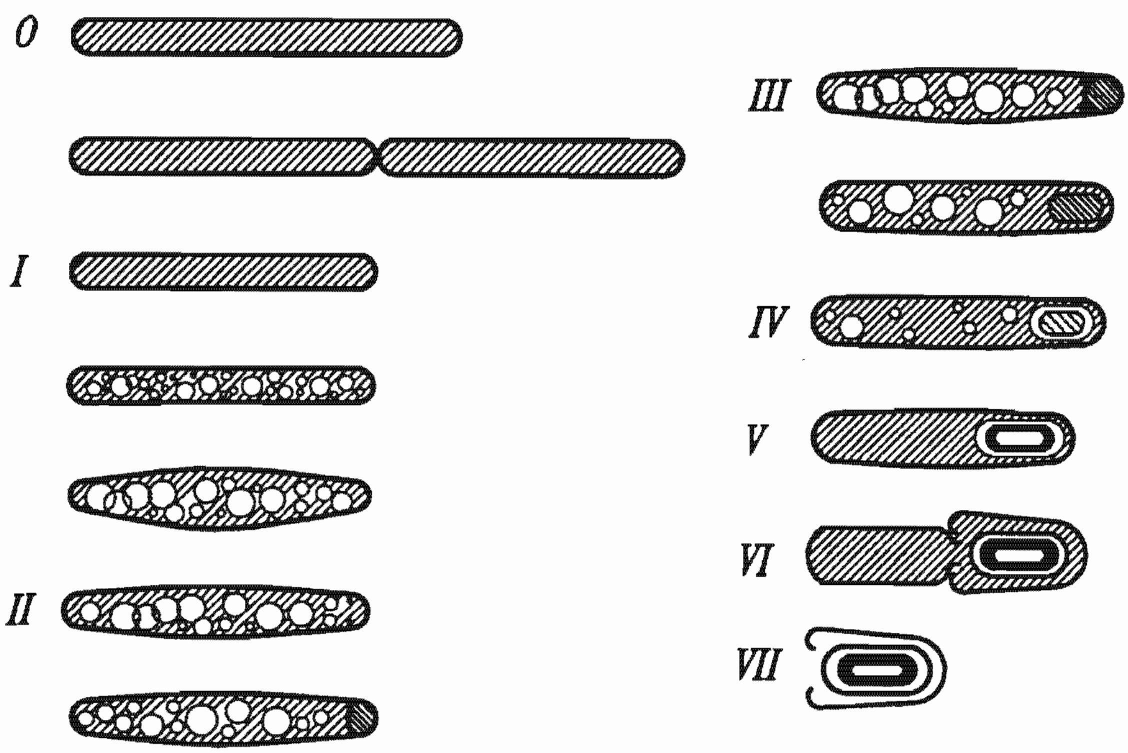

Fig. 1-1. Stages of developmental morphology as *C. acetobutylicum* P262 sporulates, adapted from electron micrographs [Long, 1984]. Sporulation stages are indicated with Roman numerals and described in the text.

Sporulating cells are unable to revert to the vegetative, replicative form. However, a proportion of cells in culture degenerate without forming spores [Jones and Woods, 1986]. It is not clear whether this is due to imbalances in sporulation regulation, the inability to begin the differentiation process or whether this is a strategy to ensure that some spores survive. The growth rate of *C. acetobutylicum* in continuous cultures is fine-tuned to maximize solvent production by preventing the cells from degenerating (at a high dilution rate) or sporulating (at a low dilution rate) [Bahl and Gottschalk, 1985]. In addition, the choice of carbon source has a significant effect on the level of sporulation as well as on the fermentation products [Awang *et al.*, 1992]. *C. acetobutylicum* strains generally sporulated well in a medium that contained glucose and starch, but *C. acetobutylicum* P262 was atypical in this regard [Awang *et al.*, 1992].

The physiological controls of sporulation in *C. acetobutylicum* are not well understood. The organism requires carbon, nitrogen and energy sources for sporulation, and sporulation is not induced by limiting any of these requirements [Long, 1984]. Initiation of sporulation in *B. subtilis* is induced by starvation and is controlled by a series of proteins that act as a phosphorylation relay [Burbulys *et al.*, 1991]. An analogous enzyme from *C. acetobutylicum*, DnaK, acts as a protein kinase and phosphorylates a 50kDa protein [Balodimos *et al.*, 1990]. The importance or relevance of this protein has not been established in sporulation induction in *C. acetobutylicum*. In other well studied systems (*Streptomyces*, *B. subtilis* and *Saccharomyces cerevisiae*) there is a drop in guanosine triphosphate (GTP) levels at the onset of sporulation [discussed in Rogers and Gottschalk, 1993]. This drop in GTP levels has also been found for *C. acetobutylicum* P262 in batch cultures at the switch to solventogenesis [Santangelo *et al.*, 1989], but there was a drop in ATP concentrations in continuous cultures of *C. acetobutylicum* DSM 1731 [Grupe and Gottschalk, 1992]. It appears that the association of a specific nucleotide with a signal to initiate solvent production or sporulation could be strain specific or could depend on growth conditions.

1.2.3. Link between solvent production and sporulation

The link between solvent production and sporulation in *C. acetobutylicum* is not clearly defined. Industrial cultures of *C. acetobutylicum* were stored as spore stocks because high solvent production characteristics were more reliably maintained [Jones and Woods, 1986]. However, the efficiency of sporulation did not correlate with the yield of solvents [Awang *et al.*, 1992]. There have been reports that repeated subculturing of *C. acetobutylicum* results in gradual loss of solvent production and in a change in colony morphology [Jones and Woods, 1986]. This genetic alteration may not be a simple link to sporulation and it depends on the strain and culture conditions [Jones and Woods, 1986]. Two colony types have been identified in *C. acetobutylicum* P262 [Babb, 1990], but their significance is not known. Asporogenous mutants have been isolated that could produce solvents, but no mutants have been found that could from mature spores without producing solvents. This was taken to indicate that solvent production preceded sporulation [Long *et al.*, 1984b]. However, one mutant of *C. acetobutylicum* P262, generated with conjugative transposons, produced no solvents and was able to initiate sporulation [Babb, 1990].

Although the control of sporulation and solventogenesis are linked, they probably form two branches of a global regulatory network. This has been found to be the case in other clostridia which have analogous biphasic metabolisms. *Clostridium beijerinckii* produces acids (butyrate and acetate) and solvents (butanol and ethanol) in a two step process. The association of solventogenesis and sporulation is close, but not invariant and the processes can occur separately [Ahmed *et al.*, 1988]. Cytotoxin production has been linked to an early sporulation event sporulation in *Clostridium difficile* [Kaniya *et al.*, 1992]. Enterotoxin production by *Clostridium perfringens* is connected with sporulation [Garcia-Alvarado *et al.*, 1992]. The medical importance of these species enhances the necessity of understanding the genetics of sporulation in clostridia.

Cell density also plays a role in sporulation regulation. In *Clostridium butyricum*, acetate and butyrate caused product inhibition of the fermentation at concentrations of $27g.l^{-1}$ and $19g.l^{-1}$, respectively [Biebl, 1991]. Cultures at high optical density (OD) had different inhibition concentrations to those at low OD, which was taken to indicate a cell density function. Cell density plays a role in sporulation of *B. subtilis* [Grossman and Losick, 1988] and there could be an analogous factor in *C. acetobutylicum* that affects initiation of sporulation and solventogenesis.

B. subtilis and *C. acetobutylicum* have similar morphological development. It is not known how different or how similar the genetic processes are between the organisms. Very little is known about *C. acetobutylicum* sporulation genes, but a gene from *C. acetobutylicum* P262 with high homology to the *spo*IID sporulation gene from *B. subtilis* has been identified [Reid, pers. comm.]. However, the gene was unable to complement appropriate mutants of *B. subtilis*, despite the similarity between morphological changes of the organisms. It is therefore useful to discuss regulation of sporulation in *B. subtilis* at this stage.

1.2.4. Overview of sporulation in *B. subtilis*

Over fifty chromosomal loci and over 100 genes have been attributed to essential sporulation functions in *B. subtilis* [Freeze and Heinze, 1984]. The process is far from simple and many mechanisms are employed by the cell to ensure the temporal and spatial co-ordination of spore development, as well as the integrity of the dormant spore. Originally, a cascade of sigma (σ) factors was proposed to control the regulation of sporulation by sequentially promoting the expression of different sets of genes [Losick and Pero, 1981]. Now it is known that there are other factors that play an important role. This organism has been an excellent research tool for studying sporulation because *B. subtilis* is easily transformed, chromosomal mapping is possible and because sporulation can be induced. Many reviews have been published on *B. subtilis* sporulation, the most recent of which was written by Errington (1993).

There are seven distinct stages of morphological change in *B. subtilis* during differentiation:

0	vegetative and dividing cells
I	axial filamentation (not considered part of sporulation)
II	forespore septum formation
III	prospore engulfment
IV	cortex formation
V	spore coat formation
VI	spore maturation
VII	release of spore by mother-cell lysis

It is immediately obvious how similar the process appears to that in *C. acetobutylicum* (Fig. 1-1). Sporulation initiation in *B. subtilis* is, as in *C. acetobutylicum*, preceded by a drop in pH [Schlegel, 1985].

The nutritional requirements of *B. subtilis* change at different stages of differentiation [Freeze and Heinze, 1984]. Certain compounds are required for sporulation (such as thiamine) and others are needed only in vegetative phases (such as biotin). Lack of manganese ions results in cells blocked at sporulation stage *0* [Freeze and Heinze, 1984]. The choice of medium, supplements and antibiotics have a major effect on the differentiation process because differentiation is the response of the cell to environmental signals. This has been used to design media that induce rapid and efficient sporulation [Freeze and Heinze, 1984]. Starvation for carbon, nitrogen or phosphorus can induce sporulation, but energy-efficient carbon sources, such as glucose, repress the process. The standardization of sporulation induction conditions is important if the temporal expression of a gene is to be determined relative to a set time point. However, there are three sporulation induction systems in use [Errington, 1993]. The initiation point of sporulation is defined as either the end of exponential growth in nutrient limiting media, the time of addition of the sporulation-inducer decoyinine or the time of resuspension in starvation media. It has not always been possible to determine the temporal sequence of gene expression when different induction systems have been used [Errington, 1993].

Sporulation begins with a process similar to cell division in which the asymmetric physical segregation of chromosomes may be important [Errington, 1991]. The signal that initiates sporulation is critical to the whole process. The controlling mechanism of sporulation can be represented by an hourglass because many different signals can affect the critical regulator which then begins a cascade of many different events in a positive feedback loop. The SpoOA protein appears to be the critical regulator that is able to start the cascade [Errington, 1993]. It acts as the terminal receptor of a phosphate group, which has been transferred through a multicomponent phosphorelay involving proteins KinA, SpoOF and SpoOB [Burbulys *et al.*, 1991]. Other important regulators of the transition to sporulation are proteins AbrB, Hpr and Sin [Strauch and Hoch, 1993]. These proteins integrate sporulation with late stationary phase genes and help to control the timing of early sporulation genes. The genes that express these proteins are under SpoOA regulation. The SpoOA protein also controls the regulation of its own gene in a positive feedback loop [Errington, 1993].

Losick and Pero (1981) proposed that there was a sequential succession or cascade of σ factors controlling sporulation. The order of σ factor expression, in which each σ factor controls the expression of the next σ factor, has now been determined ($\sigma^H \rightarrow \sigma^F \rightarrow \sigma^E \rightarrow \sigma^G \rightarrow \sigma^K$) [Stragier and Losick, 1990]. The mother cell and the forespore undergo different morphological changes and gene expression in each compartment is controlled separately. There are σ factors specific for each compartment, but the same gene is often under the control of several different stage-specific σ factors [Doi and Wang, 1986].

There are other important factors controlling compartment specificity of gene expression. The state of the chromosome effects the expression of genes on that chromosome through several mechanisms. The chromosome in the forespore condenses rapidly in early sporulation and this reduces gene expression in the forespore [Setlow, 1991]. In addition, the location of a gene on the forespore chromosome affects its expression levels [Sun *et al.*, 1991]. It is not known how this effect is mediated. Genes in the mother-cell are also affected by their location on the chromosome. A chromosomal rearrangement in the mother cell brings two portions of a gene together, thereby allowing the gene to be expressed [Stragier *et al.*, 1989]. The discrimination of the forespore and mother-cell chromosomes may be mediated through the physical state of the chromosomes. In *Bacillus coagulans*, spore deoxyribonucleic acid (DNA) is methylated and this appears to be important in chromosome discrimination [Bueno *et al.*, 1986], but this has not been shown in *B. subtilis*.

It is important that development is synchronized between the mother cell and the forespore, which means that the compartments must be able to communicate. The prospore is surrounded by four membranes: its own double membrane and that of the mother-cell. The prospore is further isolated from the mother cell by the development of the spore coat and cortex. The post-transcription modification of precursor σ factors may be an important mechanism involved in the synchronization of gene expression. The product of the *bof*A gene has been implicated in the processing of the mother-cell specific σ^K factor while under control of the forespore specific σ^G factor [Ricca *et al.*, 1992]. This communication between the mother-cell and the forespore eliminates the possibility of the exclusive use of a simple linear set of genetic controls in sporulation regulation (such as the σ factor cascade) and it necessitates the use of branched pathways of regulatory control [Errington *et al.*, 1988].

1.3. Genetics of *C. acetobutylicum*

The availability of methods for genetic manipulation of *B. subtilis* aided research in that organism. Recently, genetic techniques have been applied to *C. acetobutylicum* and subsequent progress has been rapid.

1.3.1. Genetic methodology

C. acetobutylicum is not naturally competent and until recently, genes had to be cloned from *C. acetobutylicum* into *E. coli* to be studied. The genome of *C. acetobutylicum* is AT rich [Jones and Woods, 1986], and this leads to differences in promoter sequences and in codon bias when compared to those of *E. coli*. The *lyt* gene of *C. acetobutylicum* ATCC 824 had to be reconstructed from two overlapping clones because it could not be cloned directly in *E. coli* [Croux and Garcia, 1992], but several genes cloned from *C. acetobutylicum* were expressed well in *E. coli* [Papoutsakis and Bennett, 1993]. It is preferable to study regulation in a homologous host because of differences in cellular factors and gene structure in heterologous hosts. Therefore, it was important to develop methods to transform *C. acetobutylicum*. The presence of strong nuclease activity has hampered attempts at manipulating DNA from *C. acetobutylicum* [Papoutsakis and Bennett, 1993], and the high levels of proteolytic activity have made it difficult to study gene regulation in homologous hosts [Sass *et al.*, 1993].

The first report of transformation of *C. acetobutylicum* used protoplast transformation techniques [Reid *et al.*, 1983]. This method is inefficient and has not been used routinely. However, regeneration of protoplasts could be improved by the addition of autolysin inhibitors or by the use of autolysin-defective mutants [Reysett and Sebald, 1993].

Electroporation is being used routinely for certain strains of *C. acetobutylicum* [Reysett and Sebald, 1993]. The exact method used depends on the particular strain. The application of electroporation methods developed for one strain of *C. acetobutylicum* to other strains of *C. acetobutylicum* has proved problematic [Minton *et al.* 1993]. For example, electroporation of *C. acetobutylicum* ATCC 824 using *C. acetobutylicum*

NCIB 8052 protocols was impossible [Papoutsakis and Bennett, 1993], whereas *E. coli* methods worked well. Furthermore, electroporation of *C. acetobutylicum* P262 has not been demonstrated [Woods, pers. comm.].

Young (1993b) reviewed the conjugal transfer of DNA to *C. acetobutylicum* from heterologous bacteria. Conjugative plasmids, which have the ability to mediate conjugation between different bacteria, are generally large and circular, and maintained at low copy number per cell. Plasmid transfer is mediated through nicking of plasmid DNA at the origin of transfer, *oriT*. A single strand of DNA (starting with the 5' extremity) is transferred to the recipient where the complementary strand is synthesized. In this process, other plasmids are often co-transferred. Conjugative plasmids have been found in *C. perfringens*, and plasmids pIP501 (35kilobase pairs (kb)), pAMβ1 (26kb) and pJH4, have been shown to transfer to *C. acetobutylicum*. The efficiency of plasmid transfer is donor-dependent. For example, *B. subtilis* is a poor donor. Non-conjugative plasmids can be mobilized to *C. acetobutylicum* by conjugative plasmids. Plasmid pAMβ1 was transferred to *C. acetobutylicum* P262 and simultaneously mobilized non-conjugative plasmid pVA797 [Yu and Pearce, 1986]. Mobilization of cloning vectors from *E. coli* to *C. acetobutylicum* has been demonstrated [Williams *et al.* 1990].

Minton *et al.* (1993) reviewed *C. acetobutylicum* cloning vectors. Currently there has only been progress in two species of *Clostridium*, viz. *C. acetobutylicum* and *C. perfringens*. *C. acetobutylicum* NCIB 8052 was the first *C. acetobutylicum* strain to be electroporated with plasmid pAMβ1 and derivatives thereof. It was the early success of conjugating pAMβ1 that led to the development and use of this plasmid as a cloning vector, even though it did not replicate in *E. coli* and was unstable in *B. subtilis* [Young, 1993a]. *E. coli* origins of replication have been inserted into these plasmids to create *E. coli* / *C. acetobutylicum* vectors. Plasmid pKR13 was constructed from pIM13 from *B. subtilis* and pBluescript KS+. These vectors were ineffective for use in *C. acetobutylicum* ATCC 824 [Papoutsakis and Bennett, 1993]. *B. subtilis* / *C. acetobutylicum* vectors were created from plasmid pIM13, leading to the pFNK series of plasmids. Plasmid pIP404, from *C. perfringens*, is the best studied clostridial plasmid [Minton *et al.*, 1993], but it has not been utilized as the basis of vectors for *C. acetobutylicum*. However, no plasmid replication has been demonstrated in *C. acetobutylicum* P262 [Reid, pers. comm.]. The strains of *C.*

acetobutylicum differ with respect to their ability to allow plasmid replication and this highlights the fact that *C. acetobutylicum* strains are very different.

The best results of *C. acetobutylicum* ATCC 824 transformation were obtained with plasmids that contained few *Cac*824I restriction enzyme sites (5'-GCNGC-3') [Papoutsakis and Bennett, 1993]. This was not necessarily the case for *C. acetobutylicum* NCIB 8052 which has no detectable restriction activity [Papoutsakis and Bennett, 1993]. Several different restriction enzymes have been identified in *C. acetobutylicum*, including *Cac*II, which is methyl-specific and digests DNA at 5'-GCNNGC-3' [Azeddoug and Reysett, 1991]. The restriction of foreign DNA in *C. acetobutylicum* can be reduced by the judicious use of *E. coli* hosts that protect against methyl-mediated restriction and against restriction not protected by adenine methylation.

Different antibiotic selection markers can be inserted into *C. acetobutylicum* vectors. The choice of antibiotic selection markers in *C. acetobutylicum* is important. An erythromycin resistance (Em^R) gene, *erm*, has proved very useful in selection of *C. acetobutylicum* NCIB 8052 transformants because it is expressed constitutively and because Em can be used at high concentrations [Minton *et al.*, 1993]. Tetracycline (Tc) and chloramphenicol (Cm) were poor choices of antibiotic for this strain, because of the high incidence of spurious antibiotic resistant cells. However, different strains of *C. acetobutylicum* display different antibiotic resistance properties. *C. acetobutylicum* DSM 792 and DSM 1731 gradually develop resistance to Em, making this an unsuitable means of selection [Dürre, 1993]. *C. acetobutylicum* P262 is very sensitive to both Tc and Em [Babb *et al.*, in press], but Tc offered better selection because this strain of *C. acetobutylicum* also displayed a gradual response to Em.

Vectors need both structural and segregational stability. Therefore plasmids with theta replication modes are preferable to plasmids that have single-stranded intermediates. This improves the efficiency of cloning and facilitates the maintenance of large plasmids [Minton *et al.*, 1993]. Complete segregational stability has not been demonstrated for any plasmid in *C. acetobutylicum* [Papoutsakis and Bennett, 1993]. However, a mutant of *C. acetobutylicum* N1-4081 was obtained that did allow stable segregation [Azeddoug *et al.*, 1992]. Plasmid segregation can also be improved by the incorporation of resolvase genes in the plasmids. The addition of the *res* gene from plasmid pAMβ1 to *C. acetobutylicum* vectors improved plasmid stability, probably by resolving multimeric forms of the plasmid [Swinfield *et al.*, 1991].

Conjugative transposons offer an alternative means of introducing DNA into organisms. These transposons are able to mediate their own transfer from one organism to another and it may be possible to use them as vectors for cloned DNA fragments. They have a more immediate application in generating mutants and are discussed in the next section of this chapter.

1.3.2. Overview of conjugative transposons

The use of chemical mutagens in bacteria is restricted by the fact that the genotype of generated mutants is never absolutely clear. Transposon mutagenesis allows genetically-defined mutants to be identified and, therefore, allows the correlation of genetic information with phenotypic information. The use of transposons in clostridia has been reviewed recently [Dürre, 1993].

Many transposable elements have been discovered in Gram-positive bacteria. These are either small, resembling Tn*3* from Gram negative bacteria, or large, possessing fertility functions [Dürre, 1993]. Only three non-conjugative transposons have been found occurring naturally in the clostridia. Two were found on plasmids in *C. perfringens* (Tn*4451*, Tn*4452*) and one in *C. difficile*. Transposon Tn*917*, originally from *Enterococcus faecalis*, is the only non-conjugative transposon that has been transferred to and expressed in the clostridia [Yu and Pearce, 1986]. It was unclear whether this transposon remained on the plasmid of transfer (pVA797) or whether it transposed in *C. acetobutylicum*. Tn*925*, a conjugative transposon, has also been used as a vector for Tn*917*, but no independent transfer of Tn*917* from Tn*925* was shown [Dürre, 1993].

Conjugative transposons are able to mediate their own transfer between organisms. They have an extremely large host range and have been able to conjugate from a variety of organisms to most other organisms tested, including both Gram-positive and Gram-negative bacteria [Dürre, 1993]. Because of the broad host range of conjugative transposons, they are proving extremely useful in organisms that lack well-developed genetic systems, for example *Bacillus pumilis* and *Bacillus firmus* [Hendrik *et al.*, 1991; Guffanti *et al.*, 1991]. The conjugative transposons are all large (more than 16kb) and most carry the *tetM* tetracycline resistance gene. Tet(M) is thought to act as an elongation factor analog [Burdett, 1991]. Transposon Tn*916* is the best studied conjugative transposon, and, since the conjugative transposons display extensive DNA homology, Tn*916* DNA is able to hybridize to the DNA of other conjugative transposons, including Tn*1545* [Woolley *et al.*, 1989], Tn*925* [Bertram and Dürre, 1989] and Tn*5381* [Rice *et al.*, 1992].

A model has been proposed for conjugative transposition [Caparon and Scott, 1989]. The mechanism of transposition is similar in some respects to the mechanism of transfer of lambda phages of Gram-negative bacteria [Storrs *et al.*, 1991]. The transposon excises from its host location through a staggered cleavage that requires both integrative and excisive functions [Storrs *et al.*, 1991]. The two genes encoding these functions have been identified from Tn*1545* [Poyart-Salmeron *et al.*, 1989]. In certain cases, a host factor from the donor is also required for transposition [Bringel *et al.*, 1991]. Excision of the transposon results in a covalently closed circular intermediate with a heteroduplex at the cleavage site. The intermediate has been isolated from Gram-positive and Gram-negative bacteria [Rice *et al.*, 1992; Clewell and Gawron-Burke, 1986]. This excision process can be perfect, which means that insertionally inactivated genes can be re-activated when the transposon excises [Gawron-Burke and Clewell, 1984]. There is, however, a region of 4bp at the target site in which base substitutions can occur [Gawron-Burke and Clewell, 1984].

The mechanism of integration is probably the reverse of the excision process. However, where integrative and excisive functions were needed for excision, only the integrase activity is required for integration [Storrs *et al.*, 1991; Bringel *et al.*, 1992a]. For integration, the target site of the transposon does not need to be homologous to the heteroduplex and the target sequence is not duplicated upon insertion of the transposon [Clewell and Gawron-Burke, 1986]. The transposons rarely transpose after integration and can be stable for up to 350 generations in *C. difficile* [Mullany *et al.*, 1991]. Conjugative transposons replicate passively through chromosome duplication. Copies of the transposon in the recipient do not repress further incoming transposons [Norgren and Scott, 1991], but there is evidence for *trans* activation of conjugative transposons [Flannagan and

Clewell, 1991]. Several investigators have found that transcipients frequently inherit multiple copies of conjugative transposons [Young, 1993b]. Integration of the transposons into new chromosome loci occurred more frequently by transposition than by homologous recombination [Norgren and Scott, 1991].

The DNA sequences of the termini of transposon Tn*916* are not identical to each other and contain imperfect repeats [Clewell *et al.*, 1988]. The termini of Tn*1545* are almost identical to those of Tn*916* [Caillaud and Courvalin, 1987] and their TetM genes have more than 95% identity at the DNA level [Su *et al.*, 1992]. Transposon Tn*1545* is very similar to transposon Tn*916* but it contains a gene for Em^R, as well as a gene for Tc^R. All the conjugative transposons found to date are very similar to each other [Dürre, 1993]. This suggests that they have a common ancestor from which they have recently diverged.

Transposition is not completely random and the preferred sites of insertion are at AT rich DNA sequences [Dürre *et al.*, 1993]. It has not been ascertained how the transposons differ in their preferred target sites. However, the nucleotide sequencing of DNA junction regions where transposons have inserted has suggested the following target sites: Tn*1545*, 5'-TTTNTNNN(N)(N)TAAAAA-3' [Caillaid and Courvalin, 1987]; Tn*916*, 5'-ACTAAA-3' [Young *et al.*, 1989a]; Tn*925*::Tn*917*, partial site of 5'-AAAAA-3' [Babb *et al.*, in press]. Transposon Tn*916* had different patterns of insertion in different strains of *C. acetobutylicum*: *C. acetobutylicum* NCIB 8052 had a single preferred insertion site [Woolley *et al.*, 1989]; insertions in *C. acetobutylicum* DSM 1791 were relatively random, but all the transconjugants were asporogenous [Bertram *et al.*, 1990]; *C. acetobutylicum* strains ATCC 824 and P262 had relatively random insertions [Sass *et al.*, 1993, Babb *et al.*, in press]. Despite the similarities between the *C. acetobutylicum* recipients, the transconjugants differed greatly. This suggests the involvement of a host factor in the recipients during conjugative transposition.

There is also evidence for the involvement of a host factor in the donor organism during transfer of transposon Tn*916* by non-transpositional mechanisms [Bringel *et al.*, 1992b]. There may be involvement of host factors in the donor organisms during conjugative transposition as well. Transfer efficiency of transposon Tn*925* from *E. faecalis* was increased by growing the donor in the presence of Tc [Torres *et al.*, 1991]. However, the efficiency of conjugative transposition of Tn*925* from *B. subtilis* was not increased by growing the donor in the presence of Tc [Showsh and Andrews, 1992]. Transposition of Tn*5381* was also increased by growth of the donor in media containing Tc [Rice *et al.*, 1992].

The efficiency of transposition of conjugative transposons from locations on the chromosome appeared to be dependent on the exact location of the transposon [Flannagan and Clewell, 1991]. In general, transfer of Tn*916* was higher from a plasmid donor than from a chromosomal donor [Dürre, 1993]. When conjugative transposition occurred from a plasmid donor, no extra-chromosomal elements from the donor were detected in the recipients after mating [Dürre, 1993]. However, the possibility of plasmid transfer cannot be excluded if the plasmids were unstable in the recipients. Plasmids may be involved in conjugative transposition in other ways. Transposon Tn*925* was able to mobilize two plasmids from *B. subtilis* during conjugative transposition [Guffanti *et al.*, 1991]. Non-conjugative plasmids have also been mobilized by transposon Tn*916* [Flannagan and Clewell, 1991]. Furthermore, it has been shown that Tn*925* could mobilize chromosomal genes through a mechanism that resembled protoplast fusion [Torres *et al.*, 1991]. There was extensive recombination throughout the lengths of the chromosomes of the recipient and donor organisms. It is possible, therefore, that the transfer of conjugative transposons could be accompanied by the transfer of plasmid or chromosomal genes from the donor.

Plasmids that contain conjugative transposons may be involved directly with the transfer of the transposons. In certain cases, transposon Tn*916* was transferred to the recipient organism by plasmid functions and not by conjugative transposition [Hendrik *et al.*, 1991]. The common donor plasmids for transposons Tn*916* and for Tn*925*, plasmids pAD1 and pCF10 respectively, both carry pheromone response functions [Pontius and Clewell, 1991, Christie *et al.*, 1987]. The pheromone response function, triggered in response to events such as the presence of a pheromone, causes cell aggregation of recipient cells, which improves transfer efficiency of the pheromone response plasmid [Clewell, 1993].

The conjugative transposons may prove to be useful tools for allelic replacement of specific genes and for the direct introduction of foreign DNA especially by using deleted versions of the transposons [Mullany *et al.*, 1991]. The potential of transposon Tn*916* has been demonstrated in this regard. An amylase gene was inserted into the transposon and the new construct was able to transpose from *E. coli* to *Bacillus stearothermophilus* [Natarajan and Oriel, 1991]. A vector, pAT112, designed to integrate into chromosomes, has been constructed to take advantage of Tn*1545* [Trieu-Cuot *et al.*, 1991]. It contains the attachment sites of Tn*1545* and requires transposon functions *in trans* to integrate into the host chromosome. Another use for conjugative transposons could be in the mobilization of plasmids and even of chromosomal genes [Torres *et al.*, 1991]. Due to their large size, these transposons are useful as genomic markers and they have been used to map *C. acetobutylicum* chromosomes [Wilkinson and Young, 1993]. Conjugative transposons are an interesting group of genetic elements that may be developed into powerful tools for the genetic manipulation of Gram-positive organisms.

1.3.3. Recent advances in *C. acetobutylicum* genetics

One of the major goals of research in *C. acetobutylicum*, is to improve the yield of solvents from fermentation. This section gives an indication of how the tools and information, outlined above, have been applied for this purpose. However, it remains a challenge to actually improve butanol yield.

The expression of several enzymes is induced at the beginning of solventogenesis [butyraldehyde dehydrogenase (BAD), acetoacetate-CoA:acetate/butyrate:CoA transferase (CoAT), acetoacetate decarboxylase (AADC), butanol dehydrogenase (BDH)], whereas the expression of other enzymes is only partially induced [butyrate kinase (BK), phosphotransbutyrylase (PTB)]. BAD, AADC, CoAT and BDH are required for acetone and butanol synthesis, and BK and PTB are required for butyrate synthesis. BAD activity dropped rapidly after protein synthesis was inhibited by the addition of Cm. However, butanol production was increased by the addition of Cm [Zhou and Traxler, 1992]. The *de novo* synthesis of enzymes required for butanol production decreased after mid-solventogenesis, although the production of butanol continued [Terracciano and Kashket, 1986].

Several of the solventogenesis genes have been cloned and re-introduced into *C. acetobutylicum* [Papoutsakis and Bennett, 1993]. A cloned copy of the gene encoding AADC was transferred into *C. acetobutylicum* ATCC 824 on a pIM13-based vector, pFNK3 [Papoutsakis and Bennett, 1993]. Enzyme activity was higher in the transformed *C. acetobutylicum* and gene expression was induced in stationary phase. Other genes that have been cloned and re-introduced into the homologous host include the genes coding for PTB and BDH [Papoutsakis and Bennett, 1993]. The proteins were over expressed without debilitating the transconjugant *C. acetobutylicum* and the ratio of fermentation products was altered in the transconjugants, but the total yield of solvents was not increased.

The putative ribosome binding sites of cloned solventogenesis genes display extensive sequence homology to 3' terminal region of the 16S ribonucleic acid (RNA) molecule from *B. subtilis*: the -10 region has a consensus site close to AGGAGG [Papoutsakis and Bennett, 1993]. In addition, the solventogenesis genes that have been cloned do not have a promoter consensus sequence that is distinct from the consensus Gram-positive promoter, which would be indicative of a common regulatory protein [Gerischer and Dürre, 1992]. There has been little evidence to support the idea that there is a solventogenesis-specific σ factor. An alternative mechanism of gene control has been proposed which involves the judicious use of a rare transfer RNA molecule (tRNA), $\text{tRNA}_{\text{ACC}}^{\text{Thr}}$ [Sauer and Dürre, 1992]. Although the codon representing this tRNA is rare, it occurs in genes expressed at the end of exponential growth or genes involved in the uptake of carbon or nitrogen substrates. By controlling the expression of this tRNA, it is speculated that the cell is able to regulate the expression of those genes that contain the corresponding codon.

The onset of solventogenesis is related to stress response [Bahl, 1993]. Isolation of stress related genes and their regulatory genes may provide a handle for manipulation of solvent genes [Pich *et al.* 1990]. The so-called heat shock proteins (HSP) are the best studied stress response proteins. Several of the HSP proteins are produced in *C. acetobutylicum* in response to oxygen, solvents, pH changes, and growth rate changes [Pich *et al.*, 1990; Terracciano *et al.* 1988]. The function of HSP is not clear as some of these proteins play a role in cell physiology under non-stressed conditions. The genes for GroEL, DnaJ, GrpE, GroES and DnaK HSP proteins from *C. acetobutylicum* have been cloned by using heterologous antibodies and

DNA probes [Bahl, 1993]. This was possible because there is some homology in the *C. acetobutylicum* stress response to the system in *E. coli*, although the regulatory controls of the two heat shock responses appear to be different [Narberhaus and Bahl, 1992]. The promoter sequence of the *gro*ESL genes from *C. acetobutylicum* DSM 1791 matched the normal Gram-positive consensus promoter and, therefore, did not indicate the presence of a heat shock-specific σ factor [Narberhaus and Bahl, 1992]. Direct purification of σ factors is currently underway to try and identify global regulators. The major DNA-dependent RNA polymerase has been cloned [Pich and Bahl, 1991].

Recombinant DNA technology can also enhance the fermentative capabilities of *C. acetobutylicum* by extending the substrate range [Bronnenmeier and Staudenbauer, 1993]. Clostridia and related Gram-positive bacteria seem to lack cyclic adenosine monophosphate and the related catabolite repression system. It is therefore unclear how *C. acetobutylicum* responds to available substrates. Certain carbohydrate utilization genes, including amylases and galactosidases, have been cloned from different strains of *C. acetobutylicum* [Bronnenmeier and Staudenbauer, 1993]. Dendograms of amylase sequences show that *Clostridium* species are as closely related to *Bacillus* and *Butyrivibrio* as they are to each other. It has been shown that several *C. acetobutylicum* strains produce both β-galactosidase and phospho-β-galactosidase [Bronnenmeier and Staudenbauer, 1993]. Phospho-β-galactosidase activity was highest in acidogenic phase cells and β-galactosidase was highest in solventogenic phase at the onset of sporulation. Synthesis of both of these enzymes is repressed by glucose.

There have been several reports of transposon mutagenesis of *C. acetobutylicum* using conjugative transposons. The method of conjugation depended on the strain used, and the resulting mutants often had multiple insertions of the transposon [Bertram and Dürre, 1989, Woolley *et al.*, 1989, Babb *et al.*, in press]. *C. acetobutylicum* NCIB 8052 had a single preferred site of insertion for Tn*916* [Woolley *et al.*, 1989]. Transposon Tn*916* transconjugants of *C. acetobutylicum* DSM 792 and DSM 1731 were all asporogenous [Bertram *et al.*, 1990], but three classes of solventogenesis mutants were obtained [Bertram *et al.*, 1990]. Solvent deficient, sporulation deficient and metronidazole resistant mutants of *C. acetobutylicum* P262 have been obtained using the transposon cointegrate

Tn*925*::Tn*917* [Babb *et al.* in press]. A protease-deficient mutant of *C. acetobutylicum* ATCC 824 was obtained with Tn*916* [Sass *et al.* 1993]. Genes that have been insertionally inactivated by transposons can be cloned using the transposon as a marker. The inactivated gene can then be used to identify intact genes from wild type genomic libraries. This was how the $\mathrm{tRNA}_{\mathrm{ACG}}^{\mathrm{Thr}}$ gene was cloned [Sauer and Dürre, 1992]. Despite all the literature on conjugative transposon mutagenesis there have been very few reports of the cloning of wild type genes corresponding to insertionally inactivated genes [Young, 1993a]. This implies that there are difficulties with the use of conjugative transposons.

1.4. Aims of this thesis

C. acetobutylicum P262 is one of the best natural producers of acetone and butanol and it has been shown to be very different from the other *C. acetobutylicum* strains in genome organization [Wilkinson and Young, 1993]. Work in our laboratory is centered on improving understanding of this particular strain. In addition to studying how fermentation products may be used to induce sporulation, this thesis is a study of a particular mutant of *C. acetobutylicum* P262, mutant m5, that was generated by transposon mutagenesis using conjugative transposons [Babb, 1990]. The mutant is blocked in solvent production and is inhibited in sporulation. This implies that the mutant has been affected in a regulatory gene that controls all of solvent production and possibly in a gene that affects sporulation. The isolation and characterization of these genes is of practical and academic importance to research in *C. acetobutylicum*.

Chapter 2

Sporulation induction in *C. acetobutylicum* P262

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2.1. Summary

The ability of certain acidogenesis and solventogenesis fermentation products to induce sporulation in *C. acetobutylicum* P262 was tested. Butyric acid was less effective than acetic acid at induction, and acetone and butanol were inhibitory to development. Acetic acid accelerated sporulation more efficiently at pH 5.2 compared to pH 5.9 and 6.5 under certain conditions. In order to ascertain the optimal temperature at which to quantify mature spores, spore heat resistance was characterized. The temperature-dependent heat resistance of spores was determined for ten minute intervals up to 90°C, and the time-dependent heat resistance was determined up to 20min at 70, 75 and 80°C. The optimal level for spore quantification was shown to be 75°C.

2.2. Introduction

It has been possible to determine the temporal sequence of gene expression during sporulation in *B. subtilis* because sporulation can be induced in an efficient manner. There are three standard methods to do this [Errington, 1993], viz. nutrient limiting medium, resuspension in starvation medium, or the addition of decoyinine. Of these methods, the second is the most reproducible. The development of a sporulation induction medium would benefit studies in *C. acetobutylicum* in a similar way. However, conditions for sporulation induction in *B. subtilis* are different from those of *C. acetobutylicum* as starvation cannot be used to induce sporulation [Long *et al.*, 1984a]. In addition, glucose represses sporulation in *B. subtilis* [Errington, 1993], but allows efficient sporulation in *C. acetobutylicum* [Awang *et al.*, 1992]. The link between sporulation and solventogenesis may prove to be useful in the development of such an induction medium. Several factors are known to induce solvent production and these may also induce sporulation. In particular, the addition of acetic acid and butyric acid induces solventogenesis [Jones and Woods, 1986], but their effect on sporulation has not been documented.

In *C. acetobutylicum* fermentations, a proportion of cells degenerate and are unable to sporulate [Jones and Woods, 1986]. Sporulation is seldom greater than 30% efficient, although 80% sporulation was achieved in certain media [Long *et al.*, 1983]. The optimum sporulation induction method should maximize sporulation efficiency by inducing rapid forespore development, while minimizing cell degeneration. To monitor efficiency of sporulation as the variables in the media are altered, an accurate spore quantification method is required. Microscopic total counts of spores are inadequate as the spores may not be viable. Spores of *B. subtilis* are heated at 80°C for ten minutes to determine viable spore counts [Nicholson and Setlow, 1990]. This method was adopted in our laboratory for determining the efficiency of relative sporulation in *C. acetobutylicum* P262.

An important factor in optimizing sporulation efficiency is the achievement of synchronous induction of the initial culture. Cultures of *C. acetobutylicum* proceed through the acidogenic phase before initiating solventogenesis or the sporulation pathways [Jones and Woods, 1986]. If development was arrested at the switch to solventogenesis or to sporulation, synchronous differentiation would follow from the addition of substances required for the switch in metabolism. Alternatively, if the expression of sporulation genes was induced by a certain set of factors, the addition of those substances to a relatively asynchronous culture would allow synchronous development to proceed. Therefore, there are two possible approaches to inducing sporulation. Cultures can be synchronized with respect to their development before allowing further differentiation, or cultures can be directly induced to begin the differentiation processes.

In this study, the effect of various fermentation products on sporulation induction in *C. acetobutylicum* P262 was tested, and the highest temperature that spores could withstand, was determined. The results of these experiments will help in the design of a standard method by which rapid sporulation can be induced and by which the efficiency of sporulation can be tested.

2.3. Materials and Methods

2.3.1. Bacterial strains and media

C. acetobutylicum P262 was obtained from National Chemical Products (Pty) Ltd. Germiston. South Africa. Clostridial basal medium (CBM) has been described previously [Allcock *et al.*, 1982]. BC medium was BASOL medium [Reysett and Sebald, 1985], supplemented with 1% (w/v) xylose and 1% (w/v) casamino acids (HY-Case, Sigma Chemical Company, St. Louis, Missouri). Stock solutions of sodium acetate and sodium butyrate were made at 25% (w/v) in water and adjusted to pH 5.2, pH 5.9, pH 6.5 or pH 7.0 with sodium hydroxide. Stock solutions of acetone and butanol were made at 25% (w/v) in water. TYG medium consisted of tryptone ($30g.l^{-1}$), yeast extract ($20g.l^{-1}$), glucose ($5g.l^{-1}$), cysteine hydrochloride ($0.5g.l^{-1}$) and sodium hydrogen carbonate ($1g.l^{-1}$). All incubations were at 34°C and anaerobic manipulations were carried out in an anaerobic cabinet (Forma Scientific Inc., Marietta, Ohio).

2.3.2. Optical density, granulose detection and sporulation stage determination

Optical density at 600nm (OD) was determined using a Corning colorimeter 252 [Corning limited, Halstead, Essex]. Cells that contained granulose appeared phase bright under interference microscopy [Zeiss photo-microscope]. The presence of granulose was determined by staining with iodine [Long *et al.*, 1983].

Spore development of *C. acetobutylicum* cultures was followed microscopically. Sporulation stages *I*, *II*, *III*, and *VII* [Long, 1984] were represented by I, II, III and VII respectively. Sporulation stages *IV* to *VI* [Long, 1984], which were difficult to distinguish by light microscopy, were jointly represented by V. The sporulation stages are shown schematically in Chapter 1 (Fig. 1-1).

2.3.3. Sporulation induction

Overnight cultures of *C. acetobutylicum* were grown from spore suspensions in 10ml of CBM medium in hungate tubes by heat shocking dilutions of the spore suspension at 65°C for 2min. The spore suspension dilution was such that after 12h of growth, the culture had reached early exponential phase.

2.3.3.1. Effect of various additives

Cultures of *C. acetobutylicum* P262 were grown in CBM (or TYG) to late exponential phase, by which time the cells were starting to produce granulose (OD approx. 1.0 units). The cells were harvested by centrifugation, resuspended in 1/10th volume CBM (or TYG) and incubated for 1h (or 3h for TYG cultures) [termed "pre-induction incubation" here]. Samples of 1ml were then added to hungate tubes containing 10ml of fresh CBM (or TYG), to which buffered acetic acid (pH 6.5), buffered butyric acid (pH 6.5), acetone or butanol had been added. Different final concentrations of these additives were tested (from 0 to $10g.l^{-1}$). The differentiation of the cells through the different stages of sporulation was monitored microscopically.

2.3.3.2. Effect of the initial development stage

Cultures were grown in CBM to various stages of development (early exponential phase, late exponential granulose-positive phase, or clostridial stage) as determined by microscopy. The cells were harvested by centrifugation, resuspended in 1/10th volume CBM, and samples (1ml) were added to hungate tubes containing 10ml of fresh CBM and acetic acid (pH 6.5, $10g.l^{-1}$). The differentiation of the cells through the different stages of sporulation was monitored microscopically. A sample of the granulose-positive culture was incubated for 1h in fresh CBM prior to resuspension in CBM containing acetate, to test the effect of the pre-induction incubation step.

2.3.3.3. Effect of the pH of acetic acid

The effect of the pH of acetic acid on sporulation induction was tested in *C. acetobutylicum* P262 cultures at different initial OD's. Cultures were grown in CBM to OD 0.25, 0.4, 0.6 and 1.2. The cells were harvested by centrifugation, resuspended in 1/10th volume CBM, and samples (1ml) were added to hungate tubes containing 10ml of fresh CBM and acetic acid ($10g.l^{-1}$) at pH values of 6.5, 5.9 or 5.2. The efficiency of sporulation induction was evaluated by determining the relative number of viable spores after 20h incubation.

2.3.4. Viable spore counts

An adaptation of a *B. subtilis* method [Nicholson and Setlow, 1990] was used to calculate the relative number of viable spores per ml (VS.ml^{-1}) between two cultures. Serial dilutions of each culture were heated aerobically at 80°C for 10min, cooled on ice and plated anaerobically on CBM or BC agar plates to determine the number of surviving spores.

2.3.4.1. Heat resistance of spores

The temperature-dependent survival of *C. acetobutylicum* spores was determined. Serial dilutions of a sporogenous culture of *C. acetobutylicum*, grown in CBM for 65h, were heated aerobically for 10min at various temperatures between room temperature and 90°C, and cooled on ice. The number of surviving spores was determined by plating the samples on CBM or BC agar plates anaerobically.

The time-dependent survival of spores and vegetative cells were determined. Serial dilutions of a sporogenous culture (grown 65h in CBM) and a vegetative culture (grown overnight in CBM) were heated aerobically at 70, 75 and 80°C for various time intervals between 0 and 25min, and cooled on ice. The number of surviving cells was determined by plating the samples on CBM or BC agar plates anaerobically.

2.4. Results

2.4.1. Sporulation induction

2.4.1.1. Effect of various additives

Acetic and butyric acid are major products of acidogenesis and may be important in the control of the switches to solventogenesis and sporulation. The effect of adding buffered acetic and butyric acid (pH 6.5) to a culture of wild type *C. acetobutylicum* P262 (WT) was examined (Table 2-1). Cultures that had acids added reached a further stage of sporulation within the time period relative to those cultures to which water had been added. When both acetate and butyrate were added together, development was slower than when acetic acid was the only additive. Butyric acid by itself was less effective at inducing sporulation than acetic acid. Acetic acid was,

Table 2-1. The effect of adding buffered acetic and butyric acid (pH 6.5) on the sporulation development of *C. acetobutylicum* P262. Exponential cultures grown in CBM medium were re-suspended in fresh medium and incubated for 1h, before re-suspending in fresh medium containing buffered acetate and butyrate at the indicated concentrations. The sporulation stages reached in the various cultures are indicated with Roman numerals.

Acetate ($g.l^{-1}$)	Butyrate ($g.l^{-1}$)	Stage of development after induction		
		12h	15h	18h
0	0	I	I	-
2	2	II	II	-
5	5	II/III	II/III	-
10	10	III	III/V	V
10	0	V	V	V/VII
0	10	III	III	V

- sporulation stage not determined

therefore, more effective than butyric acid in allowing sporulation to proceed under the experimental conditions.

WT cultures grew more slowly and in a less synchronous manner in TYG than in CBM. In testing the effect of various concentrations of buffered acetic acid on sporulation initiation in this medium, the culture was incubated for 3h in fresh TYG prior to sporulation induction to help synchronize development. After 18h incubation, the cultures were examined microscopically (Table 2-2). The cultures that had more acetic

Table 2-2. The effect of adding buffered acetic acid (pH 6.5) on sporulation of *C. acetobutylicum* P262. Exponential cultures grown in TYG medium were re-suspended in fresh medium and incubated for 3h, before re-suspending in fresh medium with buffered acetate added at the indicated concentrations. The sporulation stages reached in the various cultures after 18h of incubation are indicated with Roman numerals.

Acetate ($g.l^{-1}$)	Stage of development after induction
0	0
2	0
5	I
10	II

acid added, were able to develop to more advanced sporulation stages. The most advanced development stage was sporulation stage *II*, whereas after 18h in CBM, sporulation stage *V* was reached. CBM would therefore form a more suitable basis for a sporulation induction medium.

Acetone and butanol, the major products of solventogenesis, may act as signals in the regulation of sporulation. The effect of the solvents on sporulation was compared to that of acids (Table 2-3). Development proceeded to a later stage of sporulation when the culture was re-suspended in medium containing 5g.l^{-1} acetone compared to medium with 10g.l^{-1} acetone. Acetone and butanol were relatively inhibitory to development of spores, whereas the buffered acids induced sporulation. This implied that sporulation was induced at the same time as solventogenesis and not after solventogenesis.

Table 2-3. The effect of adding buffered acids (pH 6.5) and solvents on spore development of *C. acetobutylicum* P262. Exponential cultures grown in CBM medium were re-suspended in fresh medium and incubated for 1h, before re-suspending in fresh medium with the indicated additives. The sporulation stages reached in the various cultures after 27h of incubation are indicated with Roman numerals.

Additive	(g.l^{-1})	Sporulation stage after induction
acetate	10	V/VII
butyrate	10	V
acetone	10	0
acetone	5	I
butanol	5	III

2.4.1.2. Effect of the initial development stage

Since *C. acetobutylicum* differentiates, the stage at which cells are harvested for sporulation induction is important. The effect of acetic acid on sporulation induction was examined in cultures that were initially at different developmental stages. Exponentially dividing cells (stage *0*), granulose positive cells (stage *I*) and cells that had developed to clostridial forms (mixture of late stage *I* and early stage *II*) were re-suspended in fresh CBM containing buffered acetate (10g.l^{-1}, pH 7.0). The efficiency of the 1h pre-induction incubation step, used to help synchronize culture development, was tested on the granulose-positive culture (Table 2-4). The pre-induction incubation step slowed spore development, and induction of clostridial stage cultures was found to result in asynchronous spore development. The most advanced stages of sporulation were attained without pre-induction incubation in cultures that were initially granulose positive or were in the exponential growth phase.

Table 2-4. The effect of adding buffered acetate (10g.l^{-1}, pH 7.0) on sporulation of cultures initially at different developmental stages. Cultures, grown in CBM, were re-suspended in fresh medium containing acetate to induce sporulation. Sporulation stages reached after 16h incubation are indicated with Roman numerals.

Initial stage of development	Sporulation stage after induction
exponential growth	III/V
granulose-positive	V
granulose-positive[a]	II/III
clostridial stage	mixed

a, incubated for 1h in fresh CBM prior to sporulation induction

2.4.1.3. Effect of the pH of acetic acid

Batch cultures of *C. acetobutylicum* switch to solventogenesis only after a critical pH has been reached [Jones and Woods, 1986]. The pH of the medium therefore plays an important role in the development of *C. acetobutylicum*. The effect of the pH of the acetic acid used to induce

Table 2-5. Viable spore counts (VS.ml^{-1}) from cultures induced to sporulate with acetic acid (10g.l^{-1}) at pH 6.5, 5.9 or 5.2. Cultures, grown in CBM, were re-suspended in fresh medium containing acetic acid to induce sporulation. VS.ml^{-1} were determined after 20h incubation.

	VS.ml^{-1}			
Initial culture OD	0.25	0.4	0.6	1.2
control[a]	$1x10^2$	$1.8X10^3$	$2x10^2$	$2x10^2$
pH 6.5	$3x10^4$	$2.4x10^4$	$2.4x10^6$	$2x10^4$
pH 5.9	-	$8.1x10^3$	$3.3x10^6$	-
pH 5.2	$1.3x10^5$	$1.3x10^4$	$5.4x10^6$	$4.4x10^5$

a, water only

-, not determined

sporulation was examined in cultures that were initially at different optical densities (OD 0.25, 0.4, 0.6 and 1.2) (Table 2-5). Quantitative experiments were needed to assess accurately more subtle differences in sporulation induction. To do this, the number of viable spores ($VS.ml^{-1}$) was determined after sporulation induction. For the cultures at OD 0.25 and 1.2, the pH of the buffered acetic acid affected the number of viable spores produced in that more spores were produced in the more acidic media. However, the pH had little effect on the production of spores in the cultures initially at OD 0.4 and 0.6. The culture at OD 0.4 was not induced to sporulate under the experimental conditions.

2.4.2. Spore heat resistance

The cell density of stationary phase cultures of *C. acetobutylicum* is typically in the region of $1x10^9$ cells per ml. The $VS.ml^{-1}$ was several orders of magnitude lower than this in the previous experiment. This prompted the question as to whether the method used to determine $VS.ml^{-1}$ was accurate. Therefore, the heat resistance of *C. acetobutylicum* spores was determined for 10min at various temperatures between room temperature and 90°C (Fig. 2-1). The source of the spores was a WT culture grown in CBM for 65h. Above 75°C, the $VS.ml^{-1}$ decreased rapidly, whereas the $VS.ml^{-1}$ remained constant at 10^7 for samples heated at lower temperatures.

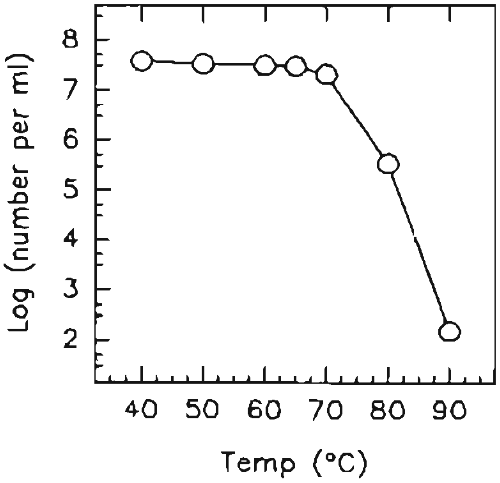

Fig. 2-1. Temperature-dependent survival curve of a sporogenous culture of *C. acetobutylicum* P262. Serial dilutions of culture samples were heated at various temperatures for 10min, prior to plating on solid medium to determine the number of heat resistant spores.

The time-dependent heat tolerance of spores was therefore determined at temperatures of 70. 75 and 80°C (Fig. 2-2). Spores were used from WT cultures grown in CBM for at least 65h and, for comparison, vegetative cells were used from overnight WT cultures (grown in CBM). At 80°C, there was a decrease in VS.ml^{-1}. The spores were therefore not heat tolerant at this temperature, but the spores were able to withstand 75 and 70°C for 20min. Vegetative cells were able to survive for short periods at 70°C, but were killed within five minutes at higher temperatures.

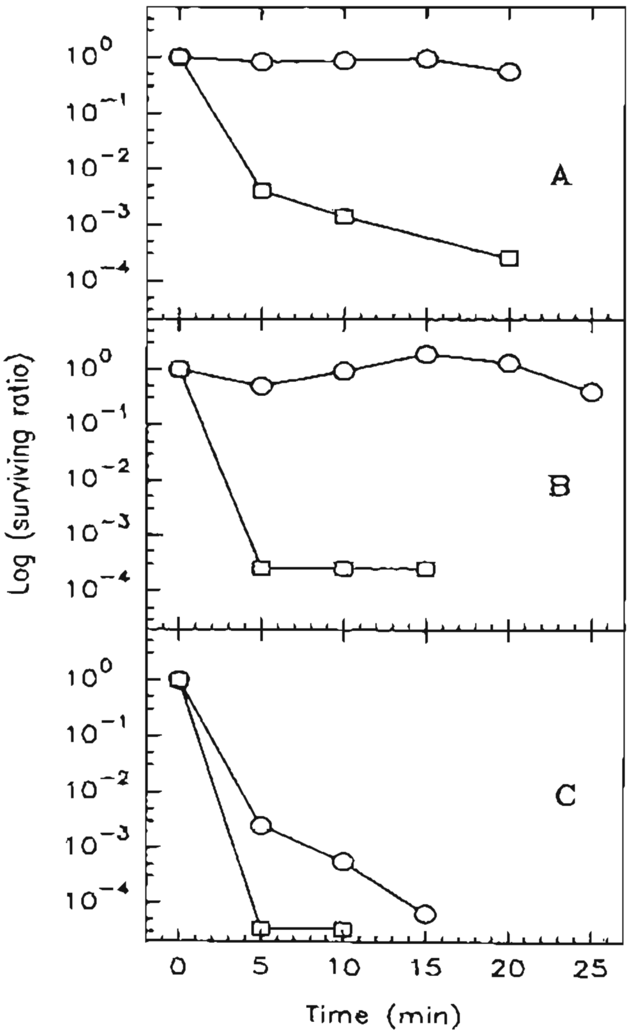

Fig. 2-2. Time-dependent survival of vegetative cells and spores of *C. acetobutylicum* P262 at 70°C, 75°C and 80°C (frame A, B and C, respectively). Serial dilutions of samples from a vegetative culture (□) and from a sporogenous culture (O) were heated for various times periods, prior to plating on solid medium to determine the number of heat resistant spores. The ratio of the number of surviving cells to their initial starting number was determined.

2.5. Discussion

The signal relays and regulatory pathways that trigger and control sporulation in *B. subtilis* are intricate [Errington, 1993] and the triggers involved in *C. acetobutylicum* sporulation are likely to be as complex. Before the genetic control of sporulation can be understood in *C. acetobutylicum*, a standard method for sporulation induction needs to be developed. The development of a sporulation induction medium and the study of sporulation requires a way to quantitate the maturation of spores. The use of counting chambers would be inaccurate because it would be impossible to determine whether a spore was fully mature. It has been shown above (Fig. 2-2) that the method which has been used routinely in our laboratory to determine relative differences in sporulation efficiency, is inaccurate in determining the exact numbers of mature spores. Spores of *C. acetobutylicum* P262 were not able to withstand incubation at 80°C, but were able to withstand temperatures of 75°C. Vegetative cells were killed at 75°C. This is therefore the optimum temperature to incubate cultures when determining viable spore counts.

Solventogenesis can be induced by increasing the concentrations of butyrate or acetate [Jones and Woods, 1986]. Sporulation of *C. acetobutylicum* P262 was induced under similar conditions, but acetate was found to allow more rapid development of spores than butyric acid. Solventogenesis of *C. acetobutylicum* ATCC 824 was not induced by acetate alone, although it was induced by butyrate alone (Hüsemann and Papoutsakis, 1990). There could be strain differences in sporulation induction, or the requirements for sporulation and solventogenesis induction could be different. No mutants blocked in solvent production have been found to sporulate normally [Jones and Woods, 1986]. Solventogenesis products, acetone and butanol, did not induce sporulation in *C. acetobutylicum* P262 (Table 2-3). This implies that solventogenesis is not important for initiation of sporulation and, since acidogenesis products induce sporulation and solventogenesis, that sporulation and solventogenesis are under the control of the same global regulator. In agreement with this, a mutant (examined more closely in chapters 3 and 4) has been identified that is blocked in solvent production and is able to initiate sporulation.

The conditions used to induce sporulation mimic the environmental conditions at the end of the acidogenic phase at the pH breakpoint. Under certain conditions, however, the pH of added acetic acid made little difference to the efficiency of sporulation induction (Table 2-5). *C. acetobutylicum* P262 is susceptible to cell lysis by lysozyme in cultures in which the OD is below 0.6, but not when the OD is greater [personal observation]. A cellular change related to the change in cell wall structure, may have affected the ability of certain cultures at similar optical densities to respond to the pH of the acetate.

The pre-induction incubation in fresh medium was thought to help synchronize cell development at the stage of granulose accumulation, because *C. acetobutylicum* is unable to switch to solventogenesis or sporulation without the correct environmental signals. It was not clear whether acetic acid itself induced sporulation genes, or whether acetic acid promoted sporulation by mimicking breakpoint conditions, which induced sporulation gene expression through signal pathway systems. However, acetic acid was able to accelerate sporulation in granulose-positive and exponential phase cultures without the pre-induction incubation (Table 2-4). Several factors that are known to effect sporulation in *C. acetobutylicum* or *B. subtilis*, have not been tested in this study. These factors include growth rate, cell density, aeration and carbon source, and these factors could account for any inconsistencies in sporulation induction. However, the results presented here show clearly that acetic acid would play a major part in the development of a sporulation induction medium for *C. acetobutylicum*.

Chapter 3

Characterization of *C. acetobutylicum* P262 mutant m5

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3.1. Summary

Mutants m5 and o6 were derived from transposon mutagenesis of *C. acetobutylicum* P262 using conjugative transposon "Tn*925*" [Babb, 1990]. Mutant m5 was previously shown to be sporulation deficient and blocked in acetone and butanol production but unaffected in ethanol production [Babb, 1990]. The sporulation development and the fermentation profiles of these mutants were analyzed in batch culture over a time period of 60h. Mutant m5 was shown to be oligosporogenous with the majority of cells blocked at sporulation stage *II*. It was also shown to be deficient in acetone and butanol production. In comparison, mutant o6 proceeded to sporulation stage VII and produced acetone and butanol at levels similar to that of the wild type. The number of transposon insertions in each mutant was determined by DNA hybridization. Using the *tet*M gene from the conjugative transposon Tn*925* as a DNA probe, an anomalous band of 5kb was identified. This provided strong evidence that the mutants had been generated not with transposon Tn*925* as originally believed, but with the Tn*925*::Tn*917* cointegrate. Mutants m5 and o6 contained three and two left transposon junctions, respectively.

3.2. Introduction

The ability to mutate the chromosome of an organism provides a powerful technique with which to study the characteristics and functions of specific genes contained in that organism. However, *C. acetobutylicum* P262 is recalcitrant to most methods of genetic manipulation, including electroporation [Woods, pers. comm.]. A method for transposon mutagenesis of *C. acetobutylicum* P262 was developed using the conjugative transposons Tn*916*, Tn*925* and Tn*925*::Tn*917* [Babb, 1990]. These conjugative transposons were able to insert at several different locations in the *C. acetobutylicum* P262 chromosome, producing mutants with a variety of different phenotypes. The mutants varied in their ability to produce the different fermentation products acetone, butanol, acetate and butyrate [Babb, 1990]. Transposon mutagenesis therefore appeared to serve as a useful tool for the isolation of genetically defined sporulation and solvent-deficient mutants of *C. acetobutylicum* P262. However, this was not necessarily the case for other strains of *C. acetobutylicum*. Transposon mutagenesis of *C. acetobutylicum* DSM 1731 and DSM 792 yielded only asporogenous mutants [Bertram *et al.*, 1990], while transposon mutagenesis of *C. acetobutylicum* NCIB 8052 involved the preferential insertion of transposon Tn*916* into a single site [Woolley *et al.*, 1989].

One mutant of *C. acetobutylicum* P262, referred to as m5, was inhibited in the sporulation process, and did not produce acetone or butanol, but did produce ethanol [Babb, 1990]. The production of ethanol was thought to be regulated separately from acetone and butanol production, because Bertram *et al.* (1990) had also obtained a class of *C. acetobutylicum* mutant that was able to produce ethanol as the only solvent product. The phenotype of mutant m5 suggested that a regulatory gene, controlling the solventogenic pathway, had been insertionally inactivated. There has been no report of a mutant of *C. acetobutylicum* that is able to sporulate and yet is unable to produce acetone and butanol [Jones and Woods, 1986]. It was not clear how the sporulation functions in mutant m5 had been affected. Therefore, the mutant was further characterized at the physiological and molecular levels.

3.3. Materials and methods

3.3.1. Strains, media and storage

C. acetobutylicum P262 mutants m5 and o6 were generated previously [Babb, 1990] using conjugative transposons obtained from Dr. P. Dürre (Institute for Microbiology, Göttingen, Germany). Results presented in this chapter indicate that transposon Tn*925*::Tn*917*, and not Tn*925*, was used to generate these mutants. The media BC and CBM have been described previously (Chapter 2). CBX was CBM containing 1% (w/v) xylose as the carbon source. Transconjugants were stored in Cooked Meat Medium (Difco, Detroit, Michigan) for short term storage, or in CBM with 50% (v/v) glycerol at -70°C. TYGM, modified TYG medium, was described by Reysenbach (1987). *C. acetobutylicum* Minimal Medium (CAMM) has been described previously [Long *et al.*, 1983]. *C. acetobutylicum* P262 was grown under strict anaerobic conditions in an anaerobic cabinet (Forma Scientific Inc., Marietta, Ohio) at 34°C. The minimal inhibitory concentration of *C.*

acetobutylicum P262 for Tc and Em was 20ng.ml^{-1} and 10ng.ml^{-1}, respectively. Transconjugants were selected on 1μg.ml^{-1} Tc or Em.

3.3.2. Plasmids

Plasmid pTet, obtained from Ms. H. Collett (Department of Microbiology, University of Cape Town, South Africa), is a 7.5kb plasmid, containing the *tet*M gene cloned on a *Hin*cII restriction enzyme DNA fragment from Tn*925* into pBluescript SK (Stratagene Cloning Systems, San Diego, California).

3.3.3. Growth curves

Mutants m5 and o6 and wild type *C. acetobutylicum* P262 were analyzed with respect to OD, cell number, viable spores (VS), sporulation stage, motility, granulose production, fermentation products and pH during a 60h fermentation in TYGM and during a fermentation in CBM. Overnight cultures of mutant m5 and mutant o6 were prepared by inoculating a 10ml CBM hungate tube with a single colony from an agar plate. Overnight cultures of the WT strain were prepared as previously described (Chapter 2). The overnight cultures were subcultured by making a 5% dilution in 20ml TYGM. These cultures were grown until early logarithmic stage was reached (OD < 0.7 units), when 10ml were used to inoculate prewarmed TYGM (1.5l). Samples of 5ml were taken during the 60h period for the various analyses, as described below.

3.3.3.1. Quantification of cells and spores

A Thoma counting chamber (Weber, England) was used to determine cell and spore numbers. Colony forming units per ml (CFU) were determined by plating different dilutions of the culture samples in water on BC or CBM agar plates. To quantitate the number of viable spores per ml (VS.ml^{-1}), 0.5ml samples of the cultures were serially diluted in water, heated at 80°C for 10min, cooled on ice and plated anaerobically on CBM or BC agar plates.

3.3.3.2. Optical density, granulose detection and sporulation stage determination

The optical density at 600nm (OD) of the culture samples was measured across a 1cm path using a Spectronic 20 (Baush and Lomb, Rochester, New York) under anaerobic conditions. Dilutions were made in water so that the measured OD was below 0.4 units. The techniques used to detect granulose and to determine the stage of sporulation development have been described in Chapter 2.

3.3.3.3. Determination of fermentation products

The concentrations of acetone, butanol, acetic acid and butyric acid were measured with a Hewlett Packard 5880A gas chromatograph as previously described [Barber *et al.*, 1979].

3.3.4. DNA extraction

Chromosomal DNA was extracted from *C. acetobutylicum* P262 using an adaptation of the method by Zappe (1988). Overnight cultures of *C. acetobutylicum* (described in Chapter 2) were subcultured into 1.2l CBM. The cultures were harvested by centrifugation (7min at 15000*g*) before reaching an OD of 0.4 units. The cells were resuspended anaerobically in 20ml CBM, containing 10% (w/v) sucrose, 12.5mM calcium chloride, 12.5mM magnesium chloride and 5mg.ml^{-1} of lysozyme (Sigma Chemical Company, St. Louis, Missouri). After anaerobic incubation at 37°C for 1h, EDTA and SDS were mixed into the suspension to final concentrations of 0.1M and 2% (w/v), respectively. This was immediately followed by the addition of 1 volume of hot phenol (equilibrated with 0.1M Tris, pH 8.0) at 50°C. The aqueous phase was separated by centrifugation (10min at 12000*g*) and residual phenol was extracted twice with one volume of chloroform:iso-amyl alcohol (24:1 v/v) and once with one volume of water-saturated ether. RNA was removed by treatment with ribonuclease A (Sigma Chemical Company, St. Louis, Missouri) [Sambrook *et al.*, 1989]. The DNA was precipitated with isopropanol [Sambrook *et al.*, 1989] and resuspended in 0.5ml Tris-EDTA buffer [Sambrook *et al.*, 1989].

3.3.5. DNA manipulation

Standard DNA manipulation techniques were used for agarose gel electrophoresis and Southern hybridization [Sambrook *et al.*, 1989]. DNA was digested with restriction enzymes from Gibco GRL (Gaithersburg, Maryland), using the suggested buffers and reaction conditions. Southern hybridization experiments were carried out at 64°C using Hybond N+ membranes from Amersham International (Aylesbury, Buckinghamshire). For Southern hybridization, DNA was transferred to the membranes by capillary action under alkaline conditions [Sambrook *et al.*, 1989]. For these experiments, non-radioactive digoxygenin (DIG) DNA labeling kits and lumigen detection kits from Boehringer Mannheim (SA) (Randburg, South Africa) were used. The plasmid pTet, linearized with restriction enzyme *Eco*RI, was labeled non-radioactively to be used as a DNA probe according to the kit manufacturer's instructions. Detection of the probe was done according to the lumigen kit instructions.

3.4. Results

In a previous study [Babb, 1990], transposon mutagenesis was used to produce transconjugants of *C. acetobutylicum* P262 of which 11.2% were sporulation mutants. Fourty-nine presumptive sporulation mutants were tested for their ability to produce mature, heat-resistant spores when grown in CBX. These mutants were either blocked in sporulation initiation, early sporulation or late sporulation functions. The mutants were classified according to the number of heat-resistant spores per ml ($VS.ml^{-1}$) that they produced after 65h incubation in CBX medium (Table 3-1): 26 mutants were classed as asporogenous (0 $VS.ml^{-1}$), 13 as oligosporogenous ($<1x10^4$ $VS.ml^{-1}$) and 6 as sporogenous ($>1x10^4$ $VS.ml^{-1}$). Mutant o6 was oligosporogenous and blocked late in sporulation. Oligosporogenous mutant m5, which was blocked early in sporulation and which was deficient in solvent production [Babb, 1990], was selected for further study.

Table 3-1. The ability of fourty-nine presumptive sporulation mutants to produce viable spores (VS). Sporulation "initiation" mutants were estimated by microscopy to be blocked at sporulation stages *O* to *I*, "early" sporulation mutants at stages *II* through *V*, and "late" sporulation at later stages.

	Number of mutants		
Sporulation stage	spo^{-} A	$spo^{+/-}$ B	spo^{+} C
initiation	17	1	2
early	2	4	0
late	7	8	4

A. Asporogenous, no viable spores detected

B. Oligosporogenous, less than $1x10^4$ $VS.ml^{-1}$

C. Sporogenous, more than $1x10^4$ $VS.ml^{-1}$

3.4.1. Sporulation characteristics of *C. acetobutylicum* mutant m5

C. acetobutylicum m5 was grown in CAMM medium for 5 to 6 days anaerobically, followed by exposure to air for 1 day to test for spore formation by microscopy. Cells blocked at stage *II* were predominant in these cultures, although a few spores were detected (<1% of all cells). Two cultures of mutant m5 that were grown in CBM for 65h, were also predominately blocked at stage *II* of sporulation. On quantification, the VS.ml^{-1} of mutant m5 varied from culture to culture but the VS.ml^{-1} of mutant m5 were at least two orders of magnitude lower than those of WT cultures (data not shown).

3.4.2. Growth curves

The phenotype of mutant m5 was analyzed during different stages of growth curves in CBM (with 6% (w/v) glucose) and TYGM. The results of the TYGM growth curve are shown, but the same results were obtained in both media. Mutant o6 and the WT strain were analyzed as controls.

3.4.2.1. Quantification of cells and spores

The results showed that there was no linear relationship between cell number and OD (Fig. 3-1). As the cells undergo morphological changes during sporulation, their optical density changes. The OD first increased and then decreased in all three cultures. OD is therefore a measure of both cell number and cell differentiation.

Viable, heat-resistant spores were produced by the WT strain and both mutant m5 and mutant o6 (Fig. 3-1). The WT strain produced 10^5 VS.ml^{-1}, whereas mutants m5 and o6 produced 10^2 and 10^3 VS.ml^{-1}, respectively. The relative VS counts showed that both mutants were oligosporogenous. The number of WT spores per ml, determined by counting in a counting chamber, were three orders of magnitude greater than the corresponding VS.ml^{-1}. This discrepancy was due to the fact that the VS testing temperature was 80°C, as described in Chapter 2.

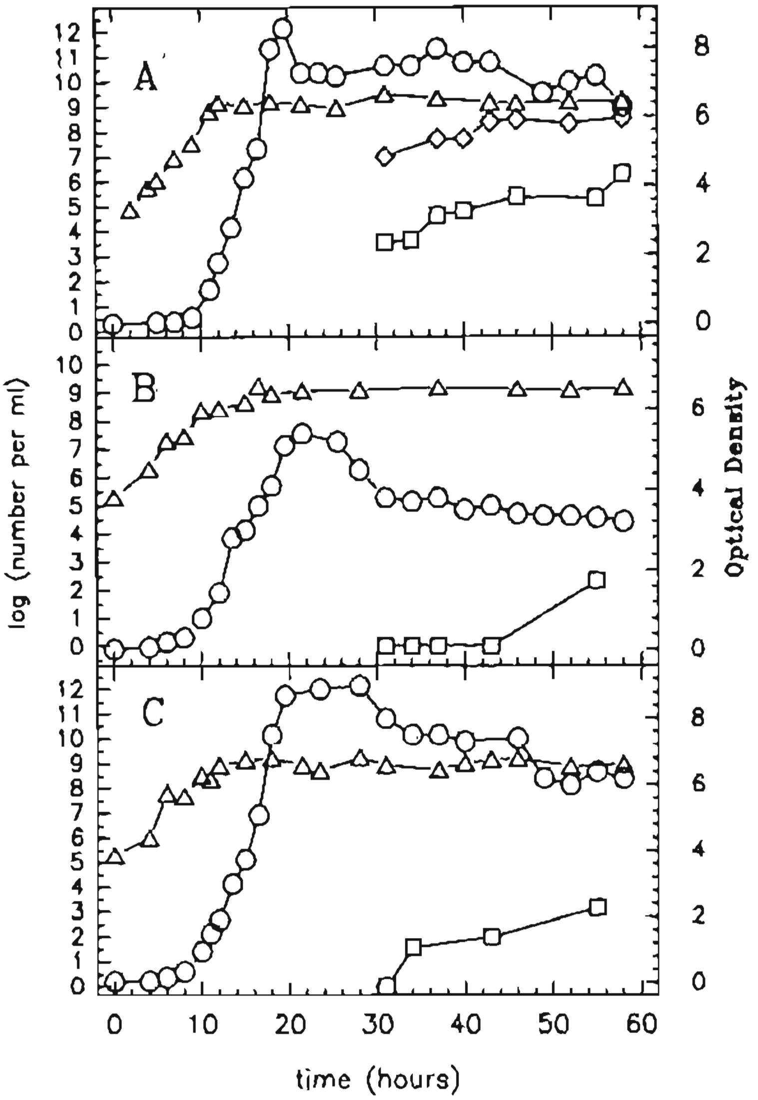

Fig. 3-1. Growth curve of *C. acetobutylicum* P262, mutant m5 and mutant o6 showing the change in OD (O), cell number (Δ), VS (□) and spore number (◊) per ml. The frames are as follows: A, WT; B, mutant m5; C, mutant o6.

3.4.2.2. Developmental stages

The development of the three cultures through the various stages of differentiation was recorded (Fig. 3-2). The proportion of cells at each stage was estimated by microscopy. In each culture, there was a sharp peak in motility at 10h, followed by the rapid accumulation of granulose. The number of cells containing granulose steadily decreased until 50h at which point most of the granulose had been utilized. The percentage of motile cells differed for each culture: 60% for WT, 50% for mutant m5 and almost

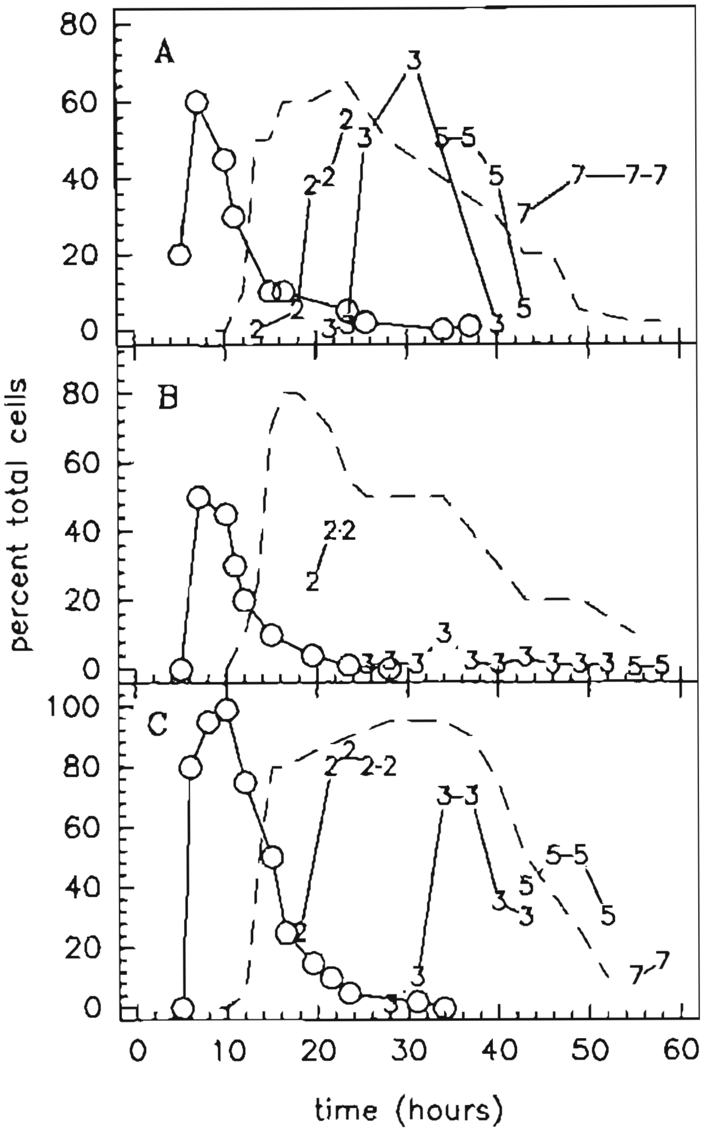

Fig. 3-2. Growth curve of *C. acetobutylicum* P262, mutant m5 and mutant o6 showing the percentage of cells at sporulation stages *II*, *III*, *V*, and *VII* (2, 3, 5, and 7 respectively), the percentage of cells that had accumulated granulose (- - -), and that were motile (O). The frames are as follows: A, WT; B, mutant m5; C, mutant o6.

100% for mutant o6. The WT and mutant o6 cultures progressed through all the stages of sporulation, but less than 10% of the cells in the mutant m5 culture were able to proceed to sporulation stage *III* and less than 5% were able to proceed beyond this stage.

3.4.2.3. Fermentation products

Acid (acetic and butyric acids) and solvent (acetone and butanol) concentrations were determined during the fermentations of the three cultures and the pH was monitored (Fig. 3-3). During the active growth phase of all three cultures, acids were produced, corresponding to a decrease in the pH. The pH reached a minimum of pH 5.0, the pH breakpoint. The pH then increased to pH 5.4 in the WT and mutant o6 cultures while the concentration of acids dropped steadily from the maximum of 3g.l^{-1}. Acid re-assimilation was accompanied by solvent production, which reached a maximum of 15 and 13g.l^{-1} for the WT and mutant o6 culture, respectively. The mutant m5 displayed a very different fermentation profile as the acid concentration and pH did not decrease beyond the pH breakpoint and no solvents were produced.

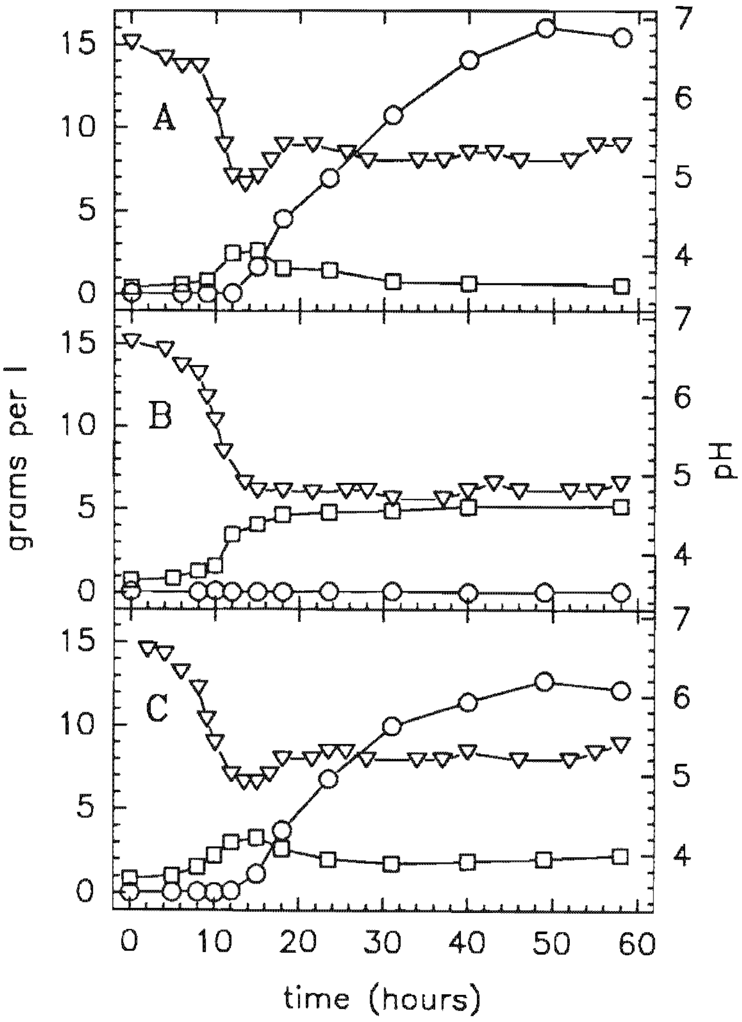

Fig. 3-3. Fermentation profiles of *C. acetobutylicum* P262, mutant m5 and mutant o6, in which acetic and butyric acid (g.l^{-1}) (□), acetone and butanol (g.l^{-1}) (O), and the pH (∇) were monitored. The frames are as follows: A, WT; B, mutant m5; C, mutant o6.

3.4.3. Number of transposon insertions

The number of transposon insertions was determined by Southern hybridization. Chromosomal DNA of mutant m5, mutant o6 and WT was digested with *Hin*dIII restriction endonuclease and probed with the DIG-labeled plasmid pTet (Fig. 3-4). This probe was specific for the *tet*M gene, which is common to most conjugative transposons. Transposon Tn*925* contains a single internal *Hin*dIII restriction site within the *tet*M gene (Fig. 3-5). Therefore, the probe would hybridize to the *Hin*dIII-DNA fragments to both the left and the right of the *Hin*dIII restriction site in the *tet*M gene. Each transposon insertion in mutants m5 and o6 resulted in two hybridization signals, the sizes of which depended on the location of the adjacent *Hin*dIII restriction sites. However, a common 5kb fragment was present, regardless of the number of transposon insertions (Fig. 3-4). This common band has been found in other mutants generated with conjugative transposon "Tn*925*" in our laboratory (H. Collett, pers. comm.). This implied the presence of another internal *Hin*dIII restriction site. The common fragment has been cloned and nucleotide sequencing showed that the DNA contained sequence that was identical to Tn*917* (H. Collett, pers.

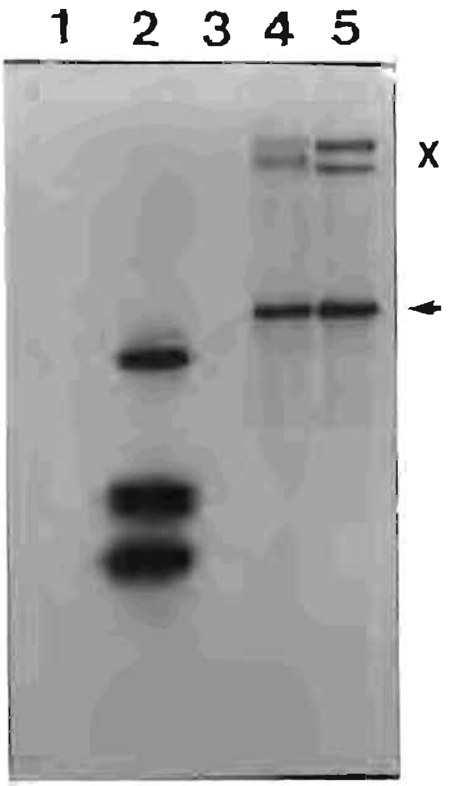

Fig. 3-4. Southern hybridization of WT, mutant m5 and mutant o6 chromosomal DNA with the *tetM* gene. Lane 1 contained λ DNA digested with restriction enzyme *Pst*I. The DNA in lanes 2 to 5 was digested with *Hin*dIII. Lane 2 contained plasmid pTet (not digested to completion). Lanes 3, 4 and 5 contained chromosomal DNA from the WT strain, mutant m5 and mutant o6, respectively. The position of the common 5kb DNA fragment is indicated with an arrow, and the positions of the DNA fragments corresponding to unique transposon insertions are indicated with an X.

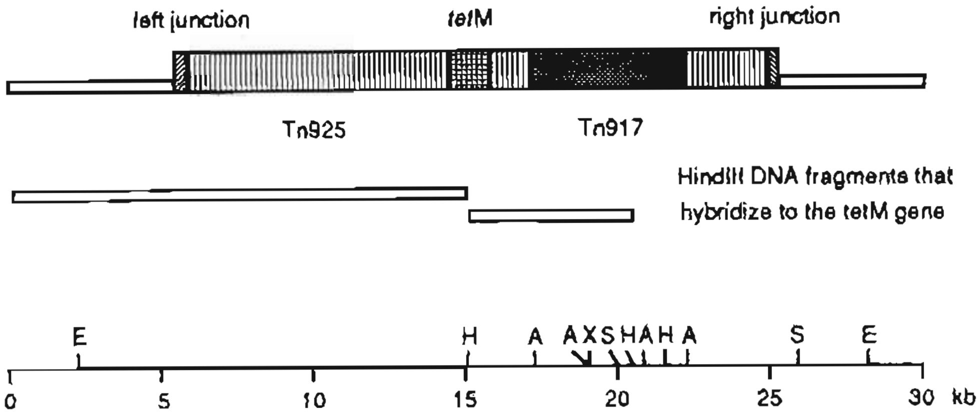

Fig. 3-5. Physical organization of Tn*925*::Tn*917*. Restriction endonucleases are as follows: A, *Ava*I; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I [Christie *et al.*, 1987]. The location of the two *Hin*dIII DNA fragments that would hybridize to the *tetM* probe are indicated.

comm.). The fact that the transconjugants were Em resistant, confirmed the presence of transposon Tn*917* in the mutants m5 and o6. The mutants were therefore generated with the Tn*925*::Tn*917* co-integrate and not with Tn*925*.

From the restriction map of Tn*925*::Tn*917* (Fig 3-5), it was evident that the *tetM* probe would hybridize to the internal 5kb *Hin*dIII DNA fragment of this transposon co-integrate and that this probe would hybridize to DNA fragments containing the left junction site. The high intensity of the hybridization signal of the 5kb band (Fig. 3-4) was due to the high relative copy number of this fragment. Use of the *tetM* probe, suggested that mutant m5 contained three transposon insertions and mutant o6 contained two transposons. The different sizes of the homologous bands, indicated that the transposon insertions were located at different loci.

3.5. Discussion

A range of sporulation-deficient mutants of *C. acetobutylicum* P262 was obtained using conjugative transposons [Babb, 1990]. These mutants were blocked at various stages in sporulation and were either oligosporogenous or asporogenous (Table 3-1). From growth curve data, mutants m5 and o6 were both shown to be oligosporogenous (Fig. 3-1). These mutants differed from the WT strain with regard to the percentage of cells that were able to develop (a measure of the level of degeneracy). Degeneracy is a complex phenomenon that is not well understood, it appears to be related to solvent production and motility [Bennett and Petersen, 1992].

Mutant m5 was blocked in sporulation at stage *II* and was unable to produce acetone and butanol (Fig. 3-2 and 3-3). This phenotype was intriguing as no solvent-deficient mutants of *C. acetobutylicum* have been isolated that are able to sporulate normally [Jones and Woods, 1986]. Sporulation is thought to be a mechanism by which *C. acetobutylicum* is able to overcome the toxic effects of acidogenesis and solventogenesis [Jones and Woods, 1986]. It is not known whether solventogenesis is a prerequisite for normal sporulation, but the phenotype of mutant m5 suggests that solventogenesis is not required for sporulation initiation.

Hybridization experiments were carried out to determine the number of transposon insertions in mutants m5 and o6. An anomalous 5kb band was observed to hybridize to the *tet*M probe in all experiments. The original donor plasmids used in the generation of the conjugative transposon mutants [Babb, 1990], were obtained from Strätz *et al.* (1990), who have also reported the presence of an anomalous band in *Acetobacterium woodii* transposon "Tn*925*" mutants. Their explanation for the anomalous band was that the Tn*925* transconjugants contained a previously unmapped *Hin*dIII restriction enzyme site in Tn*925*. Our results indicate that the transposon was not transposon Tn*925*, but the Tn*925*::Tn*917* transposon co-integrate. Mutants m5 and o6 had multiple insertions of this transposon. This is a common feature of mutants generated by conjugative transposons [Young, 1993b] as the presence of a transposon in the recipient does not impede the transfer of another transposon [Norgren and Scott, 1991].

The phenotype of mutant m5 suggested that solvent production was not a key factor in sporulation initiation. With a probe that hybridized to DNA-fragments containing the left hand junction of Tn*925*::Tn*917*, mutant m5 was shown to contain three copies of the Tn*925*::Tn*917* transposon. This has made it difficult to relate the phenotype to the genotype of mutant m5, because several explanations for the phenotype can be proposed:

1. One transposon insertion may have blocked solvent production and another insertion inhibited sporulation.

2. One of the transposon insertions may have inhibited a regulatory gene involved in controlling solventogenesis. Disruption of the sporulation process may be a secondary effect of the inability to initiate solventogenesis.
3. The synergistic effect of multiple transposon insertions may be responsible for the phenotype of the mutant without necessarily having inactivated a sporulation gene.

With regard to (2) above, several factors relating to solventogenesis (listed below) may be critical for the production of mature spores in *C. acetobutylicum* P262.

- Morphological changes associated with the switch to solventogenesis may be necessary to bring about the cascade of events involved in sporulation, as changes in morphology have been reported to control sporulation gene expression in *B. subtilis* [Stragier *et al.*, 1988].
- Solvents may be required for sporulation, because they may act as regulators during the development of *C. acetobutylicum* after sporulation initiation.
- Solventogenesis may be required during the sporulation process simply to maintain the energy and pH gradient across the cell membrane. *C. acetobutylicum* switches from acidogenesis to solventogenesis when it can no longer maintain the pH gradient across the membrane [Jones and Woods, 1986]. During the fermentation of mutant m5, the pH of the culture does not increase after the pH breakpoint (Fig. 3-3). Mutant m5 may, therefore, not have been able to support the energy requirements necessary for morphological development past sporulation stage *II*.

Further study of *C. acetobutylicum* P262 mutant m5 was undertaken in an attempt to resolve these possibilities.

Chapter 4

Genetic analysis of *C. acetobutylicum* mutant m5

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4.1. Summary

A genomic library of *C. acetobutylicum* mutant m5 was made in order to clone DNA fragments containing the transposon junction sites. Eight different plasmids with DNA homologous to the right end of transposon Tn*925*::Tn*917* were identified by colony hybridization. The plasmids contained inserts of 4.7, 7.0 and 4.8kb. Transposon junction sites of seven clones were sequenced in one direction and three different DNA sequences were found. Plasmids that had the same junction sequence did not always have the same size. There was a stretch of five adenine residues near each transposon junction, which may form part of a target insertion site for Tn*925*::Tn*917*. Computer analysis of the chromosomal sequences of the junction sites revealed that one of them contained slight homology to a DNA sequence containing a β-galactosidase from *C. acetobutylicum*. No significant DNA homology was found for the other transposon junction site DNA sequences. Polymerase chain reaction (PCR) of three plasmids representing the different junction sequences yielded product for one plasmid. This product hybridized to chromosomal DNA of WT and mutant m5. The sequences of the transposon junctions were used to design chromosome-specific oligonucleotide probes. These did not hybridize to either the mutant or the WT chromosome, but one oligonucleotide was shown to hybridize to plasmid DNA representing a different junction sequence. This suggested that the clones containing the right transposon junction were unstable in *E. coli*, and in agreement, new isolates of two of the plasmid clones showed that the plasmids had restriction enzyme sites that were not present in the original plasmid isolates. In chapter 3 it was shown that mutant m5 had three insertions based on a probe to the left end of the transposon. Southern hybridization using a probe specific for the right end of the transposon, showed many bands of homology. The cause for the anomalous hybridization pattern was neither non-specific restriction enzyme activity nor a result of independent transposition of transposon Tn*917* from transposon Tn*925*::Tn*917*. The chromosome of mutant m5 appeared to have rearranged.

4.2. Introduction

Transposon mutagenesis of *C. acetobutylicum* P262 was used to isolate sporulation mutants [Babb, 1990]. Of these, mutant m5 was blocked at sporulation stage *II* and was completely blocked in acetone and butanol production (Chapter 3). Using a probe to the left hand side of the transposon, mutant m5 appeared to contain three transposon insertions. The isolation and characterization of genes that had been inactivated in this mutant would lead to the better understanding of the genotype of mutant m5 and of the switch to sporulation and the switch to solventogenesis. This information could be vital to the rational engineering of *C. acetobutylicum* for industrial application. Therefore, it was decided to isolate all the genes that had been inactivated by the conjugative transposons and to identify the function of each gene. Several methods are available which facilitate gene identification. These include DNA sequence homology studies, gene complementation studies in defined mutants of *B. subtilis* or *E. coli*, and the allelic replacement of specific genes in *C. acetobutylicum*. For these studies, it would first be necessary to isolate clones of the insertionally inactivated genes of mutant m5 from the chromosome of the WT strain.

4.3. Materials and Methods

4.3.1. Strains, plasmids and media

C. acetobutylicum mutants m5 and o6 of *C. acetobutylicum* P262 have been described previously (Chapter 3). *C. acetobutylicum* strains were grown anaerobically in CBM (Chapter 2). *E. coli* JM105 was used [Yanisch-Perron *et al.*, 1985]. *E. coli* cultures were grown at 37°C in 2xYT medium [Sambrook *et al.*, 1989]. Plasmid pSK (Bluescript SK+, Stratagene, San Diego, California) was used to construct a genomic library of mutant m5: chromosomal DNA was digested with restriction enzymes *Hin*dIII and *Eco*RI, fragments above 4kb in size were purified from an agarose gel and ligated into pSK, which had been digested with the same restriction enzymes. Similarly, a genomic library of the WT strain was constructed using restriction endonuclease *Hin*dIII and size-selecting fragments greater than 4kb. Plasmid pTet was described previously (Chapter 3). Plasmid pTn*917'* contained the 1.2kb *Hind*III-*Xho*I DNA fragment from transposon Tn*917* cloned into pSK [H. Collett, pers. comm.]. Plasmids p3R (7.1kb) and p30R.5 (10.5kb) contained Tn*925*::Tn*917* junctions sites from *C. acetobutylicum* P262 mutants cloned into plasmid pSK using restriction endonucleases *Xho*I and *Eco*RI [H. Collett, pers. comm.]. Approximately 0.3 and 2.3kb of *C. acetobutylicum* chromosomal DNA were contained in plasmids p3R and p30R.5, respectively. *E. coli* transformants that contained derivatives of plasmid pSK were selected with 100μg.ml^{-1} ampicillin (Ap) [Sigma Chemical Company, St. Louis, Missouri].

4.3.2. DNA techniques

4.3.2.1. DNA extraction and purification

Large and small scale extractive plasmid preparations from *E. coli* cultures have been described previously [Ish-Horowicz and Burke, 1981]. The method used to extract chromosomal DNA from *C. acetobutylicum* P262 was described in Chapter 3. DNA fragments were size selected and purified from agarose gels according to kit manufacturers instructions [GeneClean, Bio 101 Inc., PO. Box 2284, La Jolla, California].

4.3.2.2. DNA restriction and transformation

DNA was digested according to restriction enzyme manufacturers instructions [Gibco GRL, Gaithersburg, Maryland]. *E. coli* was transformed with plasmid DNA by the calcium chloride method [Dagert and Ehrlich, 1979].

4.3.2.3. Labeling and detection of DNA probes

Non-radioactive digoxygenin (DIG) DNA labeling kits and lumigen detection kits for non-radioactive probes were used as instructed by the manufacturer [Boehringer Mannheim (SA), Randburg, South Africa]. The *Xho*I-*Eco*RI DNA fragment of plasmid p3R was gel purified and labeled with DIG for use as a hybridization probe (probe XE3R). Polymerase chain reaction (PCR) products were labeled non-radioactively by using the hexanucleotide labeling mix [Boehringer Mannheim (SA), Randburg, South Africa] instead of non-labeled nucleotides in the PCR reactions. On the basis of transposon junction site DNA sequence data, three oligonucleotides were designed. These oligonucleotides were synthesized in

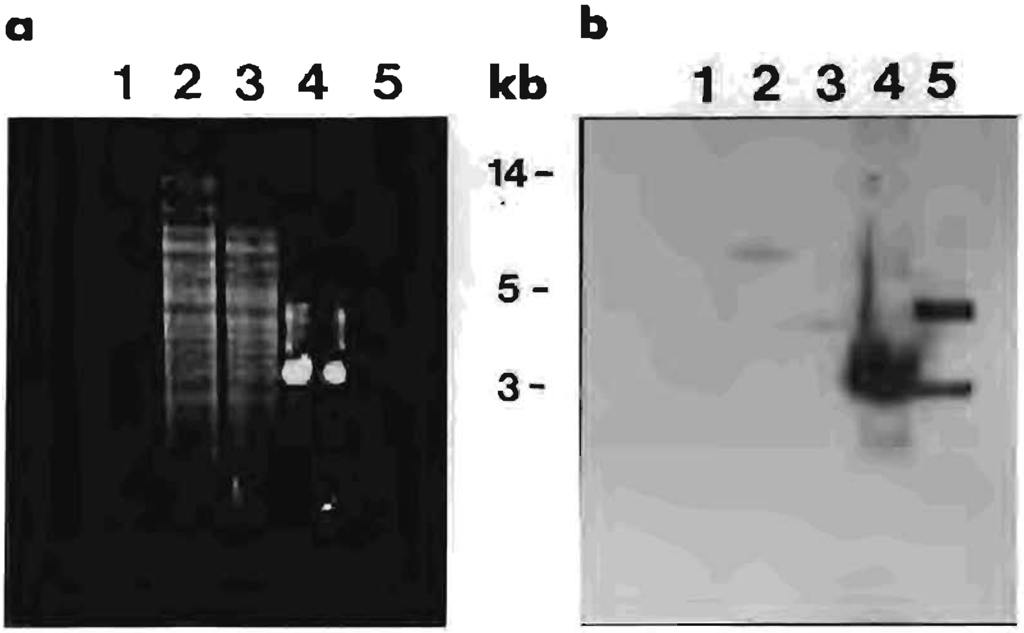

Fig. 4-5. Southern hybridization of chromosomal DNA from the *C. acetobutylicum* WT and mutant m5 strains using a probe generated by PCR of the group III plasmid pm5.4. Frame (a) shows the agarose gel used for the Southern hybridization, and the hybridization signals that were obtained, are shown in frame (b). The relative positions of DNA fragments of size 14, 5 and 3kb are indicated between the frames. Lane 1, which is not clear in this photograph, contained λ DNA digested with restriction enzyme *Pst*I. The DNA in lanes 2 to 4 was digested with restriction enzyme *Hin*dIII. Lanes 2 and 3 were chromosomal DNA of mutant m5 and WT respectively. Lane 4 contained DNA from a genomic library of the WT strain, constructed with *Hin*dIII restriction enzyme. Lane 5 was plasmid pm5.4, digested with *Eco*RI and *Hin*dIII.

the transposon insertions in *C. acetobutylicum* mutant m5. The PCR probe contained a short segment of plasmid pSK. and so was able to hybridize to the vector bands in lanes 4 and 5 (Fig. 4-5).

The PCR probe was used in colony hybridization experiments to identify clones from a genomic library of the WT strain which contained DNA that was homologous to the group III transposon junction site sequence. A WT genomic library, prepared by Zappe *et al.* (1986), was transformed into *E. coli* JM105 for the colony hybridization. Hybridization of the PCR probe against approximately 1200 colonies yielded three colonies with weak homology. Southern hybridization experiments against the plasmids from these colonies showed that the PCR probe bound more strongly to vector DNA than to the insert DNA (data not shown). This indicated that the 1200 colonies did not contain the desired clones.

4.4.3.2. Oligonucleotide probes

The three DNA sequences of the "flanking DNA" regions were used to design three oligonucleotides, one for each plasmid group (Table 4-1). The melting temperature of each probe was 57°C as estimated by the PRIMER program [WIBR, Cambridge, Massachusetts]. The three oligonucleotides were radioactively labeled and used simultaneously in colony hybridization experiments performed at 42°C. None of the 1200 colonies used above (4.4.3.1), were found to contain DNA homologous to these oligonucleotide probes. Southern hybridization of the oligonucleotide probes against WT and mutant m5 chromosomal DNA, and plasmids representative of each junction sequence group, yielded hybridization signals for the representative plasmids only and no homology to the WT and mutant m5 DNA (Fig. 4-6). Background signals were detected in lanes 1, 2 and 3 of frame (b) of Fig. 4-6, due to the cumulative effects of over-exposure of the autoradiogram and the low stringency of the hybridization washes that had to be used with these oligonucleotide probes.

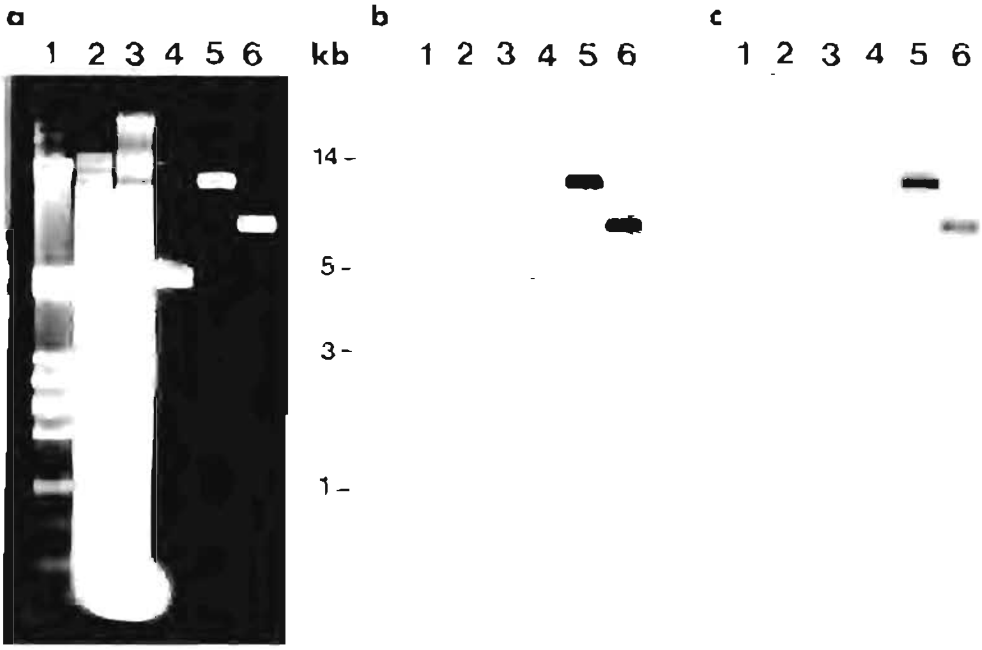

Fig. 4-6. Southern hybridization studies with oligonucleotides probes specific for the non-transposon DNA of the transposon junctions sites. Frame (a) shows the agarose gel, frame (b) shows hybridization pattern against all three oligonucleotides (radioactively labeled) and frame (c) shows the same membrane, stripped and probed with the oligonucleotide from group III junction sites (non-radioactively labeled). The relative positions of various size DNA fragments have been indicated between frames (a) and (b). Lane 1 contained λ DNA digested with restriction enzyme *PstI*. The DNA in lanes 2 to 6 was digested with restriction enzyme *HindIII*. These lanes contained WT chromosomal DNA, mutant m5 chromosomal DNA, and plasmids pm5.1, pm5.2 and pm5.4 respectively.

The group III oligonucleotide was non-radioactively labeled and used as a probe in Southern hybridization analysis against mutant m5 and WT chromosomal DNA, and plasmids representative of each transposon junction site DNA sequence. The probe was able to bind to both group II and group III plasmids, but not to the chromosomal DNA of the WT and mutant m5 strains (Fig. 4-6). These experiments suggested that the source of the flanking DNA from the transposon junction site DNA sequence was not *C. acetobutylicum* P262 or Tn*925*::Tn*917*.

4.4.4. Southern hybridization of mutant m5 chromosomal DNA

There was a discrepancy in the grouping of plasmids clones of the transposon junction sites by plasmid size and by DNA sequences analysis (Table 4-1). The expected sizes of the transposon junction site plasmids could be determined by probing digested chromosomal DNA from mutant m5 with a probe specific for the right junction sequence. Therefore, chromosomal DNA of mutant m5 and of WT *C. acetobutylicum* was digested with restriction enzymes *Hin*dIII and *Eco*RI for Southern hybridization. Probe XE3R, a probe specific for the right-hand side of Tn*925*::Tn*917*, showed no homology to the WT DNA and approximately ten bands of homology were detected for mutant m5 DNA (Fig. 4-7). Two of these bands correspond to the internal *Hin*dIII DNA fragments of transposon Tn*925*::Tn*917* (Fig. 3-5).

A probe specific for DNA fragments containing the left junction site of the transposon, had previously been shown to yield four bands of homology (Fig. 3-4). The anomalous hybridization with probe XE3R could have been due to non-specific digestion by the restriction enzymes or to independent transposition of Tn*917*. Restriction enzymes *Hin*dIII and *Eco*RI both exhibit star activity [1993/1994 Catalog, New England Biolabs, Beverley, Massachusetts]. Mutant chromosomal DNA was digested with varying amounts of restriction enzyme in Southern hybridization analysis using probe XE3R to test whether star activity caused the anomalous bands: samples of approximately 10μg of DNA, previously digested with *Hin*dIII restriction enzyme, were incubated overnight with a range of restriction enzyme *Eco*RI concentrations for under-digestion to over-digestion (0 to 10 units). As controls, samples were digested for 2h with 10 units of either restriction enzyme *Hin*dIII or *Eco*RI. There were approximately eight bands of homology in the well-digested chromosomal DNA samples (Fig. 4-8b). The number of homologous bands did not increase when excess *Eco*RI was used. Star activity was therefore not the cause of the anomalous bands.

The membrane was stripped and probed with plasmid pTet, specific for DNA fragments from the left side of transposon Tn*925*::Tn*917* (Fig. 4-8c). A 5kb internal *Hin*dIII fragment and three additional larger bands of homology were found, which matched previous results (Fig. 3-4). After restripping, the membrane was probed with pTn*917'* (specific for Tn*917*). Two hybridization signals were detected, corresponding to DNA fragments of 5kb and 1kb (Fig. 4-8d). These Tn*917*-specific bands matched the sizes

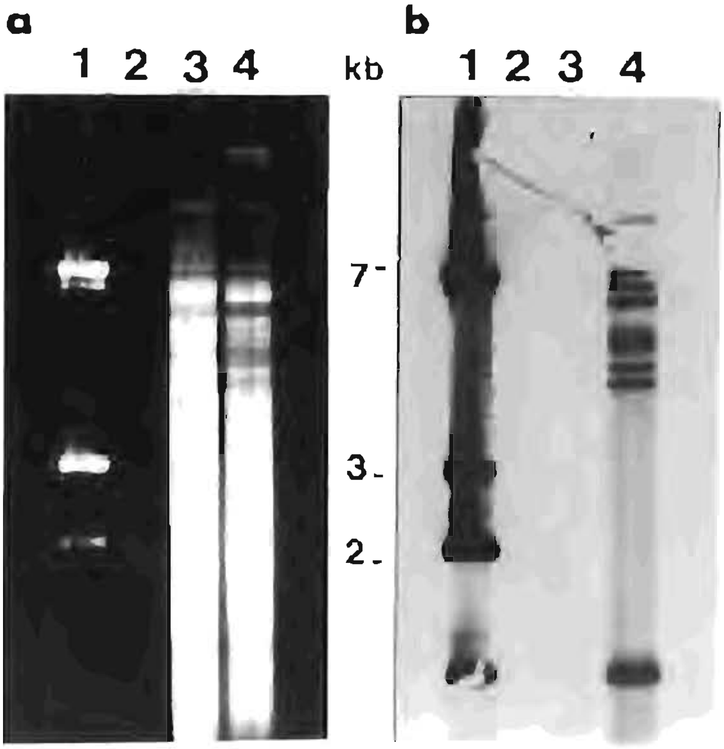

Fig. 4-7. Determining the number of transposon insertions using a probe specific for the right end of Tn*925*::Tn*917* (probe XE3R). Frame (a) shows the agarose gel and frame (b) shows the hybridization signal obtained. The positions of DNA fragments of relevant sizes have been indicated between (a) and (b). Lane 2 contained degraded λ DNA. Lanes 1, 3 and 4 contained plasmid p30R.5, WT chromosomal DNA and mutant m5 chromosomal DNA respectively. The DNA in these lanes was digested with *Hin*dIII and *Eco*RI. Plasmid p30R.5 was not digested to completion.

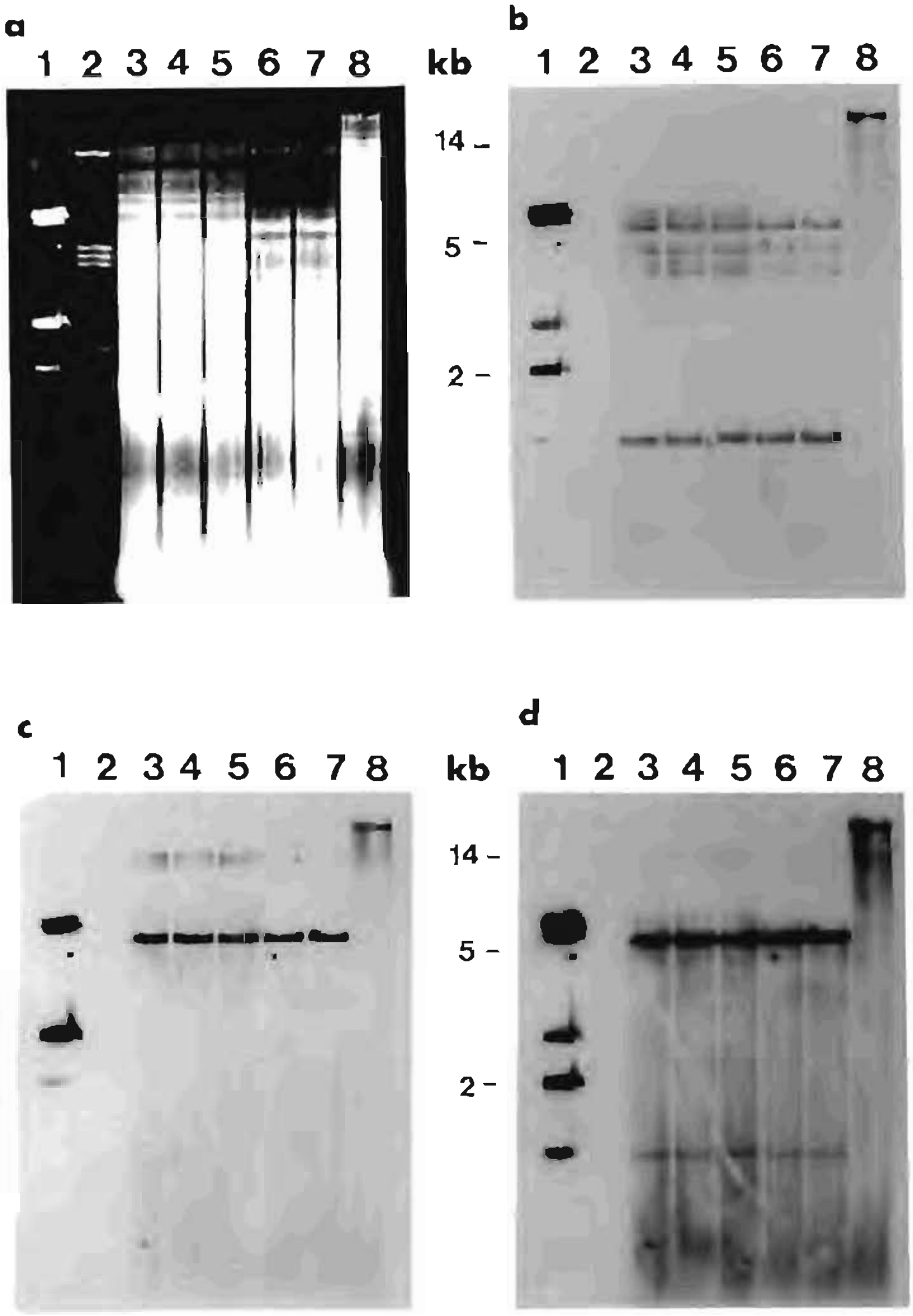

Fig. 4-8. Southern hybridization studies of *C. acetobutylicum* mutant m5 chromosomal DNA. Frame (a) shows the agarose gel of the DNA used, and the hybridization signals obtained with probes XE3R, plasmid pTet and plasmid pTn*917* are shown in frames (b), (c) and (d), respectively. The positions of DNA fragments of relevant sizes have been indicated. The same membrane was stripped for use in each subsequent hybridization experiment. Lane 1 contained plasmid p30R.5 digested with *Hin*dIII and *Eco*RI restriction enzymes. Lane 2 contained *Pst*I-digested λ DNA. Lanes 3 to 8 contained mutant m5 chromosomal DNA. The DNA in lanes 3 and 8 was digested for 2h with 10 units of *Hin*dIII and *Eco*RI respectively. The DNA in lanes 4 to 7 was digested overnight with 100 units of *Hin*dIII and 0, 0.1, 1 and 10 units of *Eco*RI respectively.

of the internal *Hin*dIII fragments of Tn*925*::Tn*917* (Fig. 3-5). Since, the size of the homologous DNA fragments would depend on the distance to the nearest *Hin*dIII or *Eco*RI restriction site, transposon Tn*917* had not transposed independently of the co-integrate transposon.

4.5. Discussion

The phenotype of mutant m5 suggested that a gene involved with the regulation of solventogenesis had been interrupted by transposon mutagenesis. The mutant was thought to contain three transposon insertions (Chapter 3). Therefore the identification of the solventogenesis regulatory gene would require that all the insertionally inactivated genes be cloned. It was hoped that this gene could be recognized on the basis of sequence homology or genetic studies on the individual genes. The initial step was to clone transposon junction sites from the mutant and then to use the junction site sequences to obtain clones of the genes from wild type *C. acetobutylicum* genomic libraries.

Eight plasmids of three different sizes were shown to be clones of transposon junction sites (Fig. 4-1). An oligonucleotide that was homologous to the right terminus of Tn*925*::Tn*917* was required to sequence the DNA of the transposon junction sites from these plasmids. A Tn*916*-specific oligonucleotide was designed to be used as the sequencing primer, because no sequence was available for the termini of Tn*925* and because Tn*925* was known to be very similar to Tn*916* [Christie *et al.*, 1987]. The termini of conjugative transposon Tn*1545* are almost identical to those of conjugative transposon Tn*916* [Caillaud and Courvalin, 1987]. Nucleotide sequence was obtained from the transposon junction site clones with the Tn*916* primer, which showed that the termini of Tn*925* were also similar to those of Tn*916*.

Three different flanking DNA sequences were obtained from the clones of the transposon junction sites (Fig. 4-2). The conjugative transposons are known to insert preferentially into AT rich DNA [Scott, 1992] and there was a stretch of at least five A residues near to the site of transposon insertion for each clone (Fig. 4-2). This feature has been verified in two other mutants generated with the same transposon [H. Collett, pers. comm.] and it could, therefore, represent the partial recognition site for Tn*925*::Tn*917* insertion. Conjugative transposons Tn*916* and Tn*1545* are thought to insert at different target sites [Young *et al.*, 1989a].

The plasmid clones of the transposon junction sites could be divided into three groups on the basis of DNA sequence data and on the basis of plasmid size (Table 4-1). However, these groupings did not correlate and this implied that the plasmids were unstable in *E. coli*. Agarose gel analysis of three plasmids representing the three DNA transposon junction site sequences, revealed that the pattern of restriction enzyme sites had changed for different extractive preparations of these plasmids (Fig. 4-1 and Fig. 4-3). The conjugative transposons are unstable in *E. coli* [Gawron-Burke and Clewell, 1984], and the transposon junction site clones contained approximately 4kb of transposon DNA, since the nearest *Hin*dIII restriction site to a transposon terminus is approximately 4kb (Fig. 3-5). The instability of these plasmid clones could, therefore, have resulted from the expression of transposon genes on the plasmids.

The instability of the plasmids meant that flanking DNA sequences of the transposon junction site clones was not necessarily *C. acetobutylicum* DNA sequence. Several factors provided evidence supporting the theory that these transposon junction site clones were unstable in *E. coli*. Oligonucleotide probes, designed from the transposon junction site DNA sequences, showed no homology to *C. acetobutylicum* WT or mutant m5 chromosomal DNA (Fig. 4-6) and the oligonucleotide probe of group III plasmids was able to hybridize to a group II plasmid (Fig. 4-6).

The DNA sequence of the transposon junction sites had been obtained by sequencing in one direction only and sequence had only been obtained for approximately 230bp. Homologies found with these DNA sequences were therefore not necessarily significant. The highest homology at the protein level for the flanking DNA of the transposon junction site sequences, was found between the group III sequence and a *C. acetobutylicum* β-galactosidase regulator gene (Table 4-2), but this was only over 70% of 17 amino acids. The highest region of homology between the two respective DNA sequences at the DNA level was found to be over 55% of 234 nucleotides at a different location (Fig. 4-4). This region of the β-galactosidase gene was homologous to various regulatory genes.

PCR amplification of the transposon flanking DNA from a plasmid with the group III transposon junction site sequence yielded a PCR product that was homologous to *C. acetobutylicum* P262 DNA (Fig. 4-5). Furthermore, the PCR product hybridized to a different sized DNA fragment in *C. acetobutylicum* mutant m5 (Fig. 4-5), which proved that this PCR product was amplified from DNA flanking a transposon insertion in mutant m5. The homology studies suggested that the transposon represented by the group III transposon junction sequence had inserted into a regulatory gene in *C. acetobutylicum* P262. However, it was not possible to draw any firm conclusions as to the nature of the flanking DNA of the transposon junction sites on the basis of the homology studies, because of the low homology scores.

The expected size of the clones of the transposon junction sites from *C. acetobutylicum* mutant m5 had not yet been determined. To address this question, digested chromosomal DNA from mutant m5 and from WT *C. acetobutylicum* P262 was hybridized with a probe for the right end of the transposon. The hybridization signals would show the sizes of the *Eco*RI-*Hin*dIII DNA fragments containing the right transposon junctions and would, therefore, give the expected sizes of the transposon junction site clones. Surprisingly, approximately ten bands of homology were detected (Fig. 4-8). Independent Tn*917* transposition and restriction endonuclease star activity were shown to not be the cause of the anomalous bands (Fig. 4-8). The difference in the number of right and left transposon junctions suggested that chromosomal rearrangements had taken place in *C. acetobutylicum* mutant m5. Because of the similarity between sequences of opposite transposon termini, a probe to one terminus of the conjugative transposon may have been able to bind to the DNA of the opposite terminus. However, this explanation does not account for the number of the anomalous bands that were obtained (Fig. 4-7). Also, if this explanation were true, the hybridization signals obtained with the pTet probe (for the left junction) would also have been obtained for probe XE3R (for the right junction), and this was not the case (Fig. 4-8).

Conjugative transposon Tn*925* is able to transfer chromosomal genes when it transposes from one bacterium to another [Torres *et al.*, 1991]. It is possible that Tn*925*::Tn*917* was able to induce chromosomal DNA transfer in *C. acetobutylicum* P262. In addition, homologous recombination could have occurred between the various transposons that were present in the

the Biochemistry Department (University of Cape Town). The DNA sequences of "group I", "group II" and "group III" oligonucleotides were 5'-GGTATCATTA-CAGCTGCACT-CG-3', 5'-TTGCGTGGAA-TGAATATTTA-TG-3' and 5'-GGTTATCATG-CTTTCATCAA-CA-3', respectively. For the construction of DNA probes, the oligonucleotides were labeled non-radioactively by following the instructions for the DIG oligonucleotide 3'-end labeling kit [Boehringer Mannheim (SA), Randburg, South Africa]. The oligonucleotide probes were end-labeled radioactively with [γ-^{32}P]ATP using bacteriophage T4 polynucleotide kinase [Sambrook *et al.*, 1989].

4.3.2.4. Southern hybridization and colony hybridization

Hybond N$^+$ membranes [Amersham International, Aylesbury, Buckinghamshire] were used for all hybridization experiments. The membranes were stripped for re-use according to the manufacturer's instructions. For Southern hybridization, DNA was transferred to the membranes under alkaline conditions by capillary action [Sambrook *et al.*, 1989]. For colony hybridization experiments, colonies were streaked directly onto Hybond N+ filters on 2xYT agar plates [Sambrook *et al.*, 1989], and the filters were prepared for DNA detection by using an adaptation of the method of Grunstein and Hogness (1975) [Sambrook *et al.*, 1989]. Hybridization of plasmid DNA and of oligonucleotide probes was done at 64°C and 42°C, respectively.

4.3.2.5. DNA sequencing

DNA was sequenced by the chain termination method using the Sequenase kit (version 2.0), following the manufacturer's instructions [US Biochemical Corporation, Cleveland, Ohio]. The sequencing primer (5'-CGAAAGCATC-TAGAATAAGG-C-3', synthesized in the Biochemistry Department, University of Cape Town) was designed from Tn*916* DNA sequence data [Clewell *et al.*, 1988]. It contained two mismatches so as to include an *Xba*I restriction enzyme site.

4.3.2.6. Polymerase chain reaction

Single specific primer PCR (SSP-PCR) was used [Shyamala and Ames, 1989]. Temperature was cycled using a JDI 8012 high performance temperature cycler [JDI Instruments, Noordhoek, South Africa] with the following program: 1) 94.0°C for 30sec; 2) 93.5°C for 15sec; 3) 43.5°C for

30sec; 4) 72.0°C for 120sec; 5) 72.0°C for 180sec (steps 2 to 4 cycled 25 times). Stock reaction buffer (10x), magnesium chloride (150mM), nucleoside triphosphate (NTP) (8mM), and primers (5μM) were diluted ten fold in de-ionized water to make up a 100μl reaction volume [Innis *et al.*, 1990], to which less than 100ng DNA and 1 unit of Taq polymerase [Promega Corporation, Madison] were added. The primers used for the SSP-PCR were the sequencing primer and the T3 primer for pSK [Stratagene cloning systems, San Diego, California].

4.3.3. Computer analysis of DNA sequences

The PRIMER program [version 0.5, Whitehead Institute for Biomedical Research (WIBR), Cambridge, Massachusetts] was used to estimate oligonucleotide melting temperatures. The BLAST network utility (BLASTX version 1.3.11MP) offered by the National Center for Biotechnology Information (NCBI) was used to search for homology between DNA sequences, translated into protein sequences in all six frames, and all available protein sequences [Altschul *et al.*, 1990; Gish and States, 1993]. Default parameters were used for the BLAST program. The FASTA program from the GCG package [version 7.0, Genetics Computer Group, Madison, Wisconsin] was used to find regions of homology between DNA sequences. Default parameters were used for the FASTA program.

4.4. Results

4.4.1. Isolation and identification of transposon junction site plasmid clones

Chromosomal DNA that flanks the transposon DNA ("flanking DNA") from clones of transposon junction sites can be used as a probe to isolate complete genes from wild type genomic libraries. A genomic library of mutant m5 was constructed to identify the "flanking DNA". Restriction enzymes *Hin*dIII and *Eco*RI were used in the construction of the genomic library, because there were no *Eco*RI restriction sites in the transposon and because the *Hin*dIII sites have been mapped [Christie *et al.*, 1987]. The *Hin*dIII restriction site that was nearest to a transposon terminus, was approximately 4kb from the right end of Tn*925*::Tn*917*. This was the minimum expected size for a plasmid clone of mutant m5 that contained a transposon junction site. Therefore, digested chromosomal DNA fragments

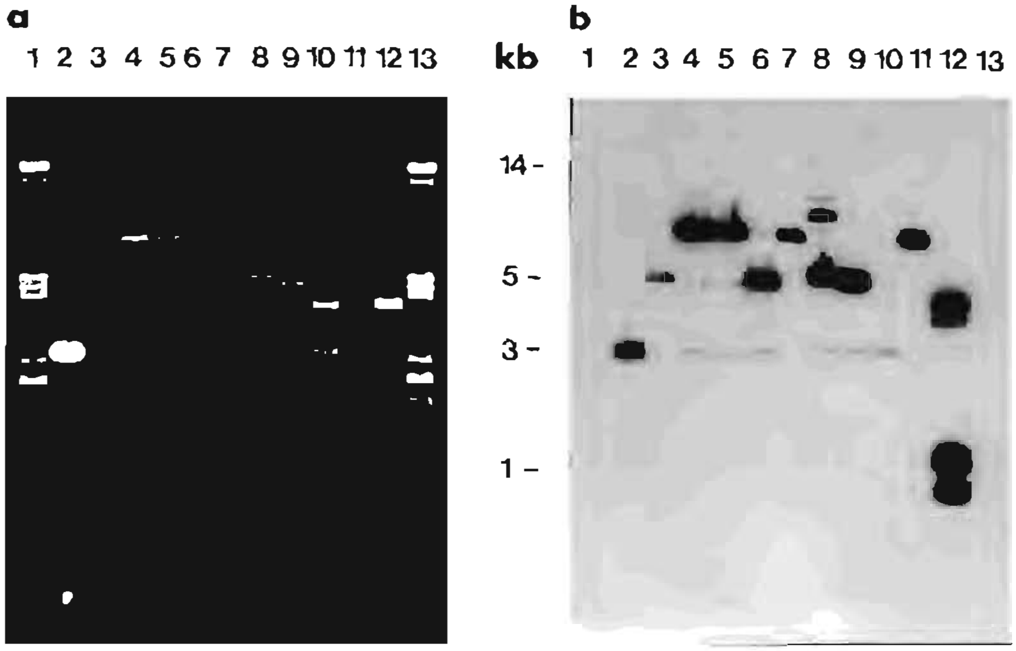

Fig. 4-1. Southern hybridization of a selection of plasmid clones from a genomic library of mutant m5, using the right terminus of Tn*925*::Tn*917* as a DNA probe. Frame (a) shows the digested DNA used for Southern hybridization and frame (b) shows the hybridization signals obtained with probe XE3R. The relative positions of various size DNA fragments are indicated between the frames. Lanes 1 and 13 contained λ DNA digested with restriction enzyme *Pst*I. The DNA in lanes 2 to 12 was digested with restriction endonucleases *Hin*dIII and *Eco*RI. Lane 2 contained the vector-only control, plasmid pSK. Lanes 3 to 11 contained plasmids pm5.1, pm5.2, pm5.3, pm5.4, pm5.5, pm5.6, pm5.10, pm5.12 and pm5.16, respectively. Lane 12 was the positive control, plasmid p3R. The DNA in lanes 4, 6, 8 and 12 was not digested to completion. The DNA bands in (a) are not clear in this photograph.

greater than 4kb in size were purified from agarose gels for the generation of a genomic library of mutant m5.

Samples of the genomic library were transformed into *E. coli* JM105 and approximately 600 colonies were picked for colony hybridization. The transposon-specific *Xho*I-*Eco*RI DNA fragment of plasmid p3R, probe XE3R, was used as a probe in the colony hybridization experiments. *E. coli* JM105 containing plasmid p30R.5 was used as a positive control. Plasmids were extracted from nine colonies that showed homology to the probe. The plasmids were then subjected to Southern hybridization against the same probe. Eight of the plasmids (pm5.1, pm5.2, pm5.3, pm5.4, pm5.5, pm5.6, pm5.10, and pm5.16) were shown to contain transposon junction sites (Fig. 4-1). The XE3R probe had small contaminating amounts of plasmid pSK DNA which was able to hybridize to the vector bands in the Southern hybridization. The eight plasmids contained three different sizes of DNA insert: 4.7, 7.0 and 4.8kb (Fig. 4-1). The plasmid DNA was transformed into *E.coli* JM105 for large scale plasmid preparations.

The DNA sequences of the transposon junction sites contained in seven of the eight transposon-positive plasmid clones, were determined using a Tn*916*-specific sequencing primer. No sequence was obtained for plasmid pm5.10, to which the sequencing primer did not bind. Three different flanking DNA sequences were found at the transposon junction sites in the seven plasmids (Fig. 4-2). The plasmids were divided into three groups according to the sequences (Table 4-1). Group I contained plasmid pm5.1, group II was represented by plasmids pm5.2, pm5.3, pm5.6, pm5.16 and group III by plasmids pm5.4 and pm5.5.

```
                           1                                                40
Junction sequence I    :   TTTATACTTT GTTTATATAC AAAAAATTTC CAAAATTTAT
Junction sequence II   :   TTTATACTTT GTTTATATAC AAAAATAGTT AAAAGAATTA
Junction sequence III  :   TTTATACTTT GTTTAGAATA AAAAAGAGAT AGCGTGGGTG

              41                                                    90
        I   : GTTCTTGTTT TTCTACTCTT CTATCATTTT TCTTATCTTG CTTATCTAAT
        II  : TATTATCAAA CAAATTCTAA AGTTATTAAA ATTTAACATA GATTACTGCT
        III : ATTGAAAAAA CGCTATCTCT ATTTCTATGT TTAGGTTATC ATGCTTTCAT

              91                                                   140
        I   : AACTTCTCTA TTTTTTCTTT GTTATCTTTC TTTGAACAAC TTCCGTCATC
        II  : AATATACGAA AATGTTTAGT GAATTTTAAT TTGCTAAATA TTATTAATTC
        III : CAACATTATT GACTATAATT TAAAACAATA TACAATAATG GGGAAAAGTA

              141                                                  190
        I   : ATTAAATTAT CACGTGGTAT CATTACAGCT GCACTCGTAG TACTTGTAGT
        II  : AATATATTTA GTATTGCGTG GAATGAATAT TTATGTTTCT AGACTCTAAA
        III : TTTAATTAAA TTTTAAAATA TATATATGAT TTCGATGAGT TACCACATAA

              191                                                  240
        I   : TCAATTGGAA CTACTACAGC TGCACCAGTG GTACTTATAG G
        II  : TCAATTAATT ATATCAATTC AAAAGTCTTT AAAATTTTGT TTAATTACAT
        III : GTCTAAGTCT TGATTTGGAT ATCTATAATT TATCCAATAA CACGTGTATA
```

Fig. 4-2. DNA sequences of the transposon junction sites determined from the transposon junction site plasmid clones of mutant m5. Italicized, underlined and bold sequences represent transposon termini, part of a putative Tn*925*::Tn*917* target sequence and the 4bp variable junction sequence [Gawron-Burke and Clewell, 1984], respectively.

Table 4-1. Sorting of transposon junction site plasmid clones according to size of DNA insert and according to junction site DNA sequence. The junction sequences I, II and III are given in Fig. 4-2.

Plasmid	Size (kb)	Junction sequence
pm5.1	4.7	I
pm5.2	7.0	II
pm5.3	7.0	II
pm5.4	4.7	III
pm5.5	7.0	III
pm5.6	4.8	II
pm5.10	4.7	nd
pm5.16	7.0	II

nd, not determined

The fact that the groupings of the transposon junction site clones according to size and according to DNA sequence did not correlate (Table 4-1), could have meant that the plasmids were unstable or that the original genomic library was made from incompletely digested DNA. Agarose gel analysis had shown that no plasmids contained *Hin*dIII-*Eco*RI DNA fragments that were smaller than 3kb (Fig. 4-1). Subsequent analysis of new preparations of plasmids pm5.1, pm5.2 and pm5.4 (representing the different sequence groups) revealed that plasmids pm5.1 and pm5.2 contained *Hin*dIII-*Eco*RI

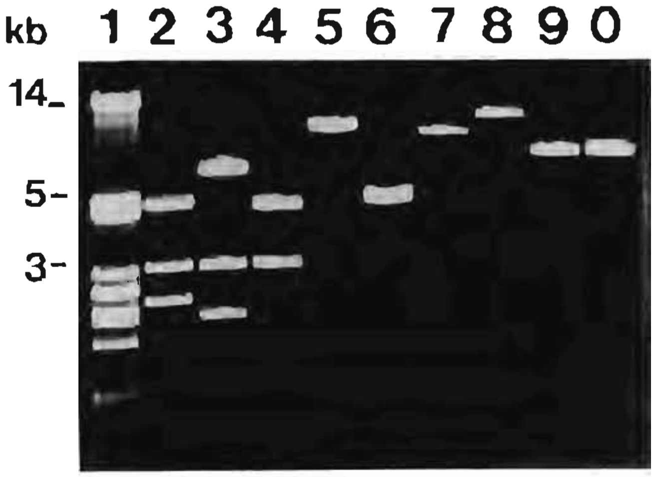

Fig. 4-3. Agarose gel analysis of plasmids pm5.1, pm5.2 and pm5.4, representing the three different transposon junction site DNA sequences. Lane 1 contained λ DNA digested with restriction endonuclease *Pst*I. Plasmids pm5.1, pm5.2 and pm5.4 were digested with restriction enzymes *Eco*RI and *Hin*dIII (lanes 2, 3 and 4 respectively), *Eco*RI (lanes 5, 7 and 9 respectively) and *Hin*dIII (lanes 6, 8 and 0 respectively). The relative positions of DNA fragments of size 14, 5 and 3kb are indicated.

DNA fragments of size 2.2kb and 1.8kb, respectively (Fig. 4-3). This suggested that the plasmids were unstable in *E.coli*.

4.4.2. Computer analysis of transposon junction site DNA sequences

The three chromosomal flanking DNA sequences of the transposon junction site DNA sequences were submitted to NCBI for homology searches using BLAST software [Altschul *et al.*, 1990; Gish and States, 1993]. For each homology search using the BLAST facility, a homology score, an expected score and a probability are calculated, where a high score and a low probability indicate close homology between the query DNA sequence, translated in all possible reading frames, and known protein sequences.

The most significant homology at the amino acid level was found between the group III transposon junction DNA sequence and the *C. acetobutylicum* NCIB 2951 β-galactosidase regulator gene (GenBank accession number M35107) (homology score 69, expected score 52, probability 0.0023), whereas little homology was detected for junction site sequences I and II (Table 4-2). The group III DNA sequence had 70% identity over 17 amino acids to position 4800bp of the β-galactosidase regulator gene (Table 4-2).

Table 4-2. The results of the most significant homologies for each of the flanking DNA sequences of the transposon junction sites found by using the BLAST facility at NCBI [Altschul *et al.*, 1990; Gish and States, 1993].

	Transposon junction site sequence		
	Group I	Group II	Group III
Sequence with highest homology	G protein-coupled receptor	Chromosomal region, 89.2 to 92.8 minutes	β-Galactosidase
Organism source	*Rattus norvegicus*	*E. coli*	*C. acetobutylicum* NCIB 2951
GenBank accession number	L09249	U00006	M35107
Homology score	62	57	69
Expected score	54	50	52
Probability	0.10	0.14	0.0023
Length of homologous region	25 amino acids	23 amino acids	17 amino acids
Percent identity	44%	39%	70%
Percent similarity	72%	73%	76%

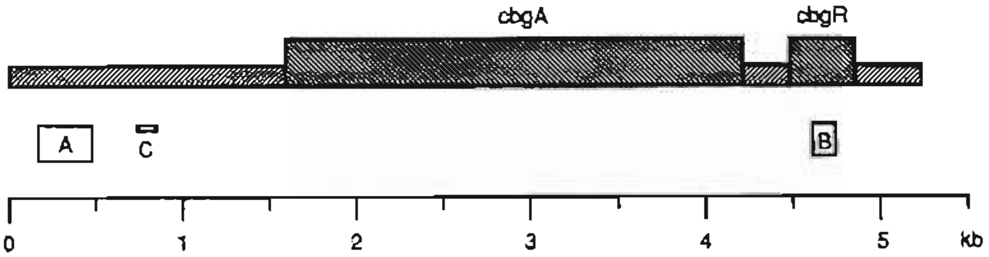

Fig. 4-4. Regions of homology between the flanking DNA sequence of the group III transposon junction site sequence and the *C. acetobutylicum* β-galactosidase gene region [GenBank accession number M35107]. The positions of the β-galactosidase (cbgA) and β-galactosidase regulator gene (cbgR) have been indicated. Regions A, B and C were the areas of highest homology to the flanking DNA sequence, the flanking DNA sequence translated into protein sequence and the DNA sequence of the group III oligonucleotide, respectively. Region A had 56% identity over 234 nucleotides, region B had 70% identity over 17 amino acids, and region C had 72% identity over 18bp.

The FASTA program from GCG (Version 7, Madison, Wisconsin) was used to compare the DNA sequence containing the β-galactosidase regulator gene and the group III transposon junction DNA sequence at the DNA level. A different region of homology was detected (56% DNA identity over 234 residues), which was located upstream of the β-galactosidase structural gene at position 300bp (Fig. 4-4). The FASTA program was also used to determine the most likely region in the β-galactosidase DNA sequence to which the group III oligonucleotide would bind. At position 780bp there was 72% DNA identity over 18bp of the 22-mer (Fig. 4-4).

The DNA sequence from position 166 to 990bp of the β-galactosidase gene was translated into protein sequence and submitted to NCBI for a BLAST homology search to identify with which gene the group III DNA sequence was homologous at the protein sequence level. This DNA region was found to bear close homology to various regulator genes (the GenBank accession numbers are given in parentheses), including the *E. coli* nitrogen assimilation regulator gene of the *gln*ALG operon (X05173), the *B. subtilis* stage 0 *spo*0F sporulation gene (M22039) and the *B. subtilis deg*U protease production enhancer gene (M21658). In each case, the expected homology score was 65, the observed homology score was greater than 138 and the probability was less than 10^{-11}. The group III transposon junction DNA sequence bears some homology to a regulatory gene, but it is not possible on the basis of these homology searches to predict which regulatory gene.

The low homology scores, the small regions of homology and the discrepancy in the location of the homologous regions at the protein and

DNA levels, indicated that the homology between the group III transposon junction site sequence and the β-galactosidase gene region was slight. Further homology searches were not undertaken as the transposon junction site DNA sequences had been sequenced in one direction only, and the accuracy of the DNA sequence data was therefore uncertain.

4.4.3. Southern hybridization of wild type chromosomal DNA

4.4.3.1. PCR generated probes

Plasmids pm5.1, pm5.2 and pm5.4 were labeled non-radioactively and used to probe against *Hin*dIII digested chromosomal DNA from WT and mutant m5 *C. acetobutylicum* cells in Southern hybridization experiments. Each probe produced only very weak hybridization signals against both WT and mutant m5 DNA, which could not be clearly distinguished from background signals (data not shown). A different method was needed to produce probes that were specific for the insertionally inactivated genes. SSP-PCR [Shyamala and Ames, 1989] was employed to specifically amplify the chromosomal DNA flanking the transposon sequences in the transposon junction site clones by using the transposon junction site sequencing primer and a vector specific primer. Since the genomic library was made by using two different restriction enzymes, the orientation of the cloned chromosomal fragment was known. The estimated melting temperature of the transposon primer was calculated to be between 55°C and 60°C [Sambrook *et al.*, 1989], but the optimal PCR annealing temperature was found to be 43.5°C. When the annealing temperature was set at 44°C, no PCR amplification was detected. PCR amplification was obtained only for the group III plasmid representative, pm5.4, for which the PCR product was 1.2kb.

To verify that the PCR product represented chromosomal DNA from *C. acetobutylicum*, the PCR product was labeled non-radioactively (PCR probe) for use as a probe against *C. acetobutylicum* WT and mutant m5 chromosomal DNA in Southern hybridization experiments. The chromosomal DNA of the WT and mutant m5 strains was digested with restriction enzyme *Hin*dIII, but hybridization signals against the chromosomal DNA were detected at different positions (Fig. 4-5). Therefore, the PCR product represented chromosomal DNA that was adjacent to one of mutant chromosome. It is therefore likely that mutant m5 contained chromosomal rearrangements. The inability to resolve the exact nature of the genotype of mutant m5 has made it impossible to identify any genes involved with the phenotype of this mutant.

Chapter 5

General conclusion

Contents

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5.1. General conclusion

Strains of *C. acetobutylicum* differ from each other with respect to genome structure, general physiology and malleability to genetic manipulation. *C. acetobutylicum* P262 is one of the best natural producers of acetone and butanol, and work in our laboratory has centered on improving understanding of this particular strain.

5.1.1. Sporulation induction of *C. acetobutylicum* P262

Little is known about the control of sporulation in *C. acetobutylicum*. Studies on sporulation in *B. subtilis* have been facilitated by the ability to induce spore formation [Errington, 1993]. The sporulation induction conditions in *B. subtilis* differ from those of *C. acetobutylicum* [Long *et al.*, 1984a]. There is a link between solventogenesis and sporulation that may be exploited in a sporulation induction medium for *C. acetobutylicum*. The addition of acids to *C. acetobutylicum* cultures, used to induce a shift to solventogenesis, has been shown to induce a shift to sporulation (Chapter 2). Conditions that induced sporulation mimicked the conditions at the pH breakpoint in the fermentation process. This implies that sporulation is induced simultaneously to the induction of solventogenesis, and that these processes may be under the control of the same global regulator.

5.1.2. Analysis of *C. acetobutylicum* mutant m5

5.1.2.1. Phenotype of *C. acetobutylicum* mutant m5

C. acetobutylicum P262 mutant m5 was blocked in acetone and butanol production, but not in ethanol production [Babb, 1990]. This implied that a regulatory gene controlling solvent production had been inactivated and, furthermore, that the regulation of ethanol was under separate regulatory control. In corroboration, mutants of *C. acetobutylicum* DSM 1731 have been obtained that were not blocked in ethanol production, but were unable to produce acetone and butanol [Bertram *et al.*, 1990].

C. acetobutylicum mutant m5 was inhibited in spore development as well as in solventogenesis (Chapter 3). That the mutant was able to initiate sporulation, suggested solventogenesis was not required for sporulation initiation and, furthermore, that the genetic control of sporulation was linked to an event earlier than solventogenesis. It is likely, therefore, that *C. acetobutylicum* does not go through a simple linear progression of the three developmental processes (acidogenesis, solventogenesis and sporulation), but rather through a branched developmental pathway. In this model, acidogenesis is followed by both solventogenesis and sporulation, which are under control of a common regulator. This is in agreement with the evidence that sporulation and solventogenesis are induced by conditions mimicking the pH breakpoint (Chapter 2). However, no mutants of *C. acetobutylicum* have been obtained that are able to sporulate normally while not going through solventogenesis [Jones and Woods, 1986]. Therefore, an event associated with solventogenesis is required for normal spore formation.

5.1.2.2. Genotype of *C. acetobutylicum* mutant m5

C. acetobutylicum mutant m5 had been generated by transposon mutagenesis with conjugative transposon "Tn*925*". When determining the number of transposon insertions in the mutant, the presence of an internal *Hin*dIII DNA fragment was detected (Chapter 3). This fragment had been ascribed to incomplete mapping of Tn*925* [Strätz *et al.*, 1990]. However, the size of the fragment corresponded to the size of the internal fragment in the cointegrate transposon Tn*925*::Tn*917*, and the presence of Tn*917* in conjugative transposon mutants was confirmed by both DNA sequencing [Collett, pers. comm.] and antibiotic resistance. The conjugative transposon which had been used to construct the mutant was Tn*925*::Tn*917*.

Hybridization studies with the Tn*925* *tet*M gene probe indicated that there were three copies of the transposon present, but the presence of more than three transposons was indicated when a probe to the right end of the transposon was used (Chapter 4). The possibilities of independent Tn*917* transposition and non-specific endonuclease activity were excluded as reasons for the anomalous hybridization signals. The anomalous signals were probably the result of chromosomal rearrangements, which could have occurred through transposon functions, or by homologous recombination between the multiple copies of the transposon. In addition, the conjugative transposons have been reported to be able to transfer plasmids and chromosomal genes [Guffanti *et al.*, 1991; Torres *et al.*, 1991]. Therefore, caution should be applied when interpreting the phenotype of a mutant that contains more than one conjugative transposon as well as the phenotype of a mutant that contains a single insertion, because both mutant types may contain unexpected genotypes. Furthermore, all the conjugative transposons are large and expression of transposon genes may affect the normal cellular processes of the transposon host cell, such as sporulation [Bertram *et al.*, 1990]. Work on mutant m5 of *C. acetobutylicum* has ceased because of the difficulty in defining the genotype.

5.1.3. Conjugative transposons

The conjugative transposons are able to transfer to most bacteria [Dürre, 1993], but there have been very few reports of insertionally inactivated genes being cloned. This probably bears testimony to the difficulty of working with conjugative transposons. The transposon junction sites of several conjugative transposon mutants have been sequenced. The right hand junction sites of insertions of transposon Tn*925*::Tn*917* in mutant m5 revealed a consensus sequence that contained five A residues. This is similar to the preferred insertion site of Tn*1545* [Caillaid and Courvalin, 1987], but different to that of Tn*916* [Young *et al.*, 1989a].

The broad host range of the transposons forms an attractive basis for the development of cloning vectors for organisms that are not easily transformable [Dürre *et al.*, 1993]. However, the exact mechanism of transfer of the conjugative transposons is not known and the transposons have not been sequenced to completion. Therefore, the interaction between the genetic complement of the transposon and that of the host bacteria has not been elucidated. The unraveling of the transfer mechanism and of the gene functions of the conjugative transposons, will allow the rational design of an exceptionally powerful cloning vector.

Conjugative transposons have been used in *C. acetobutylicum* P262 to obtain solvent-deficient and asporogenous mutants [Babb, 1990].

However, little is known about sporulation in anaerobic bacteria. Medically, the sporulation of clostridia is important as toxin production by several species is related to this process [Garcia-Alvarado *et al.*, 1992; Kaniya *et al.*, 1992]. An understanding of the transition state regulators involved with sporulation of one species may be relevant to other clostridial species. In this regard, the link between sporulation and solventogenesis in *C. acetobutylicum* may prove useful. Industrially, an understanding of how the transition state regulators work, will almost certainly aid in the optimization of ABE fermentations for butanol production. Recent genetic progress in *C. acetobutylicum* has showed the potential of cloning solvent pathway genes and introducing the genes back into *C. acetobutylicum.* The improved industrial fermentation of *C. acetobutylicum* may well prove to be a success story of genetic engineering.

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