STUDIES ON THE REGULATION OF SOLVENT PRODUCTION AND ENDOSPORE FORMATION IN CLOSTRIDIUM ACETOBUTYLICUM P262

by

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ABSTRACT

The aim of this study was to characterise the relationships between solventogenesis and endospore formation in Clostridium acetobutylicum strain P262.

Growth and endospore formation was monitored in a number of complex and minimal media and as a result of these studies a new defined sporulation medium was developed. The defined system produced high levels of solvents and supported 60 - 80% sporulation in C. acetobutylicum P262. Endospore formation occurred near-synchronously, enabling this system to be used in correlative physiological and morphological studies. Five other Type Culture Clostridium strains grew and sporulated less well in the C. acetobutylicum minimal medium (CAMM). These variations emphasise the importance of strain differences amongst the Clostridia.

Two well defined physiological phases, the acidogenic phase and the solventogenic phase, which characterise the industrial ABE fermentation process were observed in CAMM.

The morphological changes which occurred in C. acetobutylicum P262 during the production of acetone, butanol and ethanol were identified and correlated with the growth and physiological changes in CAMM. The swollen cigar-shaped

clostridial forms were associated with the production of solvents. There was a correlation between the number of cells which formed the clostridial stage and the yield of solvents in the defined medium and a complex medium. This observation was supported by the isolation of mutants which either failed to form the clostridial stage and produced no solvents, or formed reduced numbers of the clostridial stage and produced intermediate levels of solvents.

Sporulation was associated with the second fermenation phase in CAMM. Mature endospores were never observed in the absence of clostridial stage formation. The overall sporulation sequence was similar to that of other endospore forming bacteria. However, several distinctive features which have been observed in other clostridia were also observed in C. acetobutylicum P262. A presporulation stage, associated with the accumulation of starch storage granules (granulose), was observed. Although the accumulation of granulose made the detection of axial filaments difficult, axial filament formation was never detected before septation. The major difference in the developmental sequence was the initiation of spore coat formation prior to the onset of spore cortex formation.

The environmental factors involved in the initiation of the shift from the acidogenic to the solventogenic phase were investigated. The shift to the solventogenic fermentation phase

was linked to the inhibition of cell division and to the onset of the various physiological and morphological changes. Threshold concentrations of glucose, nitrogen and acid end-products and a narrow pH range were required for the initiation of the solventogenic phase. Nutrient starvation did not support endospore formation in C. acetobutylicum P262 and there was a requirement for an exogenous supply of carbon and nitrogen during sporulation. The incubation temperature markedly affected sporulation without significantly affecting the shift to the solventogenic phase. The maintenance of high partial pressures of hydrogen did not support solventogenesis, while exposure of exponential phase cells to air prompted endospore formation and the shift to the stationary phase physiology in the P262 strain.

The first sporulation-specific event which was distinguished by the requirement for chromosomal replication was the production of forespore septa. Clostridial stage formation, storage product accumulation and solvent production were not sporulation-specific and were induced in the absence of chromosomal replication. The isolation of ribosomal and RNA polymerase mutants which were unable to sporulate suggested a control of differentiation at the level of transcription and translation. In addition, studies involving physiological inhibitors and purine analogs suggested the presence of a complex internal effector system which is also influenced by nucleotide concentration.

A model has been proposed in an attempt to define the mechanism by which the various external and internal factors regulate solventogenesis and endospore formation in *C. acetobutylicum* P262.

CHAPTER I

GENERAL INTRODUCTION

1.1. The history of the acetone-butanol-ethanol (ABE) fermentation

The original observation that certain bacteria are able to produce butanol was made by Pasteur in 1861. However, the development of the acetone-butanol-ethanol (ABE) fermentation process for the commercial production of solvents was largely due to the pioneering work of Dr Chaim Weizmann which began in 1911 (Prestcott and Dunn, 1959; Ross, 1961; Walton and Martin, 1979). *Bacillus granulobacter pectinovorum*, later named *Clostridium acetobutylicum* was the strain used for the production of neutral solvents from starchy grains and a factory was established in England in 1913. During World War I, the industry expanded due to the need for acetone in the manufacture ot explosives and a number of factories were established in the United States and Canada. When the war ended, the demand for acetone decreased and as there had been little demand for butanol, many plants were forced to close.

The period between the two World Wars saw a revival of the fermentation process as the demand for both acetone and butanol increased. New plants opened in North and South America, Japan,

South Africa and in other countries. The major use of acetone was as a solvent in the production of varnishes and isoprene; butyl acetate was used for the synthesis of nitrocellulose lacquers for the automobile industry; and a by-product of the fermentation, riboflavin, also helped to maintain the commercial feasibility of the ABE process. The fermentation process flourished as a major source of solvent production until after the Second World War. During the 1950's and 1960's increasing pressure from the petrochemical industry made the microbial process less favourable economically and finally lead to the abandonment of the fermentation process.

The production of acetone and butanol was first started at National Chemical Products (NCP), South Africa, in 1936. The process at NCP was able to compete with the production of solvents by chemical synthesis for three important reasons:

1. The availability of a local supply of reasonably priced cane molasses from the sugar industry;
2. A plentiful local supply of cheap coal for the generation of steam; and
3. The high cost of imported oil.

However, the deteriorating quality of substrate due to improved sugar extraction techniques and the severe drought which caused a three-fold increase in the price of molasses, resulted in the abandonment of the commercial process at the end of 1981.

Recent developments in biotechnology and the changing economical climate have stimulated a renewed interest in the microbial fermentation (Gibbs, 1983). With modern and sophisticated techniques such as cell immobilisation and continuous culture (Häggström and Molin, 1980; Krouwel *et al*., 1980), the search for alternative fermentation substrates (Volesky *et al*., 1981; Robson and Jones, 1982; Maddox and Murray, 1983), and an increasing understanding of the physiology and genetics of the Clostridia, the production of solvents from biomass may become commercially viable once again.

The ABE process has been extensively reviewed in the literature. A major portion of the published work relates to the original industrial process and has given some insight into the fermentation abilities of the saccharolytic Clostridia (Beesch, 1952; Prescot and Dunn, 1959; Ross, 1961; Spivey, 1978; Walton and Martin, 1979). Additional studies relating to the biochemistry and physiology of solvent producing organisms have only begun to emerge in the past 10 years (Gottschal and Morris, 1981a and b; 1982; Bahl, 1982a and b; Monot *et al*., 1982; Monot and Engasser, 1983).

1.2 The physiology of the ABE fermentation

A wide variety of polysaccharides are utilised by *C. acetobutylicum* via a complex set of biochemical pathways. Carbon dioxide, molecular hydrogen, acetic and butyric acid, as well as acetone, butanol and a small amount of ethanol are the major fermentation end-products.

The batch fermentation may be divided into two distinct physiological phases (Peterson and Fred, 1932; Davies and Stephenson, 1941; Spivey, 1978; Gottschal and Morris, 1981a; Jones *et al.*, 1982).

Acidogenic phase. The first phase of the fermentation coincides with the active growth period of the culture. Carbohydrates are catabolised to acetate and butyrate and the pH of the medium decreases.

Solventogenic phase. The second phase of the fermentation coincides with the conversion of the acid metabolites to solvents and there is a further consumption of the substrate. The pH of the medium rises during this phase.

In addition to the physiological changes observed, sporulation may be initiated during the solventogenic phase. Under appropriate conditions, the sporulation process may be completed and mature spores produced.

A number of morphological changes associated with the two fermentation phases have been demonstrated in certain *C. acetobutylicum* strains. These changes are routinely used to monitor the progress of industrial and laboratory fermentation systems (Spivey, 1978; Jones *et al.*, 1982).

During the acidogenic phase, a healthy fermentation contains cells which are short, highly motile and phase-dark. Following

the pH break and the onset of the solventogenic phase, motility is lost and the rods become swollen due to the intracellular accumulation of a starch-like storage product (granulose) and the formation of cigar-shaped, phase-bright clostridial forms (Jones et al., 1982).

The morphological changes during solvent production are less obvious in other strains of C. acetobutylicum and the American Type Culture Collection strain, ATCC 824, produces solvents in the absence of apparent clostridial stage formation.

Most studies in the field of solvent production have concentrated on establishing the biochemical pathways involved in the production of acetone and butanol, or in determining the optimum substrates and environmental conditions which support good solvent yields. There is limited knowledge available with regard to the regulation of solvent production and an understanding at the molecular level is becoming increasingly important as interests turn to the bioconversion of biomass as a source of chemical feedstock.

Workers in a number of laboratories have begun to focus attention on the factors which provide the signal responsible for the initiation of solventogenesis. The biochemistry of the fermentation pathway is considered to be well understood (Fig. 1.1).

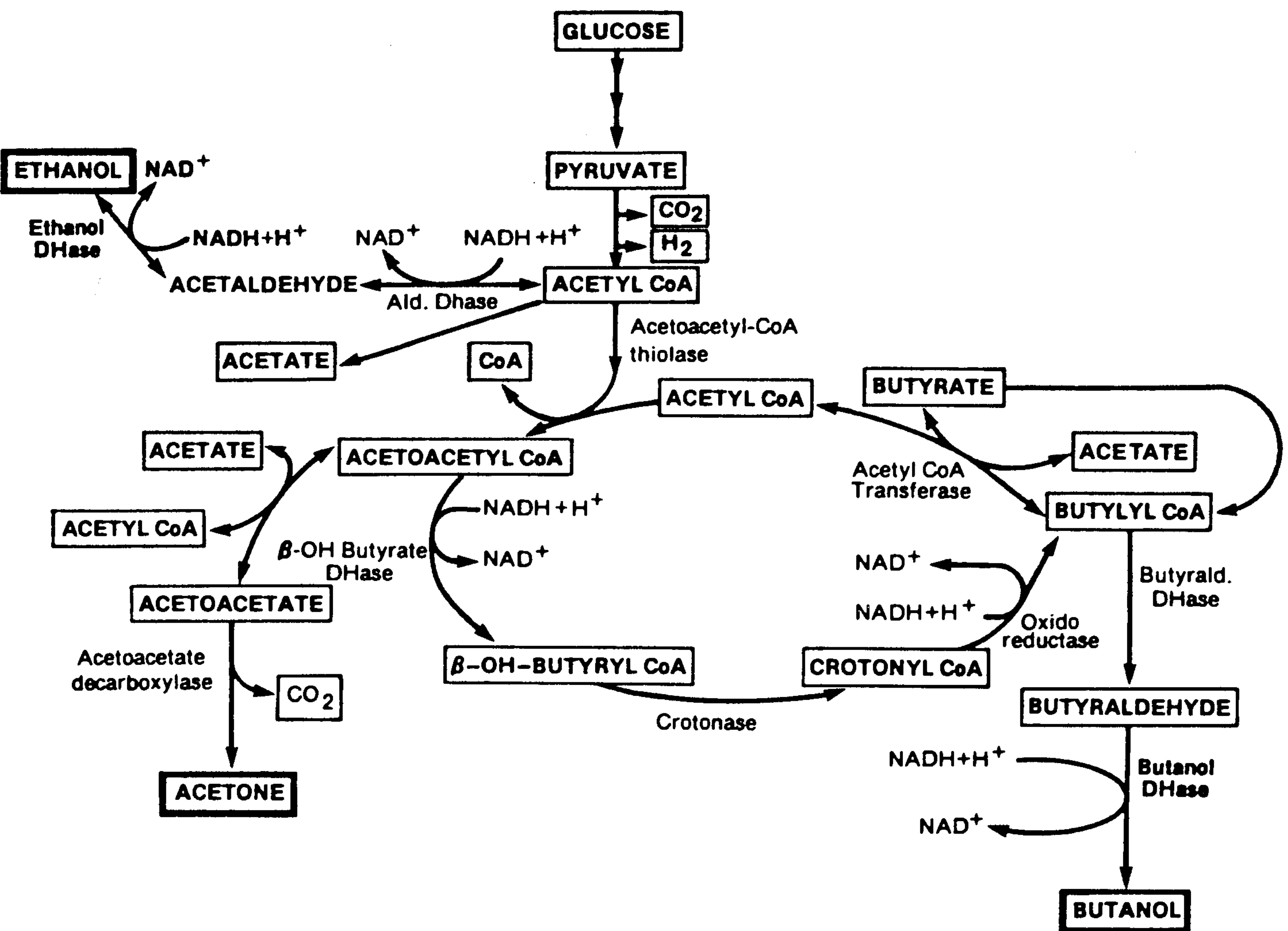

FIGURE 1.1 Main biochemical pathway leading to accumulation of acetone, butanol, and ethanol in the fermentation broth.

C. acetobutylicum ferments sugars to pyruvate via the Embden-Meyerhof pathway. Pyruvate is converted to acetyl-CoA, yielding carbon dioxide and hydrogen. This is known as the phosphoroclastic reaction. During the acidogenic phase, some acetyl-CoA produces acetate, while most enters a cyclic pathway for the production of butyrate. The process thus far yields 3 - 4 mole ATP per mole glucose consumed (Gottschalk and Bahl, 1981). During the solventogenic phase, the metabolic flux is diverted. Part of the acetoacetyl-CoA used to produce butyrate during the acid phase is converted to acetone by decarboxylation of acetoacetate. Butyryl-CoA is reduced to butanol and NAD^+ is regenerated. Ethanol is produced by a similar two-step reduction of acetyl-CoA. The commercial ABE fermentation yields solvents in the ratio of c 6 butanol : 3 acetone : 1 ethanol (Spivey, 1978). The second fermentation phase yields approximately one less mole of ATP per mole of glucose metabolised.

Early workers linked the shift from an acidogenic to the solventogenic fermentation as an adaptive response to a decreasing pH. It was suggested that solvent production would effectively detoxify the medium and prevent the accumulation of acids to inhibitory levels. However, the attainment of low pH alone was found to be insufficient to induce the fermentation changeover (Gottschal and Morris, 1981a; Nishio et al., 1983; Yu and Saddler, 1983). A recent report by George and Chen (1983) has indicated that the acidic conditions are not obligatory for the onset of solvent production by Clostridium beijerinckii, although the drop in pH during fermentation to within limits

which are strain-specific, is important in C. acetobutylicum (Gottschal and Morris, 1981a; Bahl et al., 1982; Monot et al., 1982; Nishio et al., 1983).

The production of acetate and butyrate is responsible for the lowering of the pH of the fermentation medium and evidence contained in a number of reports suggests that these metabolites may have a direct effect on the regulation of the fermentation.

The relative amounts of acetate and butyrate present during the early fermentation phase appear to determine the ratio of acetone to butanol and the solvent yield during the final fermentation phase (Nakhmanovich and Shcheblykina, 1960; Martin et al., 1983). Elevated levels of these two carboxylic acids induced solvent production in batch (Gottschal and Morris, 1981a; Martin et al., 1983), continuous (Bahl et al., 1982a; Gottschal and Morris, 1982) and immobilised (Häggström and Molin, 1980) cell cultures of C. acetobutylicum. The addition of acetate or butyrate to D-xylose grown cells resulted in an increased acetone and butanol yield which could not be accounted for solely by the conversion of the relatively low levels of acids added (Yu and Saddler, 1983).

The basis for the acetate/butyrate effect is unknown. However, it seems unlikely that acetate and butyrate (in the uncharged form) act merely to dissipate the transmembrane pH gradient whereby ensuring that the intracellular pH achieves the same low level as that in the culture medium (Gottschal and

Morris, 1981a). There is some evidence to indicate that these acids specifically influence the regulatory mechanism which directs the flow of metabolites to the acidogenic or solventogenic fermentation pathway. Bahl et al., (1982a) suggested that butyric acid entering the cell may interfere with the formation of butyrate from butyryl-CoA. Acid levels may execute their control through acetyl-CoA, the molecule which holds the most central position in the metabolism of glucose to fermentation end-products. Acetyl-CoA is the precursor form of acetate and butyrate and all three solvents produced during the fermentation. Martin et al., (1982) demonstrated that acetyl-CoA and NADH regulate NADH-ferredoxin oxidoreductase activity and determine the orientation of the metabolic pathways to acid or solvent production.

Molecular hydrogen production during the acid phase serves as a reductant of NAD^+ during solvent production. This suggests that the maintenance of high partial pressures of hydrogen should favour the accumulation of neutral fermentation end-products. The pH_2 may represent the environmental regulator of the diphasic fermentation.

Ethanol production by certain strains of Clostridium thermocellum and Thermoanaerobium brockii could be increased when hydrogen replaced nitrogen as the gaseous phase during growth (Lamed and Zeikus, 1980). Acetone, on the other hand, decreased the yield of normal reduced products in T. brockii (Ben-Basset et al., 1981). This appears to be the only direct evidence to

suggest that high pH_2 induces a shift to solventogenic fermentation and the method by which the hydrogen signal might be transmitted is unclear. Hsu and Ordal (1970) suggested that an elevated NADPH generating system would facilitate the formation of ethyl alcohol in Clostridium thermosaccharolyticum. The work of Martin et al., (1982), suggested that NADH-ferredoxin oxidoreductase determines the catabolic electron flux in C. acetobutylicum, via hydrogen formation during the acidogenic phase, or via NADH formation during the solventogenic phase. Therefore, pH_2 may have a direct influence on this control system inhibiting endogenous hydrogen production in favour of solvent production.

Specific nutrient limitations have been implicated in solvent production, although some conflicting results have been obtained. Continuous culture techniques have facilitated studies to determine the effect of glucose-, nitrogen- and phosphate-limitation on the fermentation physiology.

Gottschal and Morris (1981b) failed to observe solvent production in glucose- or ammonia-limited chemostats. Nutrient limitation appeared to select for a population of cells which were unable to produce solvents and had lost the ability to sporulate. Turbidostat cultures proved to be more useful (Gottschal and Morris, 1982). This method of continuous cultivation allowed growth in the presence of excess nutrients so preventing the accumulation of asporogenous strains and solvents were produced. At low cell densities, the steady state

fermentation was typically acidogenic, but as the culture density was increased, the fermentation gradually changed to one in which the fermentation products were predominantly solventogenic. The relationship between solvent production and cell density is probably indirect and solvent production could be induced in cultures kept at low densities, provided that acetate and butyrate were present in the inflow medium.

Bahl et al. (1982a) detected some solvent production in glucose-limited continuous cultures supplemented with fairly high levels of butyrate. However, the solvent yields were low compared with those obtained in the conventional batch culture process (Walton and Martin, 1979).

Acetone and butanol were produced by *C. acetobutylicum* grown in batch or continuous culture limited in nitrogen (Andersch et al., 1982; Monot and Engasser, 1983). The two modes of cultivation revealed similar fermentation kinetics and solvent production occurred in the nitrogen-limited chemostat when the dilution rate, and hence the specific growth rate, was low and the pH maintained at pH 5.0 (Monot and Engasser, 1983).

When *C. acetobutylicum* was grown in continuous culture under phosphate-limiting conditions, glucose was fermented and acetone, butanol and ethanol were the major end-products (Bahl et al., 1982b). However, twenty percent of the substrate was not consumed. A two-stage continuous process was developed to allow a more quantitative conversion of glucose to solvents. A decrease in the dilution rate during the second stage resulted in near complete degradation of the sugar.

It is apparent that a minimal level of essential nutrients is required for solvent production (Monot et al., 1982), and starvation conditions do not support the conversion of acids to solvents.

1.3 The relationship between solventogenesis and sporulation

A linkage between sporulation and solvent production was noted by the original investigators of the ABE process and cycles of heat shocking and outgrowth of surviving spores were used to select and maintain high solvent producing strains. This observation suggested that the shift in metabolism to solvent formation may be related to the shift of vegetative cells to a commitment to sporulation.

In C. thermosaccharolyticum (Hsu and Ordal, 1970) and in C. acetobutylicum (Jones et al., 1982), an altered fermentation pattern appeared to be related to a change in cell morphology and the ability of the cell to sporulate. Furthermore, Gottschal and Morris (1981b) found that the loss of spore forming capability during continuous cultivation of C. acetobutylicum was associated with a loss of the ability to make solvents.

The role of the developmental programme in solvent-producing strains is not well understood and the fact that Clostridium is an endospore-former, has been largely ignored by fermentation microbiologists.

The production of endospores by anaerobic bacteria is of great importance in both food microbiology (especially in the preparation of canned foods) and in medical microbiology. However, in spite of the interest directed towards the anaerobic endospore-formers from a practical and an applied aspect, the vast majority of research has been conducted using spores of the Bacillus species as these microorganisms provide the most approachable system for propagation and manipulation.

1.4 Endospore formation

The bacterial spore represents a complex cytological and chemical structure with macromolecular composition basically similar to that in other living cells. The mature spore, which exists in a state of little or no metabolic activity, also displays an exceptional resistance to a range of physical and chemical agents. Dormancy and resistance are the central function of sporulation. In the presence of subtle and specific messages from the environment, the rapid, but controlled release of the dormant condition occurs. The irreversible sequence of events that leads from the dormant spore to the vegetative cell, are divided into three distinct processes (Keynan and Halvorson, 1965): activation, germination and outgrowth. The loss of resistance, which is dependent on the dormant state and the structural integrity of the spore coat and cortex, accompanies germination. In order to complete the growth cycle, the cell must resporulate. Sporulation is initiated in the cellular population as it passes out of exponential growth and approaches

the stationary phase. The developmental process, which includes progressive morphological, physiological and biochemical changes, culminates in the formation and eventual release of the mature endospore from the mother cell. During sporulation, the vegetative genome is essentialy suppressed and the spore genome controlling the characteristics specific to the developing endospore, is expressed.

A number of unicellular, Gram-positive bacteria share the ability to form endospores. Stainer et al. (1976) suggested a primary taxonomic subdivision of the sporeformers based on their oxygen requirements:

1. The genera Clostridium and Desulfotomaculum are obligate anaerobes. The vegetative population is unable to survive exposure to molecular oxygen, although the spores are resistant. The criterion for separating the two genera is metabolic. Clostridium spp. synthesise ATP by fermentative metabolism. Desulfotomaculum spp. carry out anaerobic respiration with sulfate as a terminal electron acceptor and heme proteins associated with the electron transport chain.

2. The genera Bacillus and Sporosarcina possess an aerobic electron-transport chain coupled to oxygen as the final electron acceptor. The secondary subdivision in this case is morphological. The rod-shaped aerobic sporeformers constitute the genus Bacillus, and the genus Sporosarcina is represented by species with spherical cells.

The problems of endospore formation and germination have captured the interest of numerous researchers. The advent of new experimental systems and advanced genetic techniques has resulted in an understanding of most aspects of the subject (Murrell, 1967; Warren, 1968; Hanson et al., 1970; Piggot and Coote, 1976; Young and Mandelstam, 1979).

The developmental process in prokaryote cells has provided a relatively simple system for the characterisation of cellular differentiation compared with the process of differentiation in higher organisms. Sporulation is best understood in Bacillus subtilis, primarily because of the early development of effective systems of genetic exchange. However, it is generally accepted that the course of sporulation in the different endospore-forming bacteria have features in common. Studies on the process of endospore formation in the Bacillus group have provided an excellent structural framework for pioneering studies in other endospore-forming bacteria. In the brief review of endospore formation which follows, attention has been focused on the early sporulation stages, which are more pertinent to the initiation and regulation of differentiating systems.

1.4.1 Morphological changes

Sporulation is initiated when growth ceases to be exponential. This usually occurs after an expanded period of active growth and division. However, it is possible to proceed directly from spore to spore through microcycle sporogenesis

(Vinter and Slepecky, 1965). The first observations of highly refractile bodies contained within the cytoplasm of certain bacterial species were made by microscopists some 140 years ago (early review in Knaysi, 1948). The development of synchronous culture methods and the perfection of electron microscopy have permitted a more detailed understanding of the morphological events which constitute sporulation. The developmental process is divided into seven easily distinguishable stages (Murrell, 1967) (Fig. 1.2).

Stage 0: The vegetative cell entering the stationary growth phase contains two or more replicating chromosomes which may be seen as discrete compact entities in the cytoplasm.

Stage I: The nuclear bodies coalesce to form an axial chromatin filament conventionally representing the first stage of sporulation. The significance of the axial thread is not clear and *B. subtilis* mutants specifically devoid of axial formation have never been isolated (Mandelstam *et al.*, 1975). A comparable distribution of the cell's chromatin has been described in old cells, in cells exposed to antibiotics, and in the presence of low temperatures and high salt concentrations (Fitz-James and Young, 1969). It is plausible to assume that filamentation represents a non-specific response of dwindling DNA synthesis to adverse conditions. Axial filament formation appears to be absent in *Clostridium pasteurianum* (Mackey and Morris, 1971) and in *C. acetobutylicum* (Long *et al.*, 1983).

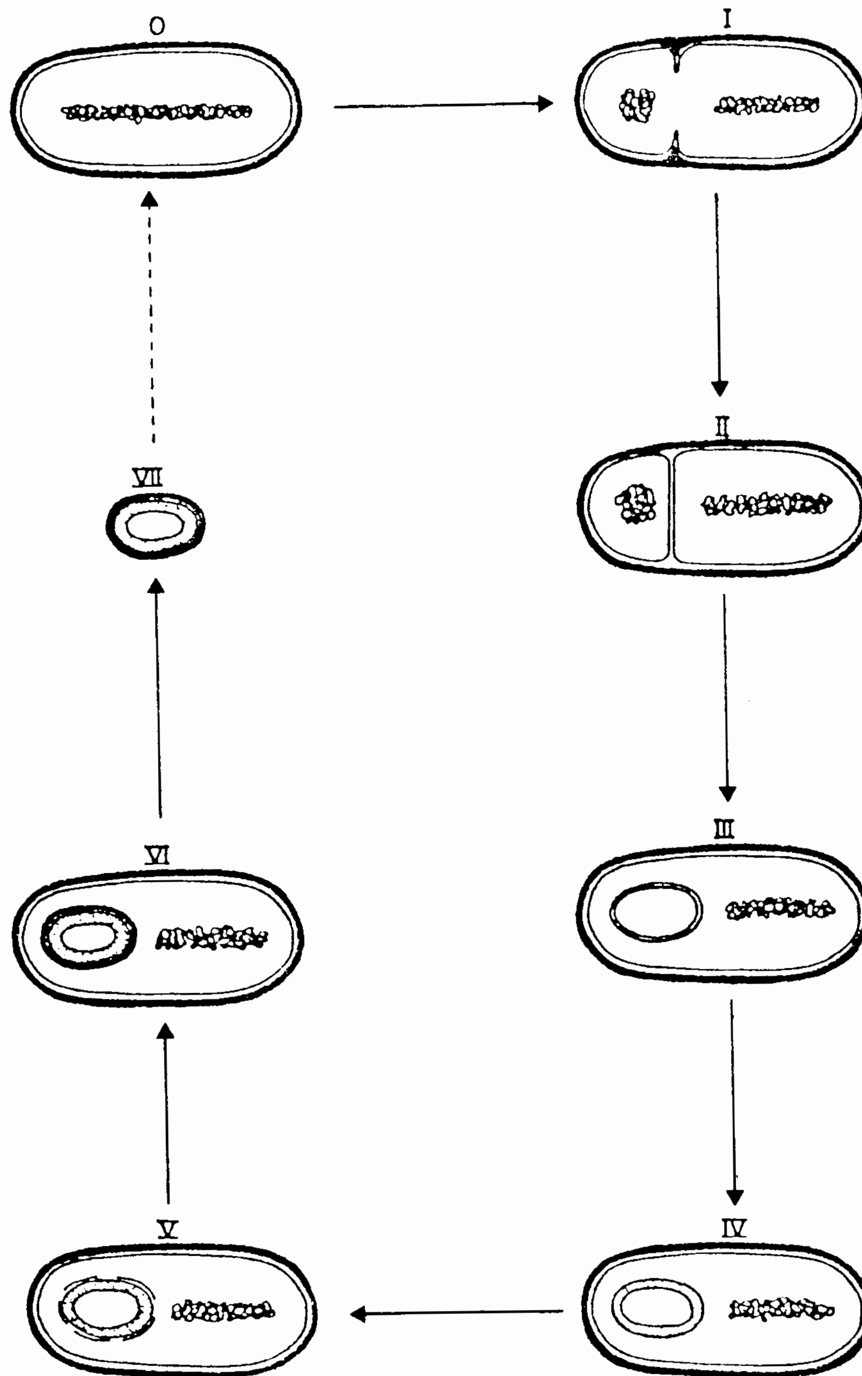

Figure 1.2

Diagrammatic representation of the morphological changes associated with the stages of sporulation.

In some species of Clostridium, the initial stage of sporulation is accompanied by considerable cell enlargement and the accumulation of intracellular storage material (granulose) (Takagi et al., 1960; Mackey and Morris, 1971; Bergère et al., 1975; Johnstone and Holland, 1977; Long et al., 1983).

Stage II: Forespore septum formation has been recognised as the first sporulation-specific event (Young and Mandelstam, 1979). A small terminal part of the condensed chromatin becomes separated from the rest of the cell contents by a transverse membranous septum. The septum is formed by an invagination of the cell membrane analogous to cell division, but it occurs in a sub-polar position. The spore septum contains no detectable peptidoglycan and is thinner than the division septum (Kay and Warren, 1968). Small mesosomes may be recognised in association with the invaginating plasma membrane (Kay and Warren, 1968; Mackey and Morris, 1971).

Stage III: The mother cell membrane extends from the periphery of the spore septum, progressively surrounding the smaller cell. Membrane fusion occurs at the pole of the sporangium and the engulfed forespore becomes detatched within the mother cell cytoplasm. The forespore is essentially a protoplast containing an inner and outer membrane which at this stage are closely apposed.

Stages IV and V: The developing spore is covered by two layers, an inner cortex and an outer spore coat. In most

species, the spore cortex is deposited before the formation of the spore coat (Murrell, 1967). However, in C. pasteurianum (Mackey and Morris, 1971), Clostridium botulinum Type A (Stephenson and Vaughan, 1972), Clostridium bifermentans (Johnstone and Holland, 1977), and C. acetobutylicum (Long et al., 1983), these events are reversed with coat production preceding cortex development.

The cortex arises from the deposition of peptidoglycan between the inner and outer membranes of the forespore. The cortex initially appears as a thin line which gradually expands and appears electron-transparent in the later stages of spore formation (Murrell, 1967). Detailed analysis of the cortical peptidoglycan in Bacillus sphaericus (Tipper and Pratt, 1970) demonstrated the presence of meso-diaminopimelic acid in place of the L-lysine found in vegetative peptidoglycan.

The spore coat is first evident as a continuous double membrane surrounding the cortex. The coat develops through deposition of successive layers of protein to give a coarse multilayered structure, the outer coat, which is electron dense and a finer, inner coat, which is also multilamellar (Kay and Warren, 1968). The coat is deposited as continuous layers in C. bifermentans (Johnstone and Holland, 1977), but in most other clostridia, the coat is produced as individual pieces deposited beyond the periphery of the outer forespore membrane (Takagi et al., 1960; Fitz-James, 1962; Santo et al., 1969; Mackey and Morris, 1971). The discrete fragments coalesce to form a continuous enveloping layer which is multilayered.

Stage VI: The endospore becomes refractile during the period of ripening which follows the completion of cortex and coat formation. The spore shrinks somewhat and becomes heat resistant, impermeable to dilute basic stains and is able to withstand adverse chemical and physical conditions.

Stage VII: Mother cell lysis and release of the mature spore conventionally represent stage VII. Young and Mandelstam (1979) do not consider this process as part of the sporulation sequence as cell lysis will occur under conditions of nutrient deprivation even in the absence of sporulation. Terminal lysis was found to be a protracted process in *C. pasteurianum* (Mackey and Morris, 1971). Spores free of mother cell debris were generally only obtained following several weeks incubation. *Clostridium perfringens* failed to execute autolysis at this stage and the spore was not released, but was encased within the contracted sporangium (Smith and Ellner, 1957).

Diversity of spore fine structure is common among different species of endospore-forming bacteria (Bradley and Williams, 1957; Robinow, 1960). Many clostridia have elaborate appendages protruding from the spore coat. The spore appendages may emanate from one (Pope and Rode, 1969; Samsonoff *et al.*, 1970) or both (Pope *et al.*, 1967) spore poles, arise laterally (Yolton *et al.*, 1968; Duda and Makaryeva, 1977), or be distributed randomly over the spore surface (Hodgkiss and Ordal, 1966).

It is postulated that the appendages may serve as attachment processes, as has been suggested for the pili and fimbriae of Gram-negative bacteria (Hodgkiss and Ordal, 1966). Alternatively, the spore appendages may act as chemo-sensory organelles during germination (Samsonoff et al., 1970).

An exosporium, which appears as a delicate balloon-like sac or as a less obvious tight-fitting membrane, frequently encloses the spores of Bacillus and Clostridium species. The exosporium of Bacillus cereus displays a complex ultrastructure of two apparent layers (Gerhardt and Ribi, 1964). The basal layer is comprised of four sublayers and the second, peripheral layer contains a fringe of hair-like projections. A similar ultrastructure has been described for the exosporium of Bacillus anthracis (Gerhardt, 1967) and species of Clostridium (Hoeniger and Headley, 1969; Samsonoff et al., 1970). Transverse sections of the exosporium of C. pasteurianum (Mackey and Morris, 1972) revealed a more simple multilamellar structure which was open at one end and resembled the exosporium of C. bifermentans (Pope et al., 1967), C. botulinum type A (Stevenson and Vaughan, 1972), a putrefactive anaerobe (Santo et al., 1969), and a pigmented Clostridium (Lund et al., 1978). The exosporium of the C. bifermentans strain reported by Johnstone and Holland (1977) consists of two unit membranes. Exosporia consisting of a single layer of subunits have been reported in other clostridia (Hodgkiss and Ordal, 1966; Long et al., 1983).

Exosporium formation is initiated during stage IV, following forespore engulfment (Murrel, 1967; Santo et al., 1969; Mackey and Morris, 1971; Stevenson and Vaughan, 1972). Initially, material is deposited laterally in the sporangium (Stevenson and Vaughan, 1972) or at the polar end (Santo et al., 1969; Mackey and Morris, 1971) progressing towards the mother cell end and around the spore. When the spore is liberated following lysis, the exosporium remains as an integument encompassing the mature spore.

The chemical composition of the mature spore will not be discussed. A detailed account of the properties of the individual spore components may be found in the review by Murrel (1967).

1.4.2. Biochemical changes

Endospore formation is the major activity of the sporulating cell, however it is not the only function associated with cell differentiation. Many events have been implicated in sporulation because of their time of appearance or their absence in sporulation mutants. However, not all the changes observed during sporulation are an integral part of the process.

The accessory events which occur in the developing cell may be divided into four categories relating to their relevance to sporulation (Young and Mandelstam, 1979).

Category I includes the processes which are both essential and specific to sporulation. Mutants with deficiencies in these events, cannot be induced to sporulate. The production and successful location of the spore septum during stage II and the subsequent engulfment of the forespore during stage III are dependent on biochemical changes which represent essential sporulation-specific events. The existence of cortexless and aberrent coat mutants (Fitz-James and Young, 1962; Wyatt et al., 1982) which complete differentiation and produce spores, suggest that stage IV and stage V, athough sporulation-specific, may be dispensable.

Category II events, which are sporulation-specific and dispensable, are not reqired for normal sporulation. Mutants defective in these functions sporulate normally. These events often serve as useful markers as their times of appearance are usually precisely controlled and closely associated with the morphological stages. Examples include metalloprotease production which coincides with the onset of sporulation, and the incorporation of dipicolinic acid during stages II and III. In view of the statement made previously, the biochemical changes responsible for coat and cortex formation conform to this category.

Category III includes those events crucial to sporulation and which may be expressed during vegetative growth, i.e. essential, but not sporulation-specific. The metabolic processes supplying energy and the precursors for macromolecular synthsis during growth and differentiation constitute Category III-type events.

Category IV events are irrelevant to sporulation and often occur as a result of nuritional step-down conditions or in response to the reduced growth rate during stationary phase. Axial filament formation appears to be an irrelevant event coinciding with the adverse growth conditions at the onset of sporulation. The enzymes of the arginine catabolic pathway and α-amylase are included in this group. Yudkin et al. (1982) compared the stationary phase protein patterns of wild-type B. subtilis and a spo OA mutant. The spo OA defect represents the earliest of all known spo mutations (Piggot and Coote, 1976). During the first three hours following resuspension, less than 50 percent (22 out of 52) of the new proteins synthesised were found to be sporulation-specific. The majority represented a generalised response to starvation.

Mutant studies are useful in assigning sporulation associated events to the various categories. The fact that the induction of sporulation is dependent on chromosomal replication, whereas the induction of vegetative functions are not, has provided an additional criterion for distinguishing events which should be considered as part of the sporulation sequence (Young and Mandelstam, 1979).

Some of the biochemical events associated with the early sporulation stages have proved to be useful markers for probing developmental biology.

Proteases: The production of a variety of proteases marks the onset of sporulation in many species of Bacillus. Two important classes of proteases have been identified (Michel and Millet, 1970).

A neutral metalloprotease, is of limited importance as it is not required for sporulation. Geele et al. (1975) described the "scavenger" action of the metalloprotease which, like the α-amylase,is believed to occur during sporulation for fortuitous reasons. Metalloprotease activity was inhibited in the presence of chelating agents such as ethylene diamine tetra-acetate and by o-phenanthroline (Millet, 1970).

The second class, alkaline serine proteases, are more useful as sporulation markers. B. subtilis produces two serine proteases during the stationary phase (Szulmajster and Keryer, 1975), one with endoproteolytic activity and the other with exoproteolytic activity. Specific serine protease inhibitors such as m-amino-benzeneboronic acid (Geele et al., 1975) and phenyl-methyl-sulphenyl-fluoride (Dancer and Mandelstam, 1975) blocked sporulation. This suggests that at least one of the serine proteases is included in the sporulation programme.

Limited knowledge is available as regards the specific functions of the individual serine proteases. However, it is generally accepted that the intracellular activity is responsible for the extensive protein turnover associated with sporulation (Hageman and Carlton, 1973; Szulmajster and Keryer, 1975;

Glenn, 1976; Cheng and Aronson, 1977). Species showing negligible protein turnover during sporulation produce little or no intracellular proteolytic activity (Slapikoff et al., 1971; Mackey and Morris, 1974a).

Leighton et al. (1972) identified a cytoplasmic serine protease in B. sutilis which was able to cleave the β subunit of RNA polymerase holoenzyme, thereby converting the vegetative polymerase into the homologous spore enzyme in vitro. However, it has since been demonstrated that the modification of the core subunit by serine protease activity is not responsible for the altered template specificity of the RNA polymerase enzyme in vivo (Linn et al., 1973; Orrego et al., 1973). It is suggested that the protease plays some other role early in the sporulation process. An intracellular protease isolated from Bacillus thuringiensis during sporulation is able to modify RNA polymerase (Lecadet et al., 1977). Intracellular proteases have also been implicated in the processing of spore coat precursor proteins (Cheng and Aronson, 1977).

Antibiotics: The onset of sporulation may be accompanied by the synthesis of a distinctive class of antimicrobial peptide antibiotics. Most are oligopeptides, often containing unusual amino acids. Peptide antibiotics are generally refractory to hydrolysis by proteolytic enzymes. Synthesis is executed by enzyme complexes rather than by ribosome mediated systems (Katz and Demain, 1977; Levinson et al., 1978).

Three types of spore antibiotics (Stanier et al., 1976) have been isolated and characterised: edines, linear basic peptides which reversibly inhibit DNA synthesis; bacitracins, cyclic peptides which inhibit cell wall synthesis; and the gramicidin-polymixin-tyrocidin group, linear or cyclic peptides which modify membrane structure or function. Antibiotics are produced exclusively by sporeformers and mutational loss of synthesis is often associated with asporogeny.

Antibiotic production and sporulation respond in parallel to specific inhibitors and to changes in culture conditions. However, certain bacteria sporulate in the absence of detectable antibiotic synthesis (Mackey and Morris, 1974a) and antibiotic production does not occur when B. subtilis is induced to sporulate by resuspension (Young and Mandelstam, 1979).

The functions attributed to the three antibiotic types suggest that antibiotics may be required for the balanced completion of the developmental programme through regulation of cellular activities during sporulation. Sekar et al. (1978) demonstrated a direct relationship between peptide antibiotic production and bacterial sporulation. Linear gramicidin isolated from Bacillus brevis inhibited the trancription of certain regions of the DNA template by specifically interfering with the binding of RNA polymerase to DNA. Gramicidin may regulate the transcription of early sporulation genes in this way. The addition of gramicidin to produce defective mutants, which were also asporogenous, restored the normal spore phenotype and

conclusively linked the two processes. A second peptide antibiotic, tyrocidine, also appears to control the transcription of sporulation genes in B. brevis (Pschorn, 1982).

A more non-specific, but nonetheless necessary role may exist for antibiotic biosynthesis during sporulation. Synthesis may reduce internal amino acid pools thereby derepressing sporulation enzymes. Antibiotic synthesis and excretion is a means of eliminating toxic products from the cell and at the same time provides a competitive advantage to the producer strain.

An apparent relationship exists between antibiotic production and spore formation, however, it is conceivable that synthesis is not obligatory for spore formation. The casual link between the two processes may be a result of independent phenomena controlled by a common regulatory mechanism.

Toxins: Lethal toxins produced by several Clostridium species first appear when the active growth phase is completed (Schaeffer, 1969).

The lethal exotoxin (α A) of Clostridium histolyticum has been implicated in spore formation (Sebald and Cassier, 1969). Asporogenous, stage 0 mutants did not produce lethal toxin, whereas strains blocked at stage III or later excreted significant levels of toxin. Sporulation and exotoxin production appeared to be incompatible in C. perfringens and exotoxin accumulation was detected during the exponential growth phase (Sebald and Cassier, 1969; Duncan, 1973).

Duncan (1973) and Labbe and Duncan (1977) demonstrated a direct relationship between enterotoxin production and spore formation in C. perfringens. It was postulated that the enterotoxin is a sporulation-specific gene product corresponding to a component of the spore coat. Enterotoxin synthesis did not begin until stage III and continued to accumulate intracellularly until lysis of the sporangium released both the mature spore and enterotoxin.

It has been shown that C. botulinum produces an intracellular and extracellular toxin (Bonventre and Kempe, 1960). The toxicity of the culture filtrate corresponded to the degree of cell autolysis suggesting that there was no distinction between the two toxin types. Although more of the protein toxin was synthesised during the period of stationary phase degeneration, no relationship could be made to sporulation.

Toxin production appears to occur less frequently in Bacillus, although a number of strains are pathogenic to insects. The δ-toxin produced by B. thuringiensis has been used as a bacterial insecticide (Wakisaka et al., 1982). The toxin is formed as a parasporal crystal during stages III and IV of sporulation, and possibly in association with exosporium formation. These findings indicate that sporulation and crystal formation are closely related. Acrystaliferous mutants which are not toxic, but are otherwise morphologically indistinguishable from the parent strain, have been described (Wehrli et al., 1980). Thus, toxin production is not essential for sporulation

in *B. thuringiensis* and the same appears to be true of the other toxin producers. Toxin accumulation may only represent an over production of a protein required for sporulation, a waste product of the sporulation process, or a selective advantage similar to antibiotic production.

The consideration of proteases, antibiotics and toxins as biochemical events associated with early sporulation provide a means of illustrating the problems encountered in determining the relevancy of certain stationary phase products to sporulation. The most convincing evidence for or against such a relationship relies on studies with sporulation mutants. A major problem is the pleiotrophic nature of many asporogenous mutants.

Schaeffer (1969) provided a set of useful guidelines for determining whether synthesis is an integral part of the sporulation process. He considered 3 post logarithmic phase products x, y and z, and proposed the following two rules:

1. A single x^- spo^+ mutant isolate rules out causality.

2. A causal relationship implies that all x^- mutants be spo^-, but also y^+ z^+, otherwise the mutants would be pleiotrophic and possibly regulatory mutants.

1.4.3. Genetic Aspects

With the advancement of new techniques for genetic exchange, it has been possible to obtain a map of the *B. subtilis* chromosome which is essentially complete with respect to the location of well-defined genetic markers (Hoch, 1978). There are estimated to be at least 40 - 60 clusters of genes that code for sporulation functions (Hranueli *et al.*, 1974; Hoch, 1978). It is not known how many genes reside within each cluster. The sporulation-specific (*spo*) loci have been named according to the morphological stages they affect. The genetic aspects of endospore formation have been explored (Hranueli *et al.*, 1974; Hoch, 1976; Piggot and Coote, 1976; Young and Mandelstam, 1979), and the different sporulation loci of *B. subtilis* have been characterised in detail (Piggot and Coote, 1976).

Examination of the morphological and biochemical properties of sporulation mutants in addition to genetic information have provided the criteria for distinguishing one locus from another. There are two classes of sporulation mutants:

1. *Asporogenous mutants* fail to produce spores.

2. *Oligosporogenous mutants* sporulate at a reduced frequency varying from about 50% to 1 in 10^8 (effectively the limit of detection by light microscopy).

Temperature-sensitive mutants can be regarded as a special class of oligosporogenous mutants in which the degree of oligosporogeny is temperature dependent.

Sporulation mutants are invariably pleiotrophic and analysis of the sporulation phenotypes associated with a series of Spo mutants has provided evidence that sporulation proceeds by a dependent sequence of events. However, it is becoming increasingly clear that the organisation of _spo_ loci expression is not simple. The following scheme was used by Piggott and Coote (1976) to illustrate this point:

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                    F → G → K
A → B → C → E → H → J → L → → SPORE
        └──→ D
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A single linear sequence of induction may well occur in the early stages of sporulation (A-B-C) but during the later stages, subsidiary sequences of dependent events may operate (F-G-K and E-H-J). It is also apparent that the protein products of certain genes expessed early during spore development (D) are not required until much later.

Genetic loci associated with the various morphological stages of sporulation have been identified and on the basis of the pleiotrophic hierarchy and the phenotypic exclusion patterns in double mutants, it has been possible to order the sequence of expression of _stage 0_ and _stage II_ loci (Piggot and Coote, 1976; Young and Mandelstam, 1979).

Piggot (1978) investigated the organisation of _spo_ loci associated with the later stages of sporulation. During _stage_

III and following engulfment, the situation becomes more complicated and certain loci are expressed in the mother cell, while others are expressed in the forespore. A novel approach for obtaining complementation of late spo loci is by protoplast fusion (Dancer and Mandelstam, 1981). Protoplasts of B. subtilis will complete sporulation if they contain fully engulfed prespores. Hence it is possible to rescue spoIII, spoIV and spoV mutations which are expressed in the mother cell.

Investigations at the molecular level provide an impression of the complexities of gene regulation during what was initially believed to be a simple differentiating system.

1.4.4. Control of gene expression

1.4.4.1. Transcriptional changes

The list of sporulation loci for B. subtilis include nine stage 0, seven stage II, five stage III, seven stage IV and five stage V loci which are transcribed at specific times during the course of sporulation (Piggot and Coote, 1976). Control at the level of transcription is necessary and could be mediated by alterations in the template specificity of RNA polymerase. Several lines of evidence support the polymerase model.

Certain bacteriophages, which respond normally in vegetative cells of B. subtilis failed to multiply in sporulating cells (Sonenshein and Roscoe, 1969; Ito et al., 1973). RNA polymerase

from sporulating cells of B. subtilis had a reduced ability to transcribe Øe DNA (Losick and Sonenshein, 1969). Transcription of the phage template was highly dependent on the sigma factor. This observation supports the proposition that the sporulation-specific RNA polymerase has initiation factor(s) different from the sigma factor associated with the vegetative enzyme, or no initiation factor at all.

Current knowledge concerning RNA polymerase modifications has been reviewed by Losick (1981). At least three sigma factors, designated σ^{55}, σ^{37} and σ^{29}, which are responsible for the distinct promoter recognition specificity of the core polymerase have been identified in B. subtilis. The σ^{55} predominates in vegetative cells, directing RNA synthesis in growing cells. The σ^{37} was shown to direct the in vitro transcription of two sporulation genes which may be involved in the initiation of sporulation. The σ^{37} as well as an additional sigma factor (σ^{28}) recently discovered by Wiggs et al. (1981), were found to be present in vegetative cells. The sporulation-specific factor, σ^{29}, may control RNA synthesis of stage II and stage III sporulation genes. The vegetative sigma protein persists within the cell during sporulation, but in an inactive form (Tjian and Losick, 1974). The multiple sigma factors may not be confined to the control of sporulation genes, but may participate in the expression of specialised gene combinations. Other yet undiscovered factors may be associated with the RNA polymerase at later stages during sporulation.

Sigma-replacing peptides associated with phage-modified RNA polymerase have been reported for the SPO1 phage system (Talkington and Pero, 1977). Infection of B. subtilis by the lytic phage involves an orderly expression of phage DNA. SPO1 genes 28, 33 and 34 encode positive regulatory proteins which change the promoter recognition specificity of the host RNA polymerase. The temporal programme of SPO1 RNA synthesis could be explained by a simple cascade of phage-encoded sigma factors which replace σ^{55} and bind sequentially to the core RNA polymerase.

Archberger et al. (1982) described a sigma-like peptide, delta, which had a molecular weight equivalent to that of the σ^{29} factor (25,000 daltons). Purified RNA polymerase lacking the delta peptide formed initiation complexes with most of the DNA restriction fragments generated by endonuclease digestion of SP82 phage DNA. The delta peptide was able to restore promoter selection efficiency to RNA polymerase. In addition, the delta subunits of B. subtilis and B. thuringiensis were completely interchangable in vitro.

No transcriptional activity was detected for core polymerase or core enzyme plus delta, in the absence of sigma. This finding would contradict the theory of a cascade of sigma factors (Losick and Pero, 1981) if in fact delta and σ^{29} are one and the same. However, it is possible that delta repesents an additional control element required for trancriptional discrimination.

An entirely different picture of RNA polymerase modulation during sporogenesis has been presented for B. thuringiensis (Klier and Lecadet, 1974). Sequential changes were shown to occur in the electrophoretic mobility of the individual core subunits, sigma was removed and in late sporulating cells, two new components, y (32,000 daltons) and z (25,000 daltons), became integrated into the enzyme structure. An intracellular serine protease has been implicated in subunit alterations (Lecadet et al., 1977). The z component may correspond to the delta peptide described by Archberger et al., (1982). The three distinct enzymatic forms of RNA polymerase, one vegetative and two sporulation-specific, transcribed B. thuringiensis DNA efficiently producing RNAs which functioned as messengers in cell-free systems. Purified sporulation form II enzyme preferentially transcribed the "stable" mRNA coding for parasporal protein and was the predominant enzymatic form active during the sporulation phase (Klier et al., 1978) . The form I enzyme appeared to transcribe a completely different set of sporulation-specific sequences.

The isolation and characterisation of drug-resistant, sporulation mutant strains of B. subtilis which display an altered RNA polymerase activity suggest that the structural integrity of the RNA polymerase molecule is essential for sporulation-specific functions (Linn et al., 1975). The resistant mutants include strains blocked at various stages of spore formation. This would support a sequential change of RNA polymerase structure and function during sporulation

(Sumida-Yasumoto and Doi, 1977). However, it has been noted that none of the Spo mutants isolated by other means have simultaneously acquired drug resistance (Young and Mandelstam, 1979).

An intriguing phenomenon of sporulation enhancement by a pseudolysogenic phage of Bacillus pumilus has been described recently (Bramucci et al., 1977). The phage, PMB1, improved the rate of sporulation by wild-type cells and corrected the sporulation defect of a select group of Spo mutants. It is possible that the phage provides an RNA polymerase or host-like polymerase subunit which correctly transcribes the sporulation genes.

A bacteriophage similar to PBS1 has been implicated in the oligosporogenous to wild-type reversion of B. cereus (Boudreaux and Srinivasan, 1981). Sporulation kinetics and the proportion of heat resistant spores were also increased in two C. perfringens strains carrying the temperate phage s9 (Stewart and Johnstone, 1977). The effect of phage infection on enterotoxin biosynthesis during sporulation was not reported.

Although the evidence provided would support a polymerase model for control of sporulation at the level of transcription, a clearer picture of the exact mechanism of regulation remains to be established. Uncertainties which exist may be resolved by studying mutants resistant to new drugs that inhibit transcription. Lipiarmycin preferentially inhibited sigma-dependent transcription in B. subtilis (Sonenshein et al., 1977). This antibiotic may benefit a search for sigma mutants.

The peptide antibiotics, gramicidin and tyrocidin, which interfere with the formation of stable enzyme-DNA complexes, regulate RNA synthesis during sporulation in B. brevis (Pschorn et al., 1982). A similar selective inhibition, in combination with polymerase modification may operate during transcriptional promotor selection and the programmed expression of sporulation genes in other bacteria.

1.4.4.2. Translational changes

The primary site of regulation of endospore formation appears to be at the level of transcription, although alterations also occur in the protein-synthesising machinery during sporulation.

Bacterial mRNA has a short half-life of two to three minutes and the rapid adjustments in gene expression are made at the level of transcription. However, certain spore-specific mRNA molecules have longer half-lives which permit their protein products to accumulate in the presence of RNA synthesis inhibitors. Dipicolinate synthetase mRNA in B. subtilis (Chasin and Szulmajster, 1969), parasporal protein mRNA in B. thuringiensis (Klier et al., 1978), the mRNA coding for enterotoxin and spore coat protein in C. perfringens (Labbe and Duncan, 1977) and the long-lived mRNA isolated by Segall and Losick (1977) during early sporulation in B. subtilis have been reported to date.

The existence of stable mRNA and its role in sporulating cells has been debated (Aronson, 1965; Sterlini and Mandelstam, 1969; Tipper and Pratt, 1970; Leighton and Doi, 1971). However, if stable mRNA is important, then is expression must be regulated at the level of translation in the sporulating cell.

Structural and functional changes in the ribosomes of sporulating cells have been reported. There is little agreement with respect to variations in the physical and chemical properties of the ribosomal proteins, although subtle changes may occur during sporulation. Several criteria support a functional alteration in the ribosomes following the induction of sporulation (Fortnagel et al., 1975).

Mutants of B. subtilis resistant to fusidic acid as a result of a defective elongation factor G have been isolated (Fortnagel and Freese, 1977). These mutants are oligosporogenous and partially blocked at stage 0 of sporulation. The parent strain became refractory to fusidic acid at the time of spore septum formation. It was also at this stage that ribosomes no longer bound the drug in vitro. This data suggests a functional change in ribosomes which is of significance to sporulation. However, it was not established whether the mutation causing fusidic acid resistance was allelic with the lesion causing defective sporulation and Guha and Szulmajster (1974) have questioned the validity of such findings.

Chambliss and Legault-Demarne (1975) found that washed early sporulation ribosomes were functionally identical to washed vegetative ribosomes. Initiation factors, which were modified upon induction of sporulation, were suggested to be solely responsible for the altered specificity of the translation machinery during sporulation. However, the asporogenous nature of certain aminoglycoside antibiotic-resistant mutants, specifically altered in ribosomal proteins, suggests that the ribosomes are modified during the course of sporulation (Leighton, 1974; Domoto _et al_., 1975; Tipper _et al_., 1977; Bott _et al_., 1978).

Rhaese and coworkers (1975) detected the synthesis of unusual nucleotides by the ribosomes of sporulating cells. The highly phosphorylated adenosine nucleotides (HPN) were different from the phosphorylated guanosine nucleotides synthesised by vegetative ribosomal preparations. Asporogenous mutants containing a single mutation in the _spo_ OF locus, which encoded adenosine bis-triphosphate synthetase, were unable to synthesise the sporulation-associated nucleotides (Rhaese _et al_., 1978). Sporulation and specific nucleotide synthesis were also linked in temperature-sensitive mutants of the same type.

The work of Rhaese _et al_. (1975 and 1978) supports a functional change in the translation apparatus during the transition from the growth phase to sporogenesis and it has been suggested that adenosine pentaphosphate (HPNIV) is essential for sporulation.

Changes in the proportions of iso-accepting tRNA species and the appearance and disappearance of specific tRNAs during sporulation in B. subtilis have been observed. There is a change in the percentage of iso-accepting lysyl-tRNA (Vold, 1975) and glutamyl-tRNA (Lapointe, 1975) species during sporulation. Buu et al., (1981) reported a correlation between the expression of spore-specific enzymes and the thiomethylation of tyrosine-tRNA during the stationary phase. The tRNA changes observed are related to cell development, but appear to be regulated independently of sporulation.

Transfer-RNA synthetases may also serve in a regulatory capacity during sporulation. A sporulation-specific modification to the lysyl-tRNA synthetase has been reported (Steinberg, 1975). The altered aminoacyl activity associated with each of the two forms of valyl-tRNA synthetase was implicated in protein biosynthesis during sporulation (Ohyama et al., 1981).

The limited amount of information which is available is inconclusive with regard to the functional significance of ribosomal, t-RNA and t-RNA synthetase changes and the existence of long-lived mRNA species during sporulation. In considering these observations, it is important to distinguish between modifications which are a consequence of cell differentiaton or the nutrient starvation conditions which initiate sporulation, and those modifications which may be involved in regulating differentiation. Graef-Dodds and Chambliss (1978) used a cell-free protein synthesising system in an attempt to measure

translational changes during sporulation. This type of experimental approach is essential for determining the fidelity of sporulation regulation by the protein-synthesising machinery.

1.4.5. Induction of sporulation

The definition by Knaysi (1948) that, "Spores are formed by healthy cells facing starvation" suggested that two important factors influence the initiation of sporulation.

Firstly, the nutritional state of the culture will determine the proportion of cells in the population that will be induced to sporulate. The signal by which the cell recognises the nutritional deficiency may be reflected in the concentration of an intracellular biochemical effector which influences the induction of sporulation.

The second factor to be considered is superimposed on the first and refers to the "healthy cells" in the quotation by Knaysi (1948). At any particular moment, the bacterial cell must be in the right physiological condition to respond to the biochemical effector. This is determined by the state of chromosomal replication at the time of nutritional step-down.

Sporulation in B. subtilis and related species is induced when cells growing in liquid culture are exposed to a limited supply of carbon, nitrogen and sometimes phosphate. A small proportion of cells may sporulate during exponential growth in various media (Schaeffer et al., 1965) and it was initially believed that the probability of a cell becoming committed to sporulation was dependent on the nitrogen and carbon sources used. Dawes and Mandelstam (1970) investigated spore formation during nutrient limitation by continuous culture techniques. They found that the probability of sporulation taking place was largely influenced by the growth rate although media composition was important. At any particular growth rate, a definite fraction of cells was induced to sporulate. The lowest growth rates yielded the highest percentage sporulation when either nitrogen or carbon were limiting. Single amino acid, magnesium ion or inorganic phosphate limitation induced sporulation less well. These results were consistent with the notion that sporulation is a repressible function and that the repressor molecule is present at high concentrations in cells growing rapidly in a carbon and nitrogen rich medium. This suggestion subsequently led to the search for a suitable repressor of sporulation.

The regulation of sporulation is different from the catabolite repression which controls the synthesis of inducible enzymes in Gram-negative bacteria. Neither cyclic AMP nor the associated cyclase and phosphodiesterase activities have been detected in appreciable amounts in Bacillus species (Ide, 1971;

Setlow, 1973). The structurally analogous cyclic GMP has been identified and may play a role in the catabolite repression of inducible systems in Bacillus and in the repression of sporulation (Hanson, 1975). The isolation of mutants defective in cyclic GMP metabolism will provide a suitable test for this speculation.

Catabolite repression-resistant mutants of B. subtilis which are able to sporulate in the presence of ammonium salts and a carbon source have been isolated (Takahashi, 1979). The catabolic-resistant markers mapped at six distinct loci on the chromosome. The biochemical reasons for the sporulation characteristic are not known. The repression of sporulation appears to involve several metabolic steps and there was no obligatory relationship between catabolite repression of an inducible enzyme and the repression of sporulation (Takahashi, 1979).

Freese (1981) reported the existence of mutants which were able to sporulate continuously in media containing excess glucose, but not if the medium also contained carbon compounds which feed into the tricarboxylic acid cycle. A slight reduction in the rate of aspartyl-tRNA synthesis rather than a change in the concentration of any particular nucleotide appeared to be responsible for the increased sporulation frequency during growth (Endo et al., 1983).

The change in ATP level or total adenylate charge which occurs during energy starvation by carbon or phosphate limitation may be the signal which initiates sporulation (Hutchinson and Hanson, 1974; Hanson, 1975). Although ATP may act directly, phosphorylation or adenylation of an aporepressor protein is a possible mechanism for effecting the repression. After the initial signal has been received, it is postulated that the ATP level is restored (Hutchinson and Hanson, 1974).

A series of highly phosphorylated nucleotides (HPN I-IV) appear at the start of sporulation (Rhaese *et al*., 1975). HPN I and HPN II are synthesised by the ribosomes of sporulating cells and reflect a functional change in the translation machinery during sporogenesis. HPN IV, which appears to be sporulation-specific (Rhaese *et al*., 1978), is not synthesised by the ribosomes, but by plasma membrane vesicles *in vitro* (Rhaese and Groscurth, 1976). Synthesis, via ATP phosphorylation, occurs only in the presence of depleted sugar-phosphate levels. These workers suggest that HPN IV is the "chemical messenger" released from the membrane, which induces sporulation when the growth medium becomes depleted.

The central position held by glutamine synthetase in nitrogen and carbon metabolism, makes it an excellent candidate for the regulation of metabolism and sporulation in *Bacillus*. Reysset and colleagues (1978) investigated the role of glutamine synthetase in *Bacillus megaterium* and *B. subtilis*. They isolated mutants which were glutamine dependent and defective in glutamine

synthetase production. These mutants were often deficient in their ability to sporulate. The majority of glutamine-independent revertants recovered normal sporulation properties and improved glutamine synthetase activity. However, the pattern was not always consistent and *B. megaterium* revertants selected primarily for their ability to sporulate, still required glutamine for growth. It was hypothesised that the glutamine synthetase molecule acts as a regulatory protein with an important role in the regulation of sporulation which is quite separate from is catalytic role in glutamine biosynthesis. The confusing experimental results obtained could be explained by assuming that mutations in the glutamine synthetase molecule impair either the regulatory site or the catalytic site or both sites. The details of such a model remain to be tested.

It has been postulated that glutamine synthetase is the first enzyme of a pathway which leads to the production of a small effector molecule which represses sporulation(Elmerich and Aubert, 1975). The alternative, that glutamine synthetase activity prevents the accumulation of an effector with inductive action, cannot be excluded. Glutamine itself does not seem to be the effector but may represent a precursor form of the true molecule. Possible candidates for the effector role include ATP (or energy charge), cyclic GMP, HPN, or an intermediate in the purine biosynthetic pathway as suggested by Elmerich and Aubert (1975).

The synthesis and activity of glutamine synthetase is rigidly controlled in Gram-negative bacteria by the recently discovered ntr genes (Mc Farland et al., 1981). It is feasible that any relationship between glutamine synthetase and sporulation is also very complex.

Neither the enzymes nor the metabolites of the nitrogen assimilation pathway appear to be the signal which regulates the control of sporulation in Bacillus licheniformis (Donohue and Bernlohr, 1978; Schreier et al., 1981).

Amino acid limitation, which initiates sporulation, also evokes the stringent response. This response includes the synthesis of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) and a subsequent decrease of guanine nucleotides, GTP and GDP (Lopez et al., 1981). Sporulation is absent in relaxed (relA) mutants which lack the stringent response. Ochi et al. (1981) demonstrated that this was a consequence of an insufficient decrease in the level of GTP which did not suffice to initiate sporulation. Direct nucleotide measurements have also shown a decrease in GTP and GDP under other conditions which initiate sporulation by nutrient deprivation (Lopez et al., 1979). The activity of other nucleotides decreased under certain sporulation conditions and increased in others.

Freese et al. (1978) were able to induce sporulation in a nutrient rich environment by the partial reduction in synthesis

of purine nucleotides. This condition of sporulation was achieved by purine removal in leaky purine-requiring mutants or by the addition of inhibitors of the purine biosynthetic pathway. A preferential decrease of GTP (and GDP), but not ATP, was observed in the sporulating cultures. The inhibition of pyrimidine nucleotide synthesis was shown to be without effect.

It has been suggested that the partial starvation for purine creates a new biosynthetic balance which is condusive to the positioning of a septum in a polar rather than a central position within the cell (Mitani et al., 1977).

The data which has been summarised, identifies several small molecules which may be involved in the induction of sporulation during nutrient starvation. However, it is difficult to determine whether any one of these potential effectors has a primary or secondary effect on gene expression, whether the effector acts in combination with a receptor molecule, and whether the control of sporulation is positive or negative. In addition, the intermediary metabolism of the bacterial cell, which is a network of interdependent pathways, further complicates the search for the trigger event which initiates sporulation.

There is strong evidence to suggest that the initiation of sporulation is tied to the cell cycle and that induction can only occur while DNA is being replicated (Young and Mandelstam, 1979).

When cells growing in a chemostat were exposed to intermediate periods of nutrient starvation, only a fraction of the population was induced to sporulate during each exposure (Dawes an Mandelstam, 1970). In addition, sister cells sharing the same micro-environment behaved identically when tranferred to a sporulation medium, dividing and either proceeding through a cycle of vegetative growth together, or sporulating with near perfect synchrony (Dawes et al., 1971). These observations infer that cultures of B. subtilis challenged by a sporulation inducing stimulus only respond if a critical stage of the cell cycle has been reached.

Inhibition of chromosomal replication by thymidine starvation of a thymidine-requiring auxotroph of B. subtilis prevented the earliest known sporulation-associated event, extracellular serine protease production, and subsequent sporulation from occurring in a sporulation medium (Mandelstam et al., 1971). The replication requirement prior to the earliest sporulation event may be viewed as a DNA replication requirement for the initiation of spore formation.

Induction of sporulation appears to occur as the replication fork passes a particular segment of the bacterial chromosome. A B. subtilis mutant, temperature-sensitive for the initiation of DNA replication, was used to obtain a cell population synchronised in DNA replication (Mandelstam and Higgs, 1974). Culture samples removed at intervals were transferred to a poor medium in order to test the ability of the population to

sporulate. The capacity for sporulation was low initially, reached a peak about 15 min after the initiation of DNA replication, and then declined.

More recently, experiments were performed using the drug 6-(p-hydroxyphenylazo)-uracil (HPUra) which is believed to be a specific inhibitor of the replicative DNA polymerase (pol III) in a number of Gram-positive bacteria (Brown, 1971; Cozarelli, 1977). The incorporation of HPUra into the sporulation medium prevented endospore formation in B. subtilis (Dunn et al., 1978). When the drug was added at various times after the nutritional step-down, the population began to escape the inhibitory effect and became refractory after about 35 min in the sporulation medium (Dunn et al., 1978; Young and Jeffs, 1978). This corresponds to the time at which the first cells completed the round of replication which was in progress when the nuritional step-down conditions were experienced. Since the chromosomal replication time in B. subtilis is 50 - 55 min, the first cells to escape had completed the first 15 - 20 min replication at the time the step-down was imposed. This is in full agreement with the results obtained with the temperature-sensitive mutant.

The critical region of the chromosome corresponds roughly with the time at which the spo OH and spo OJ loci are replicated (Mandelstam and Higgs, 1974; Piggot and Coote, 1976). It has been suggested that the expression of these loci are only activated during replication under step-down conditions. This could be mediated by one or more of the effector molecules

considered in the previous section. Furthermore, it has been proposed that the segregation of the presumptive prespore and mother cell chromosomes before the formation of the polar spore septum is dependent upon chromosomal termination (Young and Mandelstam, 1979), as is chromosomal segregation during vegetative cell division (Miyakawa *et al*., 1982).

The requirement for DNA replication during the initiation of sporulation has been interpreted by another quite different model. It is postulated that the origins of the chromosome, which are attached to the membrane, move towards the cell poles and initiate asymmetric septum formation before duplication of the chromosomal terminus permits symmetric septation (Freese, 1981).

1.5. Aims of this thesis

The induction of sporulation and solvent production have features in common. It was this observation which stimulated a study of the relationship between the two processes in *C. acetobutylicum*. The near-synchronous nature of the defined sporulation medium and the distinct morphological features displayed by the P262 strain used in this investigation, provided an ideal experimental system with which to determine the morphological changes associated with the shift to solventogenesis and the spore developmental programme, as well as the external and some internal factors which influence the induction of both processes.

CHAPTER II

THE DEVELOPMENT OF A CHEMICALLY DEFINED MEDIUM FOR THE SPORULATION OF CLOSTRIDIUM ACETOBUTYLICUM P262

Summary

The growth and sporulation of *Clostridium acetobutylicum* P262 in a number of complex and minimal media was investigated, and a new chemically defined medium was developed. This medium supported 60-80% sporulation in a semi-synchronous manner. The defined medium was used in subsequent studies to characterise solventogenesis and formation in *C. acetobutylicum* P262. Five other *Clostridium* strains showed marked variations in their ability to grow and sporulate in the various complex and minimal media. This observation accentuates the importance of strain differences in the genus *Clostridium*.

2.1. INTRODUCTION

The study of sporulation in *Clostridium* has lagged behind sporulation studies in aerobic endospore-formers for two important reasons:

1. The technical difficulties of working with strict anaerobes.

2. The complex nutrient requirements displayed by most of the medically important clostridia.

Clostridia are unable to use oxygen as a terminal electron acceptor and members of the genus exhibit varying degrees of intolerance towards molecular oxygen. Clostridium haemolyticum requires a pO_2 of less than 0.5% for growth; Clostridium novyi type A will tolerate a pO_2 of up to 3% (Loesche, 1969). However, rigid methods to exclude oxygen from the culture environment have been recommended in all cases (Gottschalk et al., 1981). Improved anaerobic techniques (Hungate, 1969; Holdeman et al., 1977) and the development of the anaerobic glove box to create strict anaerobic conditions have augmented the isolation and propagation of clostridia which show extreme sensitivities to oxygen.

The majority of early studies related to Clostridium sporulation involved those species which were a potential hazard to public health. Many of the more harmful clostridia are proteolytic. Their growth requirements are complex and for many it has not been possible to determine the individual nutrient requirements necessary for cultivation.

In general, clostridia will sporulate in common anaerobic media containing protein hydrolysates and yeast extract (Gibbs and Hirsch, 1956; Perkins, 1965; Roberts, 1967). However, sporulation frequencies vary significantly (0 to > 80%) from one strain to the next. It is apparent that there is no one medium which will routinely support the sporulation of a wide range of clostridia.

The interpretation of data obtained from studies on the cellular process of spore formation is frequently complicated by the complexity of the culture medium. The ability of B. subtilis to sporulate in defined media has facilitated, in particular, the sophisticated genetic studies performed in this organism. Other areas, including the chemical and biochemical analysis of growing cells, the application of continuous culture techniques and the isolation of auxotrophic mutants, also require media of known chemical composition. Defined media specifically developed to determine the nutritional and physiological requirements of sporulating clostridia have been reported.

The synthetic medium of Ting and Fung (1972) supported the growth of 9 out of the 11 strains of C. perfringens which were tested. However, only 2 strains sporulated and the level of spore formation was low (c 8%). The D-medium (Sacks and Thompson, 1978) represented an improvement of the Ting and Fung medium. A more slowly metabolisable energy source, dextrin, partially replaced glucose. The carbohydrate concentration and the buffering capacity were adjusted to maintain a pH level which allowed sporulation. Calcium, which is generally regarded as essential for the production of healthy spores, was incorporated together with zinc and copper ions. A methylxanthine stimulated sporulation and prevented the formation of a precipitate which was undesirable during the recovery of purified spores.

The D-medium supported the growth and sporulation of 7 strains of C. perfringins including one which had failed to

sporulate in the Ting and Fung medium. The heat resistant spore yields compared favourably with those obtained in a complex medium (Duncan and Strong, 1968).

Frank and Lum (1969) defined the nutritional requirements of a putrefactive anaerobe growing in a complete synthetic medium. Some compounds essential for spore development were not necessary for vegetative growth. Extensive autolysis which occurred during the stationary phase was a major disadvantage of the sporulation medium. Approximately 10% of the vegetative cells resisted lysis and sporulated. Cultures of *C. bifermentans* followed a similar fate when grown in a synthetic medium (Holland and Cox, 1975).

The synthetic media described thus far all contain most of the commonly occurring amino acids and an unwieldy supply of vitamins and minerals. Less complicated minimal media have been developed for the saccharolytic clostridia where the amino acids may be replaced by a single inorganic nitrogen source.

Cultures of *C. pasteurianium* were found to sporulate rapidly in a simple defined medium containing salts, glucose, cysteine and ammonium ions (Mackey and Morris, 1971). The sporulation developmental stages occurred near - synchronously rendering the system amenable to correlative morphological and biochemical studies.

A glucose-mineral salts-biotin medium incorporating ammonium sulphate as the sole nitrogen source has been recommended for the growth and sporulation of *Clostridium butyricum* and *C. beijerinckii* (Holdeman *et al*., 1977).

It has been known for several years that C. acetobutylicum is able to grow in a defined minimal medium (Oxford et al., 1940). Recently, attention has focussed on synthetic media designed specifically for solvent production in batch and continuous culture (Gottschal and Morris, 1981b; Andersch et al., 1982; Monot et al., 1982). Current literature does not appear to contain any reports relating to the minimal sporulation requirements of C. acetobutylicum.

The growth and sporulation of C. acetobutylicum P262, a strain used in the industrial ABE process, was investigated in a number of different media. These studies lead to the development of a defined sporulation medium (Long et al., 1983). The defined medium provided the basis for detailed morphological and physiological studies (Chapter III) and has facilitated an investigation of the environmental factors which influence sporulation in C. acetobutylicum P262 (Chapter V).

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains

The *C. acetobutylicum* P262 strain was supplied by National Chemical Products Limited Germiston, South Africa. This strain is thought to have been derived from one of the original strains isolated by Weismann (Spivey, 1978). It is one of the few strains of *C. acetobutylicum* to be used recently in the industrial production of acetone and butanol (Spivey, 1978; Robson and Jones, 1982). Other strains of *C. acetobutylicum* (ATCC 824 and 10132 and NRRL 527) and *Clostridium butylicum* (NRRL 592 and 593) were used in comparative studies. All strains were maintained as spore suspensions in sterile distilled water stored at 4°C. Spore suspensions were prepared from cultures grown anaerobically at 34°C on buffered *Clostridium* basal medium (CBM) agar (O'Brien and Morris, 1971). After 3 days incubation, the spores were scraped into distilled water, incubated in lysozyme (1 mg ml^{-1}) at 37°C for 30 min, and then washed 3 times in sterile distilled water.

2.2.2. Culture media

All culture media are described in Appendix A. Growth and sporulation was studied in:

2.2.2.1. Complex media

1. *Clostridium* basal medium (CBM).
2. Reinforced *Clostridium* medium (RCM) (Biolab Chemicals, Pretoria, South Africa).

2.2.2.2. Chemically defined and semi-defined media

1. Glucose-mineral salts-biotin medium (GSMM) (Holdeman *et al.*, 1977).
2. *C. pasteurianum* minimal medium (CPMM) (Mackey and Morris, 1971).
3. Semi-defined fermentation medium (SFM).
4. *C. acetobutylicum* minimal medium (CAMM).

CPMM and GSMM were used with and without the following additional salts and growth factors (per 100 ml of medium): 20 mg of $MgSo_4.7H_2O$; 1 mg of $MnSO_4.4H_2O$; 1 mg of $FeSO_4.7H_2O$; 0.1 mg of p-amino benzoic acid; 0.1 mg of thiamine hydrochloride; and 5 ug of biotin.

2.2.3. Inoculation and Growth

CBM was inoculated with spores activated by heat shock at 75°C for 2 min and then cooled on ice for 45 sec. Samples (5 ul) of the spore suspension was used to inoculate 10 ml medium. Cultures were incubated at 34°C until the cells had reached mid-exponential phase (*c* 5 x 10^7 cells ml^{-1}; OD 0.4 - 0.5 at

600nm). A 5% (v/v) inoculum was used to inoculate all test media except CAMM. CAMM (50 ml) was inoculated with 20 ml exponential phase cells (c 5 x 10^7 cells ml^{-1}) harvested from CBM and washed once with sterile distilled water. Manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma-Scientific Inc., Marietta, Ohio) containing an atmosphere of oxygen-free N_2, CO_2 and H_2 (70:20:10 [v/v/v]).

2.2.4. Growth Measurements

1. Growth was measured turbidometrically at 600 nm with a Corning Colorimeter 252.

2. Total cell counts were made using a Thoma counting chamber (Weber Scientific International, Lancing, England) and a Zeiss photomicroscope fitted with phase-contrast optics.

3. Viable cell counts were determined as colony forming units of culture samples grown anaerobically at 34°C on buffered CBM agar.

2.2.5. Physical Measurements

2.2.5.1. pH

pH measurements were performed using a Beckman Ø 70 pH meter.

2.2.5.2. Measurement of fermentation end-products

Acid end-products and solvent prodution was monitored with a Hewlett-Packard 5880A gas chromatograph. Column specifications and conditions for use are detailed in Appendix A.

2.2.6. Methods for Characterisation of Cell Morphology

Clostridial stage and spore counts were determined with a Thoma counting chamber and a Zeiss photomicroscope fitted with phase- and interference-contrast optics. The ratio between the number of spores obtained at the end of development and the maximumn number of cells present during the stationary growth phase was used to compute the percentage sporulation in the test media.

The presence of capsules was determined by negative staining with India ink, the presence of granulose was determined by staining with iodine, and the presence of forespores was determined by the methods of Smith and Ellner (1957) and Hoeniger and Headley (1968).

The experimental data reported in this chapter are results typical of each experiment. However, experiments were repeated at least three times.

2.3. RESULTS

Cultures of *C. acetobutylicum* P262 sporulated well when grown on solid media. Up to 65% of the cells formed mature endospores when grown on CBM agar plates. Growth on solid media provided a practical method for the production of spores, but for the study of the sporulation process, liquid media are more suitable.

2.3.1. Growth and Sporulation of *C. acetobutylicum* P262 in Complex Liquid Media

Several complex anaerobic media will support the growth of *C. acetobutylicum* P262. These include beef liver medium (see Appendix A); peptone yeast extract-glucose medium (Holdeman *et al.*, 1977); potato/glucose broth and molasses fermentation medium (Barber *et al.*, 1979). However, these media were not suitable for sporulation studies due to the low sporulation frequencies (< 10%) and/or the particulate nature of the medium. Two clear laboratory media, CBM and RCM, which supported sporulation in *C. acetobutylicum* P262, were used in the studies involving complex culture media.

2.3.1.1. Clostridium Basal Medium (CBM)

Cell growth in CBM was determined tubidometrically and by total cell count (Fig. 2.1). A short lag period (1 - 2 h) was followed by a phase of exponential growth (3 to 6 h). The cells

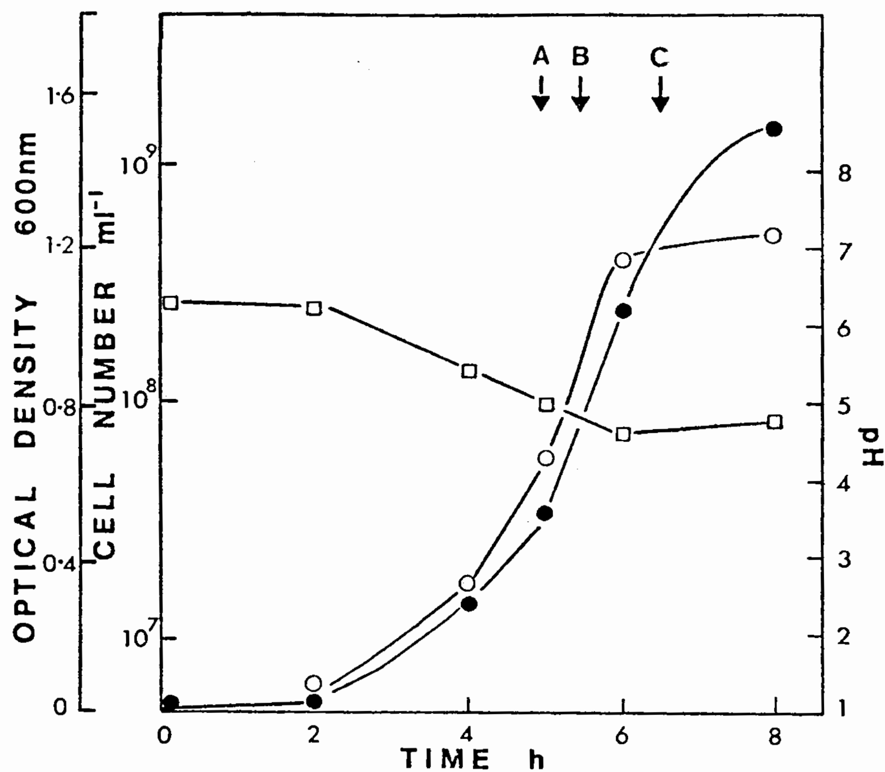


Fig. 2.1

Growth of C. acetobutylicum P262 in CBM. Total count (○); Optical density (●); pH (□). Times are shown for the peak of motility (A), onset of granulose accumulation (B), and the initiation of clostridial stage formation (C).

were motile during this stage and the generation time was about 30 min. Towards the end of the growth phase (from 5.5 h) the cells began to accumulate granulose. The optical density of the culture continued to rise after cell division had halted. This increase was related to cell enlargement and the formation of cigar-shaped clostridial forms. During the stationary growth phase (after 6 h), 50 - 60% of the cells were swollen and phase bright. By 20 h, mature spore formation had occurred. The sporulation frequency was low and mature spore yields in excess of 30% were rarely obtained. Attempts were made to improve endospore formation by altering various components of the complex medium.

Varying the glucose concentration (10 - 40 g l^{-1}) did not affect endospore formation. However, CBM which contained 40 g l^{-1} sucrose as the carbon source stimulated sporulation and spore yields of 30 - 40% were obtained (Table 2.1). Cultures grown in the presence of sucrose tended to form heavy flocculates of capsular material which interfered with the microscopic examination of culture samples.

A reduction in the organic nitrogen level from 4 g l^{-1} to 2 g l^{-1} failed to improve the production of endospores.

The metabolism of glucose with the resulting production of acid end-products during growth in CBM caused the pH of the medium to drop (Fig. 2.1). At the end of the exponential phase (6 h) the pH was pH 4.8. A reduced buffer concentration which permitted a pH decrease to below pH 4.3 inhibited endospore formation (Table 2.2).

TABLE 2.1

Effect of glucose concentration and sucrose on endospore formation in *C. acetobutylicum* P262 in CBM. The number of cells which formed endospores is expressed as a percentage of the maximum cell count ($4 - 5 \times 10^8$ cells ml^{-1}).

Carbohydrate	Concentration ($g\ l^{-1}$)	Endospores (%)
Glucose	10	20 - 30
	20	20 - 30
	40	20 - 30
Sucrose	40	30 - 40

TABLE 2.2

Effect of buffering agents on endospore formation in *C. acetobutylicum* P262 in CBM. The number of cells which formed endospores is expressed as a percentage of the maximum cell count ($4 - 5 \times 10^8$ cells ml^{-1}).

Buffer	Concentration	pH (0 h)	pH (25 h)	Endospores (%)
$NaHCO_3$	12 mM	6.4	4.8	20 - 30
	6 mM	5.8	4.4	20 - 30
	none	4.8	4.2	< 5
$CaCO_3$	100 mM	6.3	5.5	20 - 30
$KHPO_4$	50 mM	6.4	5.0	20 - 30

In an attempt to improve sporulation by increasing the buffering capacity of CBM, alternate buffering agents were used (Table 2.2). In the presence of $CaCo_3$, CBM broth was maintained above pH 5.0 during growth. However, endospore formation was not enhanced. Potassium phosphate buffer, under a nitrogen atmosphere, also maintained a favourable pH (pH 5.0) without affecting the sporulation frequency.

Incubation temperature had a marked effect on growth and sporulation in CBM (Table 2.3; Fig. 2.2). Although 37°C was the optimum temperature for growth, proportionately more spores were formed at the lower temperatures. An incubation temperature of 34°C was used for all sporulation studies.

2.3.1.2. Reinforced Clostridium Medium (RCM)

Inocula of *C. acetobutylicum* P262 grew well in the commercially available complex medium. A doubling time of *c* 30 min was obtained during the exponential phase (2 to 5 h). Growth and development in RCM (Fig. 2.3) was similar to that obtained in CBM. The pH of the medium was maintained close to pH 5.0 during growth. The sporulation frequency was low, averaging between 10 and 30%. No attempt was made to modify this medium.

2.3.2. Growth and sporulation of *C. acetobutylicum* P262 in defined and semi-defined liquid media

TABLE 2.3

Effect of incubation temperature on endospore formation in C. acetobutylicum P262 in CBM. The number of cells which formed endospores is expressed as a percentage of the maximum cell count ($4 - 5 \times 10^8$).

Temperature (^{o}C)	Endospore[a] (%)
30	40 - 50
34	40 - 50
37	10 - 20
40	5 - 10

[a], The improved sporulation frequencies reported are a reflection of later improvements made to CBM broth through changes in the gas atmosphere and the supplier of yeast extract.

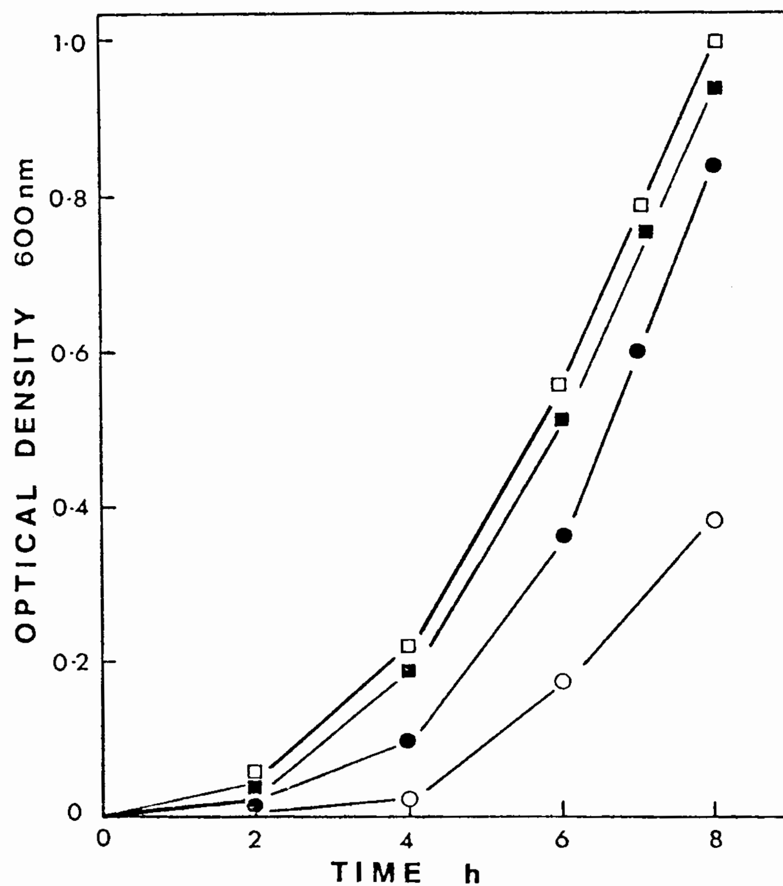


Fig. 2.2

Effect of incubation temperature on growth of C. acetobutylicum P262 in CBM. 30°C (○); 34°C (●); 37°C (□); 40°C (■).

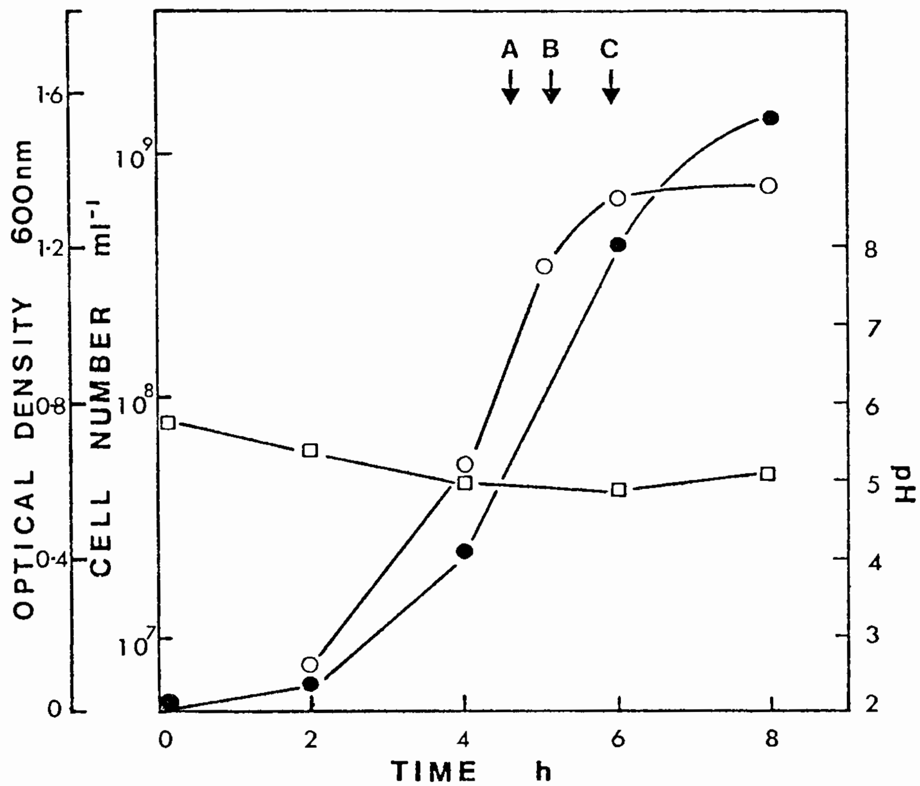


Fig. 2.3

Growth of C. acetobutylicum P262 in RCM. Total count (○); optical density (●); pH (□). Times are shown for the peak of motility (A), onset of granulose accumulation (B), and initiation of clostridial stage formation (C).

A number of chemically defined media supported the growth of C. acetobutylicum P262. In addition to the media used to develop a minimal system for the sporulation of the P262 strain, growth was also obtained in the defined media of Monot et al. (1982) and Leung (1980).

2.3.2.1. Glucose-mineral salts-biotin medium (GSMM)

C. acetobutylicum grew poorly in a defined glucose-mineral salts-biotin medium (Holdeman et al., 1977). Growth was improved when the medium was supplemented with additional salts and growth factors (Fig. 2.4). A doubling time of 160 min was obtained during the exponential phase (4 to 12 h). The final cell yield (c 10^8 cells ml^{-1}) was lower than that obtained in CBM (c 5 x 10^8 cells ml^{-1}). The pH of the medium dropped to a minimum level (pH 4.2 - pH 4.4 at 10 h) before the onset of the stationary phase (12 h). A few swollen, phase-bright clostridial forms (< 5%) were observed at 12 h, but these, together with the rest of the cell population, showed degenerative changes and no sporulation was detected.

A superior GSMM medium which contained ammonium acetate (2 g l^{-1}) as the nitrogen source supported better growth of C. acetobutylicum P262 (Fig. 2.5). A doubling time (between 6 and 16h) of 145 min and a final cell yield of c 2.5 x 10^8 cells ml^{-1} was obtained. Most significant was the improved buffering capacity of the modified medium. The pH was maintained above pH 5.0 during growth. The pH breakpoint (pH 5.1 at 16 h) coincided

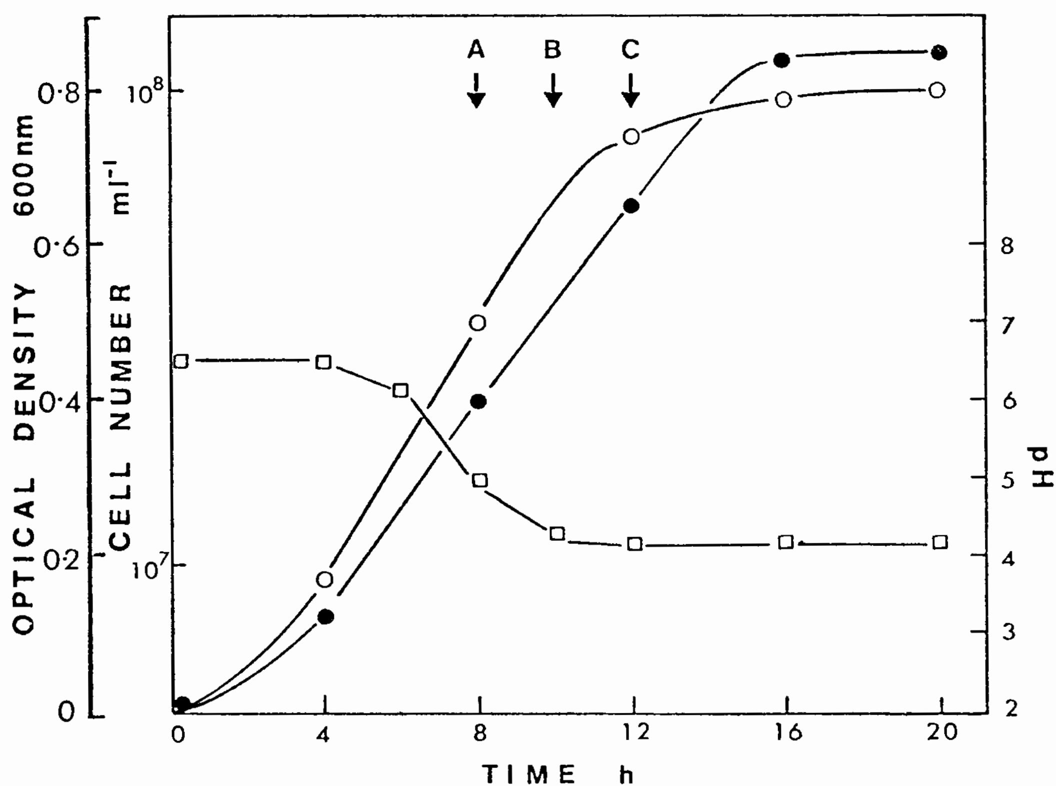


Fig. 2.4

Growth of C. acetobutylicum in GSMM. Total count (○); optical density (●); pH (□). Times are shown for the peak of motility (A), limited granulose production (B), and limited clostridial stage formation (C).

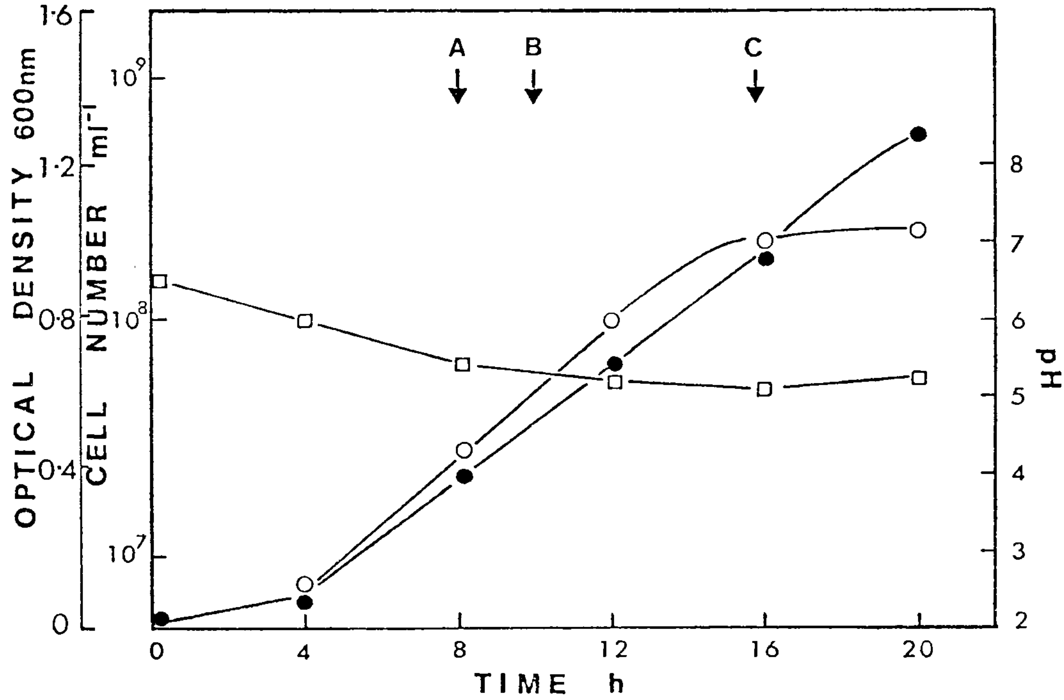


Fig. 2.5

Growth of C. acetobutylicum P262 in the modified GSMM medium. Total count (○); optical density (●); pH (□). Times are shown for the peak of motility (A), onset of granulose accumulation (B), and the initiation of clostridial stage formation (C).

with the cessation of cell number increase and the onset of the stationary phase. Between 16 and 24 h, 70 - 80% of the cells formed swollen clostridial forms. Sporulation was induced in the modified GSMM and 20 - 30% phase bright spores were observed after 48 h. The pH recovered to c pH 5.4, approximately 8 h after the pH breakpoint and 2.87 g l^{-1} solvents were produced in the medium.

2.3.2.2. C. pasteurianum Minimal Medium (CPMM)

The *C. acetobutylicum* P262 strain grew in the *C. pasteurianum* minimal medium of Mackey and Morris (1971), but growth was improved when additional salts and growth factors were added (Fig. 2.6). A $CaCO_3$ precipitate made it difficult to measure growth by absorbance. Total cell counts were used to monitor the extent of cell proliferation. The vegetative rods tended to become elongated and motility was sluggish during the exponential phase (between 10 and 30 h). The cell doubling time was 240 min during this period. The pH of the medium did not vary much during growth. The lowest pH (pH 6.0) was obtained at 30 h and coincided with the end of the growth phase. The clostridial forms which were produced after 30 h were thin and did not have the characteristic cigar shape observed in other media. None of the clostridial stage cells developed endospores. A low level of solvents (c 5 g l^{-1}) were produced during the stationary growth phase. After 40 h the culture began to lyse.

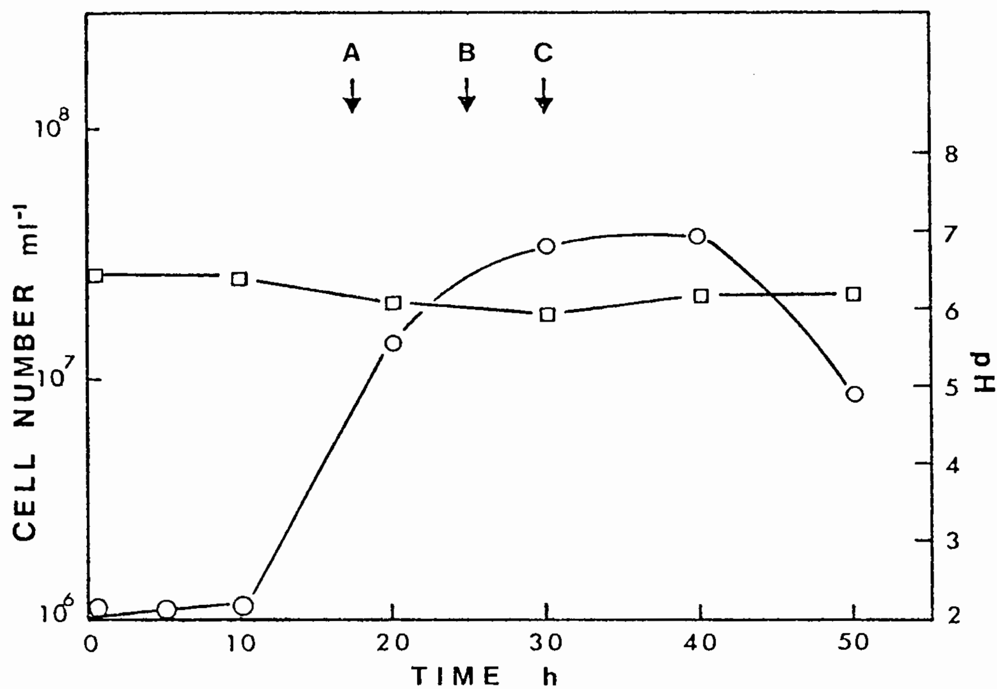


Fig. 2.6

Growth of C. acetobutylicum P262 in CPMM. Total counts (○); pH (□). Times are shown for the peak of motility (A), the onset of granulose accumulation (B), and the initiation of clostridial stage formation (C).

2.3.2.3. Semi-defined Fermentation Medium (SFM)

The physiological and morphological changes which occurred when *C. acetobutylcium* P262 was grown in SFM were comparable to the changes observed during the industrial ABE fermentation (Spivey, 1978) (Fig. 2.7). Short, highly motile rods predominated during the early part of the fermentation (5 to 25 h). The culture was actively dividing and a generation time of 130 min was obtained. The pH of the medium decreased during this phase (acidogenic phase). After 28 - 30 h, cell proliferation ceased and the pH recovered from pH 5.8 at 30 h to pH 6.5 at 50 h. The majority of the vegetative rods (80 - 90%) formed swollen phase-bright clostridial forms and 14 - 18 g l^{-1} solvents were produced during the solventogenic phase. After 50 h, the clostridial forms began to lose their phase-bright appearance. A few cells formed forespores (< 10%) but further spore development was inhibited.

2.3.2.4. *C. acetobutylicum* Minimal Medium (CAMM)

A new chemically defined medium was developed by testing different combinations of constituents of GSMM, CPMM and SFM. The growth and development of *C. acetobutylicum* P262 in CAMM (Fig. 2.8) followed a pattern very similar to that which occurred in the laboratory fermentation medium (SFM).

Growth commenced 10 - 12 h after inoculation. The exponential growth phase (12 to 28 h) was characterised by a

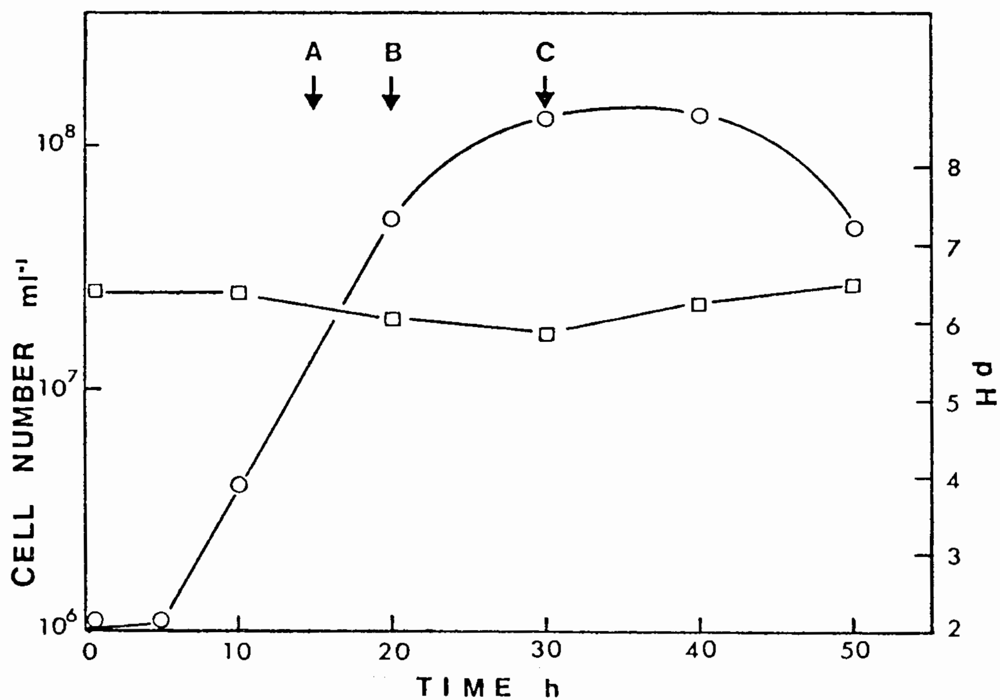


Fig. 2.7

Growth of C. acetobutylicum P262 in SFM. Total counts (○); pH (□). Times are shown for the peak of motility (A), the onset of granulose accumulation (B), and the initiation of clostridial stage formation (C).

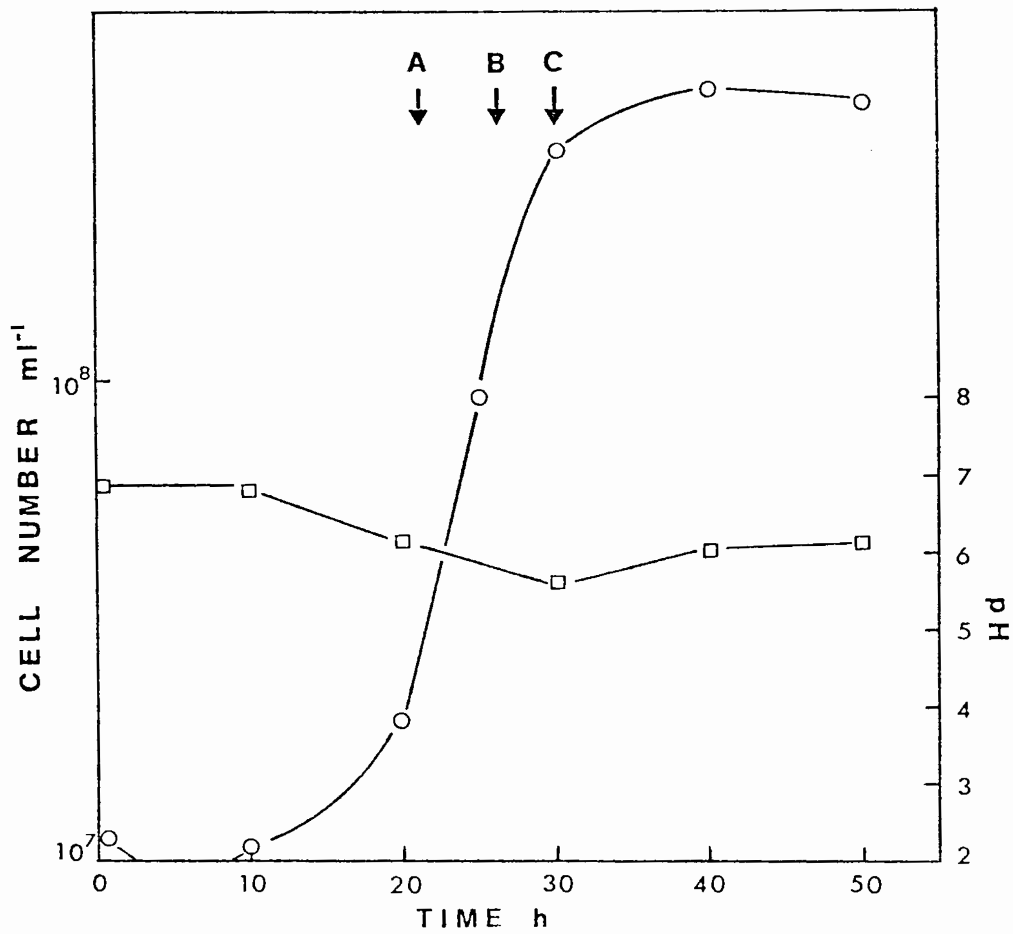


Fig. 2.8

Growth of C. acetobutylicum P262 in CAMM. Total counts (○); pH (□). Times are shown for the peak of motility (A), the onset of granulose accumulation (B), and the initation of clostridial stage formation (C).

generation time of 110 min and a pH decrease from pH 6.8 to c pH 5.6. The onset of clostridial stage formation coincided with the pH breakpoint and the cessation of cell number increase at 28 - 30 h. Clostridial stage formation reached a maximum at 44 h when 70 - 80% of the cells were swollen and phase-bright. CAMM grown cells did not show the degenerative changes which occurred in SFM. Sporulation was initiated and 60 - 80% mature spores were released into the medium. Substantial solvent production (10 - 14 g l^{-1}) was measured after 60 h in the sporulating cultures.

A number of carbohydrates supported sporulation in CAMM (Table 2.4). It was also possible to vary the nitrogen component in CAMM (Table 2.5). Diammonium hydrogen phosphate (DAP), ammonium nitrate (NH_4NO_3) and yeast extract supported 70 - 80% sporulation. Two other organic nitrogen sources (casamino acids and peptone) were less effective in the promotion of endospore formation. However, in cultures containing part organic nitrogen and part DAP, a higher proportion of sporulation occurred.

A combination of potassium phosphate and $CaCO_3$ provided the optimum buffering system for CAMM. Alternate buffering agents, sodium bicarbonate, sodium phosphate and calcium lactate, did not support growth. Potassium lactate promoted growth, but the cells did not sporulate. $CaCo_3$ could not be replaced by K_2CO_3.

The effect of individually omitting 5 salts and the 3 growth factors from the defined medium was determined (Table 2.6). The $MgSO_4$ was essential for the growth of C. acetobutylicum P262. No

TABLE 2.4

Effect of carbohydrate source on endospore formation in *C. acetobutylicum* P262 in CAMM. The number of cells which formed endospores is expressed as a percentage of the maximum cell count ($5 - 6 \times 10^8$ cells ml^{-1}).

Carbohydrate	Concentration (g l^{-1})	Endospores (%)
Glucose	30	5 - 10
	60	60 - 80
Fructose	60	80 - 90
Galactose	60	5 - 10
Sucrose	60	1 - 5
Maltose	60	1 - 5
Lactose	60	1 - 5
Raffinose	30	40 - 50
L-arabinose	30	40 - 50
D-xylose	30	40 - 50

TABLE 2.5

Effect of nitrogen source on endospore formation in *C. acetobutylicum* P262 in CAMM. The number of cells which formed endospores is expressed as a percentage of the maximum cell count ($4 - 5 \times 10^8$ cells ml^{-1}).

Source	Concentration ($g\ l^{-1}$)	Endospores (%)
DAP	6	70 - 80
NH_4NO_3	7.2	70 - 80
$(NH_4)_2SO_4$	6	10 - 25
NH_4 acetate	7.2	5 - 60
NH_4Cl	7.2	-[a]
Yeast extract	6	70 - 80
Casamino acids	6	20 - 30
Peptone	6	5 - 10
Yeast extract + DAP	4 + 2	70 - 80
Casamino acid + DAP	4 + 2	60 - 70
Peptone + DAP	4 + 2	70 - 80

[a] - no growth

TABLE 2.6

Effect of omitting individual salts and growth factors from CAMM on endospore formation in C. acetobutylicum P262. The number of cells which formed endospores is expressed as a percentage of the maximum cell count.

Component omitted	Maximum cell count ($x10^6$ ml^{-1})	Endospores (%)
None	600	70
$MgSO_4.7H_2O$	-[a]	-
$MnSO_4.4H_2O$	528	20
$FeSO_4.7H_2O$	98	< 1
$ZnSO_4.7H_2O$	156	< 1
$Na_2MoO_4.2H_2O$	568	< 1
Biotin	10	0
Paba	254	20
Thiamine	620	20

[a] - no growth

growth occurred when Mg^{2+} was limiting. Biotin markedly stimulated growth in CAMM. Growth was stimulated to a lesser extent in the presence of $FeSO_4$, $ZnSO_4$ and p-aminobenzoic acid (paba). Thiamine hydrochloride, $MnSO_4$ and Na_2MoO_4 were not required for growth, but stimulated sporulation.

Continuous agitation of CAMM resulted in an increased growth rate, but the cells lysed during the stationary phase. However, agitation by gentle mixing every 6 - 8 h was essential for the complete utilisation of the substrate and good sporulation. Intermittent mixing also prevented cell clumping and ensured a more synchronous developmental profile.

2.3.3. Sporulation of other strains in complex and defined media

Five other *Clostridium* strains were tested for their ability to grow and sporulate in the complex and defined media (Table 2.7). All strains grew well in the complex media, but the levels of endospore formation were low (0 - 30%). The 5 strains grew on GSMM but sporulation was poor or absent. CPMM was able to support the growth of only one strain (NRRL 592), which did not sporulate in this medium. The ATCC 824 and the NRRL 592 strains were able to grow in CAMM and 10 - 30% sporulation was obtained.

TABLE 2.7

Sporulation of Clostridium strains in various media.

Medium	Strain (% phase-bright spores)					
	P262	ATCC 824	ATCC 10132	NRRL 527	NRRL 592	NRRL 593
CBM	20	0	20	15	15	15
Sucrose CBM	30	0	25	30	30	25
RCM	20	0	20	5	5	5
GSMM	<1	<1	<1	0	<1	0
CPMM	0	-[a]	-	-	0	-
CAMM	70 - 80	10	-	-	20 - 30	-

[a] - no growth

2.4 DISCUSSION

Complex laboratory media such as CBM and RCM provide a suitable culture medium for many purposes. The growth rate is rapid and viable vegetative rods may be obtained within a few hours after inoculation. However, due to the relatively low sporulation frequency (20 - 30%) and the complex nature of the medium, neither system was ideally suited to sporulation studies. Initial attempts to increase sporulation frequencies in CBM by manipulating chemical and nutritional factors, met with limited success. The pH did not influence sporulation when levels were maintained above pH 5.0 during growth. Spore yields were increased 3 to 4-fold when the incubation temperature was reduced from 37 to 34°C, but no further stimulation was observed at 30°C.

Sporulation studies in chemically defined culture media were more advantageous. Comparative studies performed in 3 defined systems (GSMM, CPMM, SFM) gave an indication of the nutritional and physiologial conditions which were required for the growth and sporulation of C. acetobutylicum P262.

In B. subtilis, the incidence of spore formation was shown to be a function of the growth rate, although media composition was important (Dawes and Mandelstam, 1970). Cultures of C. acetobutylicum P262 grew more slowly in the defined media (doubling times of 110 - 240 min) as compared with growth in the complex media (doubling times of c 30 min). The highest degree of sporulation was not associated with the longest generation time and additional factors were required for spore induction.

Anaerobic bacteria are strongly inhibited by the end-products of substrate metabolism (Leung, 1980; Herrero, 1983). In GSMM, the pH of the medium dropped rapidly and reached a minimum value when the culture density was still low. Maximum acid production was correlated with the inhibition of growth. There was a relationship between autolysin production under low pH conditions and the degenerative changes typical of GSMM cultures (Reysenbach, 1982). This observation suggests that it is important that the pH of the culture medium is prevented from falling to unfavourably low levels during growth.

The development of low pH conditions in GSMM was prevented when ammonium acetate was used as a source of nitrogen. Acetate appeared to enhance the buffering capacity of the medium and the pH breakpoint was delayed until the end of the active growth phase. Clostridial stage formation coincided with the pH breakpoint and the onset of the stationary growth phase. Spore formation was induced, but the developmental stages were not sufficiently synchronous for correlative sporulation studies. The modified GSMM proved to be a suitable culture medium for the isolation of auxotrophic mutants for genetic studies (unpublished results).

A suitable pH level (above pH 5.0) was maintained during the growth of C. acetobutylicum P262 in CPMM and the SFM. Clostridial stage degeneration in SFM was associated with the production of relatively high concentrations of fermentation end-products (8 - 10 g l^{-1} butanol) and resulted in the

inhibition of endospore formation. The toxic effects of butanol production on clostridial stage cells has been demonstrated by van der Westhuizen (1982). During the ABE process, the production of up to 14 g l^{-1} butanol appeared to be responsible for clostridial stage degeneration and the lack of mature spore formation in the molasses fermentation medium (Robson and Jones, 1982; van der Westhuizen, 1982).

The clostridial forms produced in CPMM did not have the characteristic swollen appearance which is predominant in industrial ABE fermentations producing high levels of solvent (Spivey, 1978). Butanol production (< 3 g l^{-1}) did not reach toxic levels in CPMM. Therfore, some factor other than solvent toxicity prevented sporulation from occurring in this system.

The chemically defined sporulation medium (CAMM) developed for C. acetobutylicum P262, routinely supported 60 - 80% mature spore formation. The elevated spore yield appeared to be a consequence of the improved buffering capacity of the CAMM and the production of levels of solvents (8 - 10 g l^{-1} butanol) which, in this medium, were not toxic to the cell. Occasionally, high solvent levels (16 - 17 g l^{-1} total solvent; 12 g l^{-1} butanol) were obtained and sporulation was reduced (20 - 40% mature spores).

Substitutions made to the carbon and nitrogen components did not severely affect the frequency of spore formation in CAMM. However, when the buffering components were altered, no

sporulation occurred. A requirement for stringent pH conditions during sporulation has been reported in other acid-producing clostridia (Bowen and Smith, 1955; Labbe and Duncan, 1974; Hickey and Johnson, 1981).

There was a stringent requirement for all the metal ions and growth factors present in CAMM. No attempt was made to determine whether these compounds were present in concentrations which were optimum for sporulation.

The major disadvanagtes of the CAMM were the protracted growth and development stages, the need for a large cell inoculum to initiate growth, and the presence of a $CaCO_3$ precipitate which hindered optical density measurements and restricted the isolation of particle-free cell samples. It was possible to remove most of the precipitate by acid treatment with dilute H_2SO_4.

The variatons observed when other Clostridium strains were grown in CAMM, emphasises the importance of strain differences and supports a recent statement by Gottschalk et al. (1981) that the taxonomy of the genus Clostridium is still in an unsatisfactory state. The taxonomy of the butyric acid clostridia is based largely on their fermentation end-products (McCoy et al., 1930; Holdeman et al., 1977). Studies in modern taxonomy have begun to rely more on cell wall sugar composition, DNA homology, nutritional requirements (Cummins and Johnson, 1971) and rRNA homologies (Johnson and Francis, 1975) as a more accurate means of grouping strains.

The CAMM has been specifically developed for C. acetobutylicum P262. Although perfect synchrony of the sporulation process was not obtained, the time sequence of the major structural changes was sufficiently distinct and constant to enable the system to be used in subsequent correlative studies. The defined medium enabled a detailed investigation of the factors which trigger endospore formation in the P262 strain.

CHAPTER III

CHARACTERISATION OF GROWTH, SPORULATION AND OTHER STATIONARY PHASE EVENTS IN LIQUID MEDIA

Summary

A study of the relationship between the morphological changes and the growth dynamics of C. acetobutylicum P262 in CBM and CAMM suggested that the clostridial stage is involved in the production of acetone, butanol and ethanol. Clostridial stage formation and sporulation were linked under the conditions tested. However, three other C. acetobutylicum strains did not show this association.

Electron microscopy revealed a sporulation sequence with distinct differences from those of other endospore-forming bacteria. An extracellular enzyme with proteolytic activity was produced in low levels during growth and sporulation in the defined system, but was not detected in CBM. Sporulation-specific protease activity was not observed.

3.1. INTRODUCTION

Light and electron microscope studies of sporulating cells have revealed a sequence of well-defined morphological events in Bacillus (Murrell, 1967; Kay and Warren, 1968).

The recognised sporulation stages are:

I preseptation or axial chromatin formation

II forespore septation

III protoplast envelopment

IV cortex formation

V coat formation

VI maturation

VII free spore release

Five of these sporulation stages can be distinguished by microscopic examination using phase- and Nomanski interference-contrast optics (Hitchins et al., 1968): septation, engulfment, phase-dark forespore (corresponding to cortex formation), phase-bright forespore (corresponding to coat formation) and the free spore.

Similar morphological stages have been observed in clostridia (Fitz-James, 1962; Mackey an Morris, 1971; Eller and Ordal, 1972; Johnstone and Holland, 1977).

An understanding of the anatomy of spore development has provided a framework for physiological studies. However, it is necessary that the sporulation process be synchronised in order to correlate functional and morphological changes. The development of resupension techniques (Sterlini and Mandelstam, 1969), techniques employing heavy culture inocula of vegetative cells (Halvorsen, 1957; Zoha and Sadoff, 1958; Day and

Costilow, 1964a), and the repeated filtration and transfer of cultures to fresh media (Hoffman et al., 1978) have been used to bring cell division and sporulation into synchrony which is sufficient for correlative studies.

The physiology of sporulation in the anaerobic bacteria has received far less attention. Hsu and Ordal (1969a) were able to separate the process of sporulation from that of active cell turnover in C. thermosaccharolyticum cultures grown in continuous culture with glucose as the limiting substrate. Differences in glucose metabolism under various physiological conditions which induced or repressed sporulation were studied (Hsu and Ordal, 1970). An altered fermentation profile from acid to alcohol production was first observed as the cells began to elongate and form sporangia. The induction of sporulation and the concomitant metabolic shift was associated with the increased activity of three dehydrogenases and the accumulation of lipid within the cell.

The physiological changes associated with solvent production by C. acetobutylicum in the ABE process have been documented (Petersen and Fred, 1932; Davies and Stephenson, 1941; Walton and Martin, 1979). The production of solvents was correlated with a change in the cellular morphology and the appearance of swollen clostridial forms in C. acetobutylicum P262 (Jones et al., 1982). During the fermentation of molasses, sporulation proceeds slowly. The factory process is usually stopped before endospore formation is complete. However, in fermentations which

are allowed to continue, clostridial forms containing forespore septa do not usually develop further, but degenerate in the presence of the high levels of solvents (van der Westhuizen, 1982).

The accumulation of cellular reserve material is another physiological change associated with the stationary growth phase and the sporulation process in a number of bacilli and clostridia (Slepecky and Law, 1961; Hsu and Ordal, 1970; Mackey and Morris, 1971; Bergère et al., 1975). The generic name, granulose, has been suggested for the α-polyglucans found in the saccharolytic clostridia (Darvill et al., 1977). The presence of granulose is revealed by treatment with a solution of iodine in potassium iodide which stains polyglucoses a dark blue to brick red colour.

Granulose production has been studied extensively in C. pasteurianum (Mackey and Morris, 1974b; Robson et al., 1974; Robson and Morris, 1974; Darvill et al., 1977). Very little granulose was synthesised during vegetative growth in a glucose-rich minimal medium (Robson et al., 1974). Reserves were deposited at the onset of sporulation. A mutant which accumulated only half the amount of granulose laid down by the wild-type strain, formed forespores, but failed to sporulate (Mackey and Morris, 1974b). When the rate of glucose supply was reduced to a level which sustained sporulation of the parent strain without concurrent granulose biosynthesis, the mutant sporulated well. It was suggested that granulose synthesis in C.

pasteurianum may serve to moderate the accumulation of a potentially harmful metabolite, including sporulation repressor(s), in the presence of excess glucose and ATP (Mackey and Morris, 1974b). Under starvation conditions, granulose may be mobilised to serve as an endogenous supply of carbon and energy (Robson and Morris, 1974).

In *C. acetobutylicum* P262, granulose accumulation is associated with the swollen phase-bright clostridial stage (Jones *et al*., 1982).

The *C. acetobutylicum* minimal medium (CAMM) has provided a defined system in which the development of the solventogenic phase and sporulation in *C. acetobutylicum* P262 occur concurrently. This medium was utilised to determine the relationship between the physiological events which occur during the fermentation, the pattern of cell growth, and the morphological changes associated with the conversion from actively growing rods to the sporulation stage. Growth and sporulation dynamics in *C. acetobutylicum* P262 were also monitored in a complex medium (CBM) and compared with five other *Clostridium* strains. Preliminary studies were performed to determine whether protease production could be used as a marker event to indicate the onset of sporulation in *C. acetobutylicum* P262.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial strains

The *Clostridium* strains used were described in 2.2.1.

3.2.2. Culture media

All culture media are listed in Appendix A.

3.2.3. Inoculation and growth

The CBM and CAMM media were inoculated as specified previously (see 2.2.3). Growth was measured by total and viable cell counts as outlined in Chapter II.

3.2.4. Physical measurements

The determination of acid end-products, solvents and pH was described in 2.2.5.

Residual glucose levels in culture filtrates were monitored using a Beckman Glucose Analyser 2.

3.2.5. Methods for characterisation of cell morphology

3.2.5.1. Light Microscopy

Determinations using the light microscope were described in 2.2.6. In addition, the Gram reaction of the bacterial cell wall was determined by the method of Cruickshank _et al._, (1969). Percentage motility was estimated by differential count of motile and non-motile cells in a given microscopic field of a wet slide mount.

3.2.5.2. Electron Miroscopy

The method of Kellenberger _et al._, (1958) was used to fix cell samples (_c_ 5 x 10^8 cells). The fixed cells were dehydrated by passage through a series of acetone solutions of increasing concentration. The organisms were embedded in NC1010 Spurr-low-viscosity embedding material (Polaron Equipment Ltd., Hertfordshire, England). Sections were cut with a glass knife on an ultramicrotome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined in a Zeiss 109 electron microscope at 80 kV.

EYE.6. Estimation of Protease Activity

3.2.6.1. Liquid protease assays

Culture supernatants (1 ml) and cell extracts (_c_ 10^9 cells) were assayed for protease activity by the following methods:

1. Azocasein (Sigma) at a concentration of 1% in 0.1 M phosphate buffer pH 7. Samples (1 ml) were added to 2 ml of the azocasein solution. After 30 min incubation at 37°C,

the reaction was stopped by the addition of 2 ml of 10% trichloroacetic acid. The mixture was filtered and 2 ml of 0.4 M NaOH was added to 2 ml filtrate. Absorbance was measured at 440 nm against a water blank after 5 min.

2. Azocoll (Calbiochem) at 1% concentration in 0.1 M phosphate buffer pH 7 was assayed as above. Absorbance was recorded at 520 nm.

3. The liquid casein assay was that of Rippon (1968). A 0.6% casein (BDH) solution in 0.1 M phosphate buffer pH 7 was assayed as described for the synthetic substrates. Absorbance was measured at 280 nm.

4. Gelatin assay. A 0.5% gelatin solution (1 ml) in 0.1 M phosphate buffer pH 7 was incubated at 37°C with 1 ml of the enzyme sample. After 1 h, the unhydrolysed gelatin was precipitated with 2 ml of 15% $HgCl_2$ (acidified) and allowed to stand at room temperature for 5 min. The absorbance of the assay mixture was measured at 600 nm. Controls were boiled culture supernatants. The activity of the culture samples were recorded in arbitrary units (AU)

$$AU = 1 - \frac{\text{Sample OD}}{\text{Control OD}}$$

3.2.6.2. Qualitative well plate assay

Wells (10 mm diameter) were punched out of 10 ml agar plates (1% w/v) containing 0.5% gelatin. Supernatant samples (25 ul) were added to the separate wells. The plates were incubated for 4 h at $37^{o}C$ and then flooded with a 15% $HgCl_2$ solution (acidified). The plates were examined for zones of clearance around the wells.

3.2.7. Protease inhibitor studies

EDTA (Merck); PMSF (Sigma) and pCMB (Sigma) were used.

The experimental data presented in this chapter are results typical of each experiment. However, experiments were repeated at least three times.

3.3. RESULTS

3.3.1. Solvent production and sporulation in CAMM

3.3.1.1. Growth and physiological changes

The growth and physiological changes associated with glucose fermentation were monitored in CAMM (Fig. 3.1a and 3.1b). The total cell counts used to measure growth represent a combination of vegetative cells, sporangia (at various stages of development) and free refractile spores. Viable counts, pH, granulose production and fermentation end-products were also determined.

Exponential phase cells (OD 0.4 - 0.5) in CBM were diluted 10^2-fold to give an initial cell concentration of approximately 10^6 cells ml^{-1} in CAMM. A lag period of 8 - 10 h occurred initially. During this period, the cell concentration fell a further 10^2-fold as a result of cell lysis.

During the exponential phase (10 - 25 h), the doubling time of the culture was about 100 min. Acid production occurred between 10 and 30 h. The accumulation of c 5.5 g l^{-1} acetate and butyrate was associated with a pH decrease from pH 6.5 to pH 5.5 and the consumption of about 12 g l^{-1} glucose. Granulose biosynthesis, determined by iodine-staining, commenced as the doubling time began to increase (between 25 and 30 h). After 30 h, there was no further increase in the total cell number (c 5×10^8 cells ml^{-1}) and solvents were detected in the medium. The pH

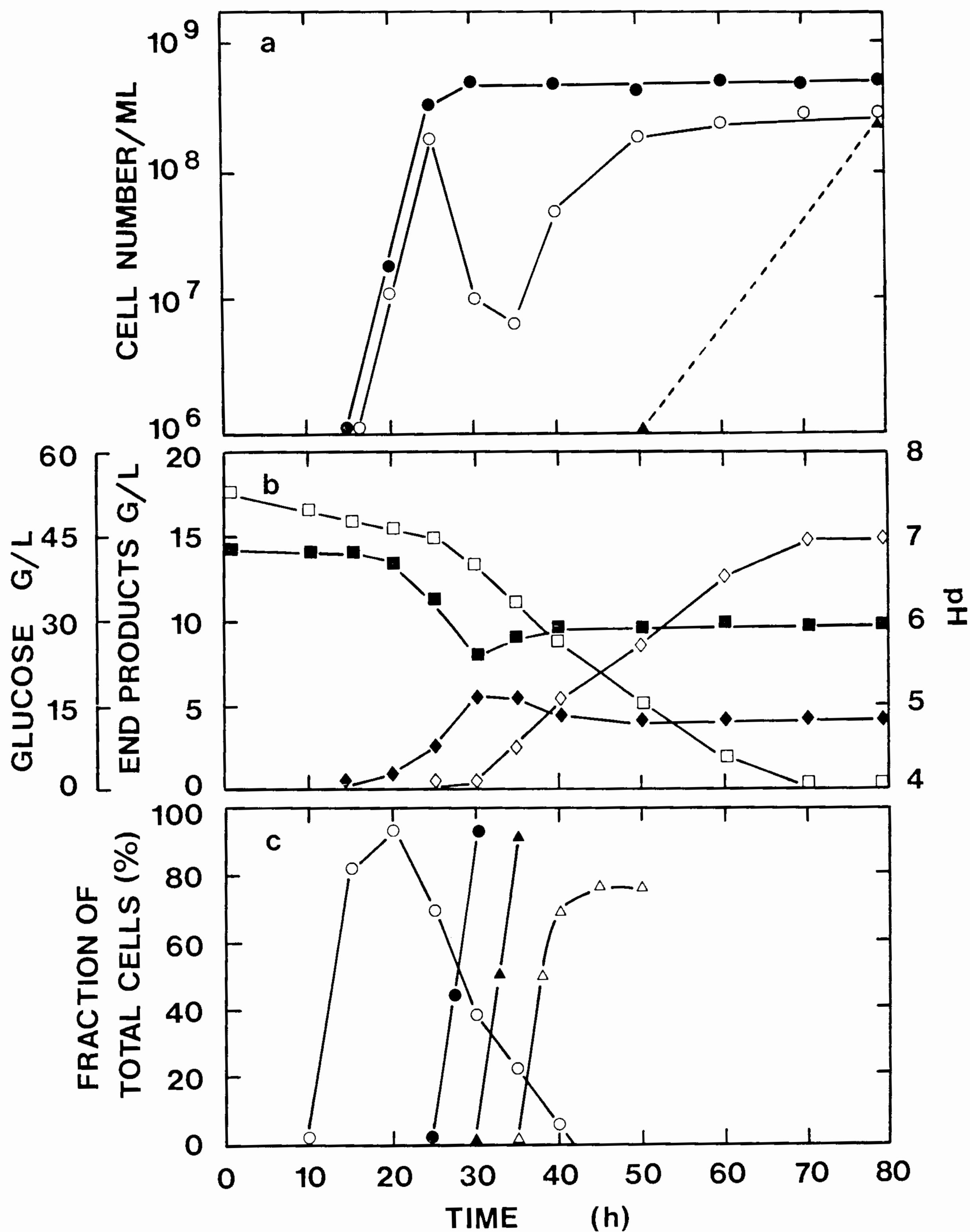


Figure 3.1

Growth (a), physiological (b) and morphological (c) changes in C. acetobutylicum P262 grown in CAMM.

(a) (●), total cell count; (○), colony forming units; and (▲), mature endospores.

(b) (□), residual glucose; (■), pH; (◆), total acids; and (◇), total solvents.

(c) (○), motility; (●), granulose; (▲), clostridial forms; and (△), forespore septa.

increased to pH 6.0 during the solventogenic phase (30 - 80 h). Following the initation of solvent production, the acid level remained constant. However, a decrease or small increase in the total acid concentration was sometimes observed after the pH breakpoint. Ten to fifteen grams per litre solvent were produced as the remaining 38 g l^{-1} substrate was utilised. The final solvent ratio (after 80 h) was c 7:2:< 1; n-butanol (9.780 g l^{-1}): acetone (2.745 g l^{-1}): ethanol (0.423 g l^{-1}).

Viable counts, which coincided with the total bacterial counts during the first 25 h, decreased during the period 25 to 35 h. The colony forming units increased concomitantly with the initiation of sporulation at 35 h and plateaued after 50 h.

3.3.1.2. Morphological and cytological changes

The time course of the morphological and cytological changes observed in CAMM grown cultures is represented in Fig 3.1c and Plates 3.1 and 3.2.

Initially, the *C. acetobutylicum* P262 cells appeared as elongated, sluggishly motile rods which developed division septa and formed chains of up to 6 cells per chain. Approximately 12 h after inoculation, the chains began to separate and release individual rods which were highly motile. The vegetative cells stained strongly Gram-positive and were phase-dark (Plate 3.1 A). Cell proliferation continued during the motile period (12 to 25 h) and the growth rate was at a maximum. Termination of active

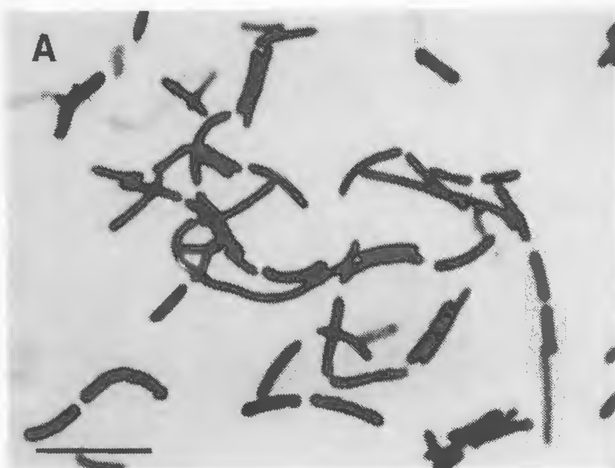
growth between 25 and 30 h was correlated with a decrease in motility. At 30 h, residual motility was observed in less than half the population.

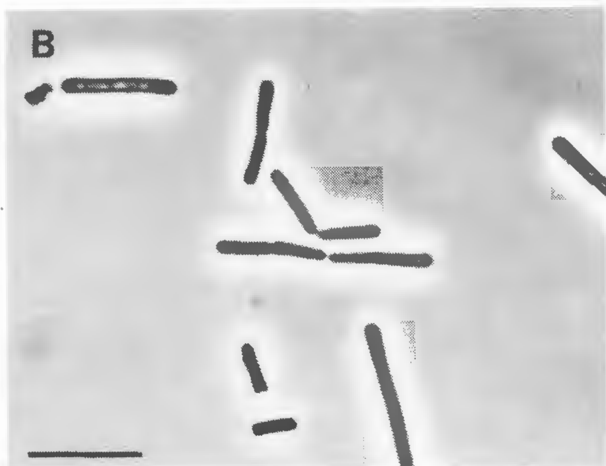
Granulose deposition within the cell was first detected c 2 h before the pH breakpoint. Incipient accumulation occurred as discrete storage granules which stained a reddish-brown with iodine. As the cells synthesised more granulose (between 30 and 40 h), the cytoplasm began to stain a uniform brown colour. The cells were phase-grey with a granular appearance during the early stage of granulose biosynthesis (Plate 3.1 B). By 35 - 40 h, the majority of cells (> 80%) had formed the typical swollen, phase-bright clostridial forms, which were Gram-positive (Plate 3.1 C). A small proportion of the culture (< 20%) which did not enter the clostridial stage, underwent degenerative changes and became Gram-negative.

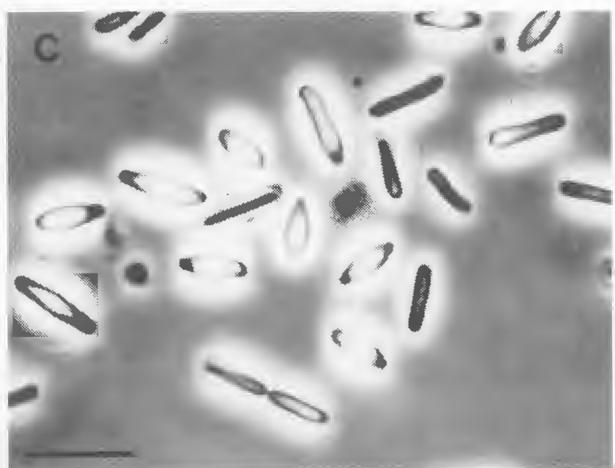
The excretion of a voluminous capsule consisting largely of polysaccharide monomers (unpublished data) embedded the clostridial forms (Plate 3.1 D).

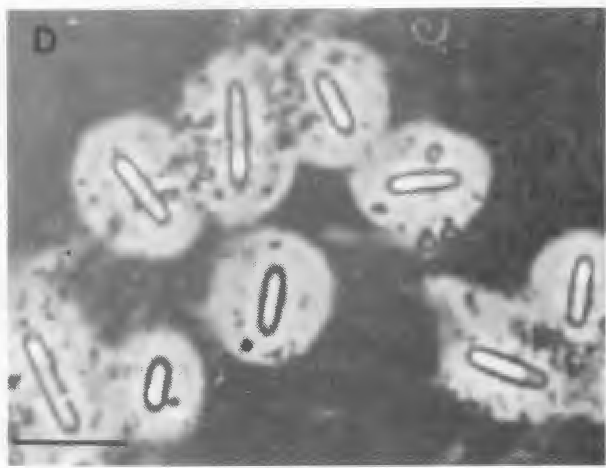
The formation of phase-dark forespores at 35 h was the first sign of incipient sporulation (Plate 3.1 E). Early forespore formation was also apparent in clostridial forms stained with $KMnO_4$ and safranin (Smith and Ellner, 1957).

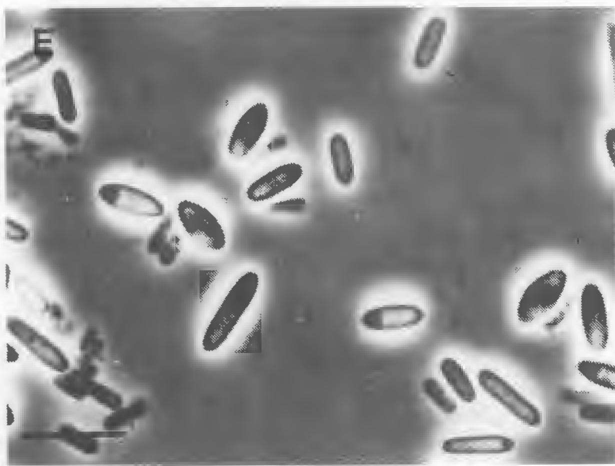
Between 44 and 50 h, the forespores became phase-bright and by 50 h, more than 80% of the clostridial forms had refractile

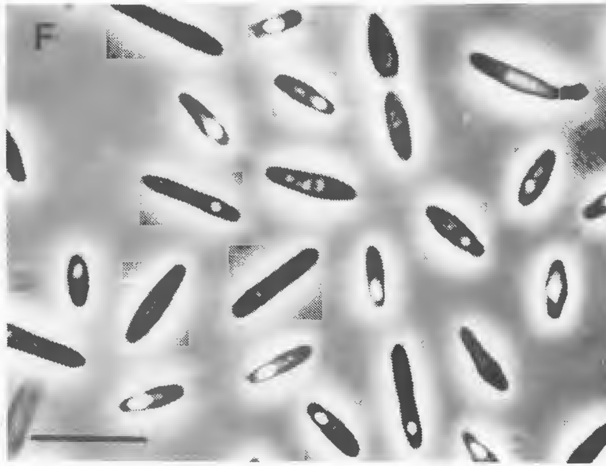


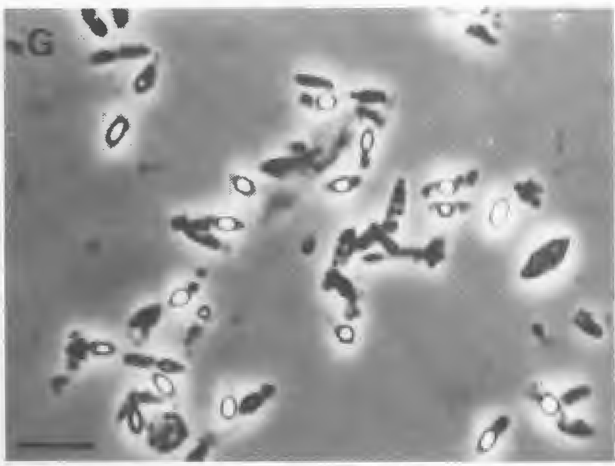












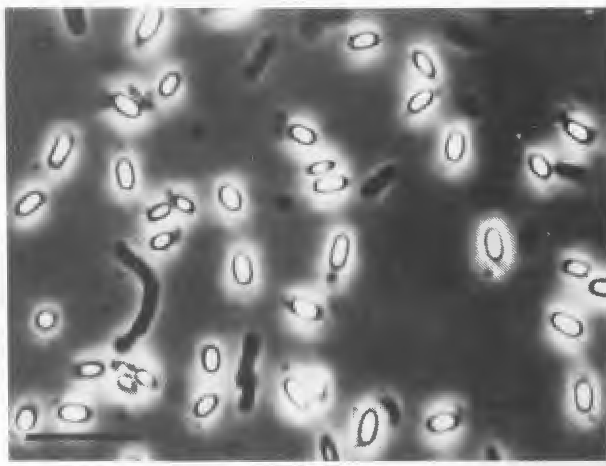


PLATE 3.1

PLATE 3.1 Photomicrographs of cytological changes in *C. acetobutylicum* P262 during growth in CAMM.

(A) Actively growing vegetative rods, stained Gram-positive (12 to 25 h)

(B) Phase-grey rods showing an early stage of granulose accumulation (30 h)

(C) Typical swollen phase-bright clostridial forms (35 h)

(D) Negatively-stained clostridial forms showing voluminous capsule deposition (35 h)

(E) Clostridial forms with phase-dark forespores (40 h)

(F) Clostridial forms with phase-bright forespores (45 h)

(G) Maturation spore release and degeneration of the mother culture (60 h)

(H) Released cell-free spores (80 h)

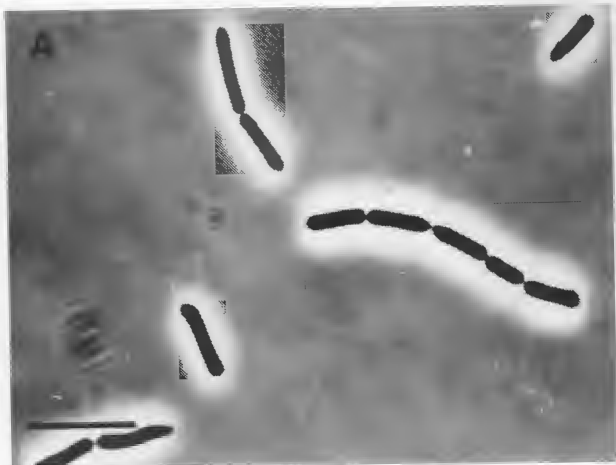
Bars: 10 um

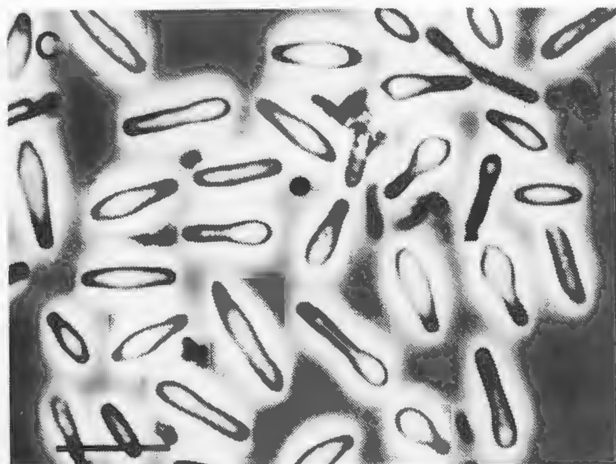
terminal ends (Plate 3.1 F). At this stage, the cell cytoplasm became less dense and the population predominantly more Gram-negative. Autolysis and spore release occurred after 50 h (Plate 3.1 G). About 3 - 4 x 10^8 spores ml^{-1} were obtained after the completion of mother cell lysis (60 - 80 h) (Plate 3.1 H). Bipolar sporulation at a low incidence (1 - 5%) was found in all CAMM cultures. Cells with multiple spores were usually elongated.

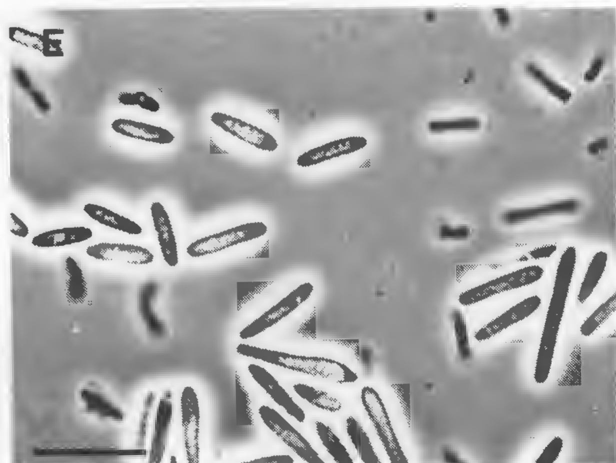
3.3.1.3. Comparison of sporulation by phase- an interference-contrast microscopy

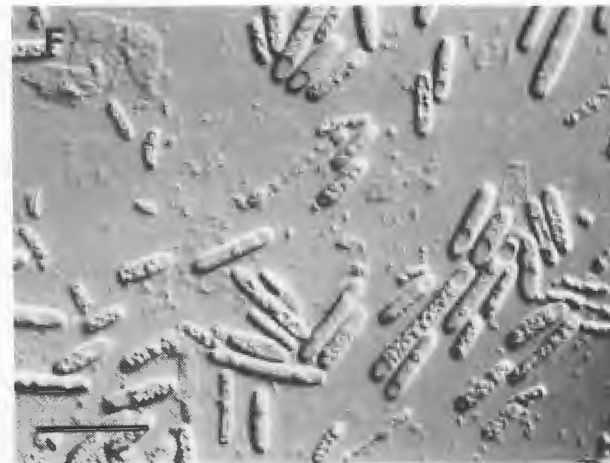
The gross morphological changes which occur when cells of the same age were viewed by phase- and interference-contrast optics are depicted in Plate 3.2.

The Nomanski system provided more cell surface detail, but otherwise had little advantage over the phase system. The vegetative rods had a smooth appearance which was retained during the presporulation stage (30 - 35 h) (Plates 3.2 A and B). The accumulation of phase-bright storage granules was not easily discernable by interference-contrast, although cell swelling was apparent (Plates 3.2 C and D). Degenerating cells which were phase-dark, had a rough appearance under interference-contrast optics. Phase-dark forespore formation was manifested as a terminal indentation of the sporulating cell viewed by interference-contrast microscopy (Plates 3.2 E and F). This was the earliest sporulation stage visualised by both optical









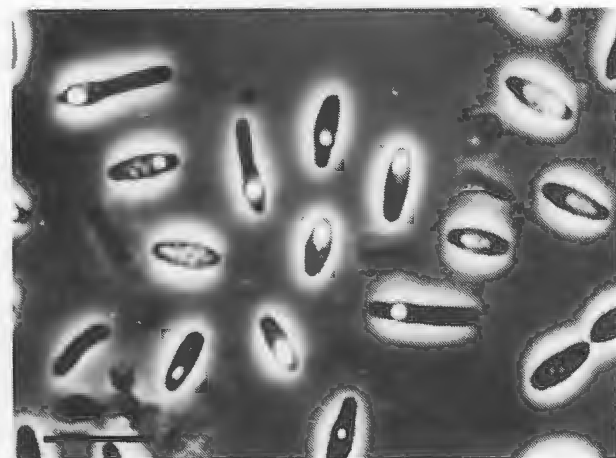


PLATE 3.2

PLATE 3.2 Comparison of sporulation in CAMM as revealed by phase-contrast and interference-contrast microscopy.

(A, C, E, G) Cells viewed with phase-contrast optics

(B, D, F, H) Cells viewed with interference-contrast optics

(A, B) Vegetative rods

(C, D) Swollen clostridial forms

(E, F) Forespore formation showing the terminal indentation of the sporulating cell viewed by interference-contrast optics.
The rough appearance of the degenerating mother cell is apparent

(G, H) Refractile spores showing the shadowing effect of the developing spore viewed by interference-contrast optics

Bars: 10 um

systems. The development of refractility as seen with phase-contrast, was paralleled by an enhancement of the shadowing or three-dimensional effect of the spore with interference-contrast optics (Plate 3.2 G and H). During the period of spore maturation and release (after 50 h), the clostridial forms became phase-grey and had a granular, ill-defined outline under Nomanski optics.

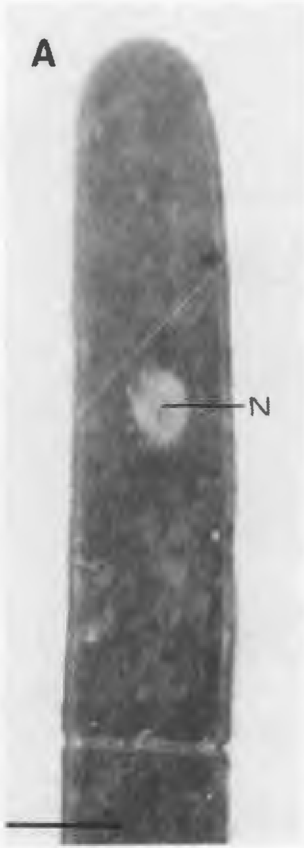
3.3.1.4. Ultrastructural changes

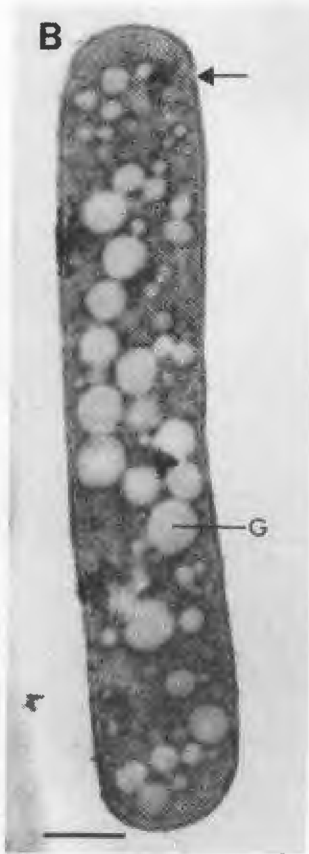
Electron microscope examination of ultra-thin sections of cells harvested from CAMM at regular intervals revealed the intricate detail of the morphological changes which occurred during spore formation. The sequence of events will be discussed by reference to the seven developmental stages used to describe sporulation in other endospore-forming bacteria (Murrell, 1967).

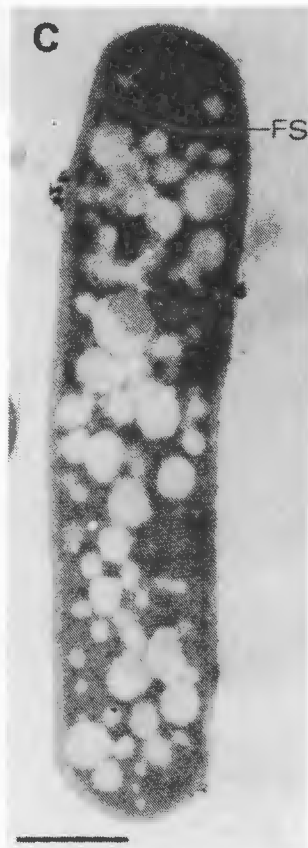
Stage 0: Vegetative cells approaching the stationary growth phase contained discrete replicating chromosomes (Plate 3.3 A).

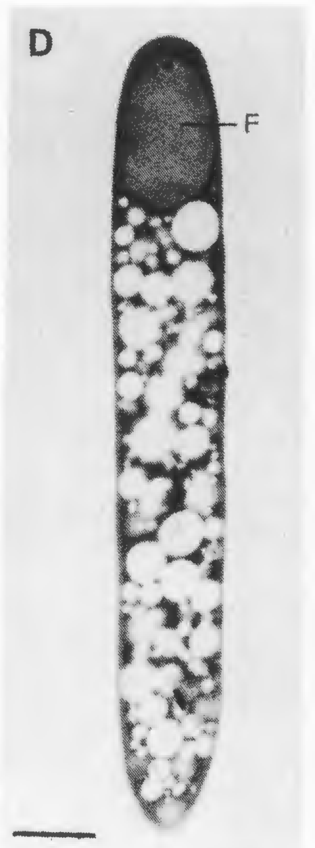
Stage I: The storage granules containing granulose (Plate 3.3 B) which accumulate at this stage, made the detection of axial filament formation difficult. However, no fibrous chromatin body was observed before septation.

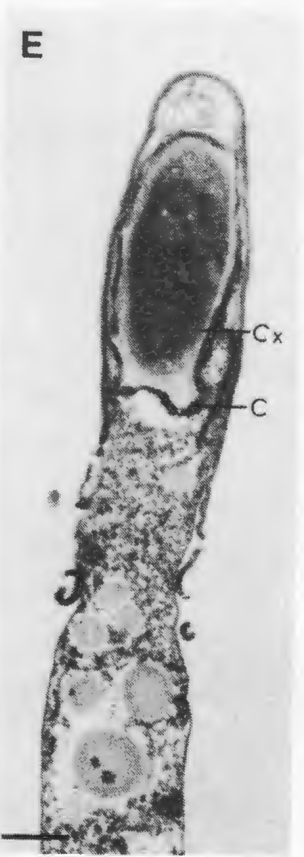
Stage II: The development of the forespore septum was the first indication of sporulation in *C. acetobutylicum* P262. Invagination of the mother cell membrane occurred close to one

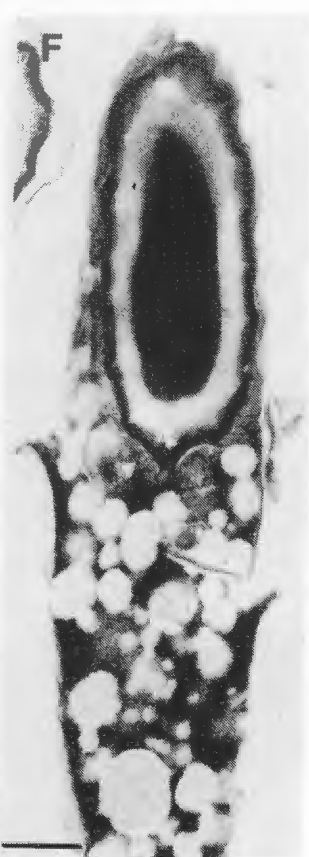












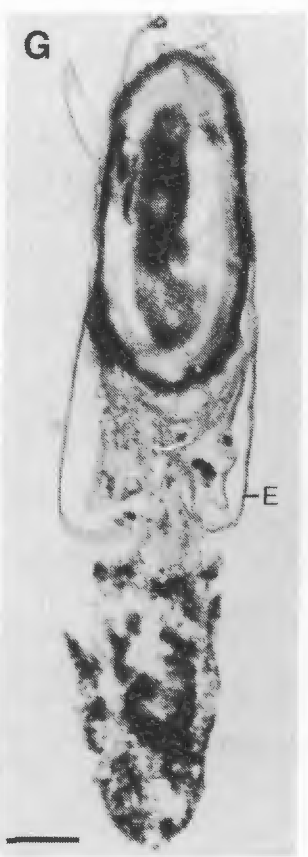


PLATE 3.3

PLATE 3.3 Electron micrographs of C. acetobutylicum P262 showing the stages of spore development in CAMM. Abbreviations: N, nuclear material; G, granulose; FS, forespore septum; F, forespore; C, spore coat; Cx, cortex; S, spore; E, exosporium.

(A) Vegetative rod

(B, C) Granulose accumulation and the formation of the spore septum by invagination and inward growth of the septum (arrowed)

(D) Completion of engulfment of forespore

(E) Spore coat and cortex formation

(F) Spore maturation

(G, H) Mature spore with its triangular exosporium.

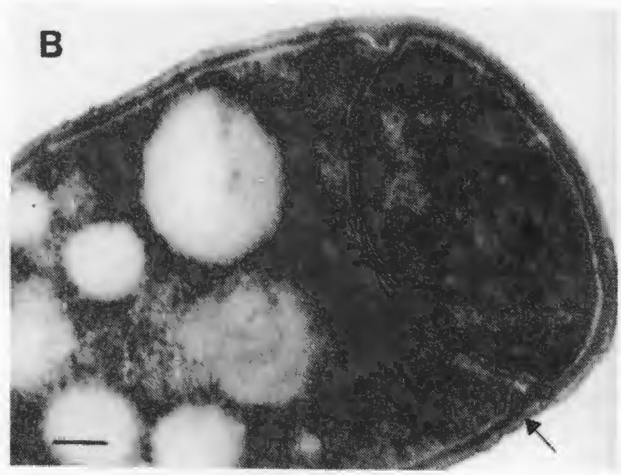
Bars: 0.5 um.

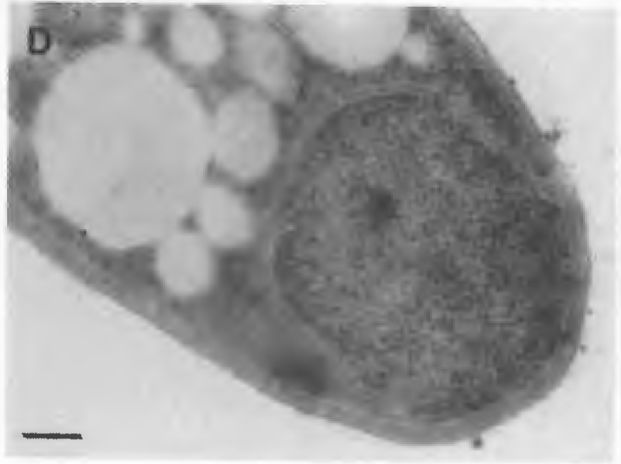
pole of the cell (Plate 3.3 B). Forespore septum formation was completed following central fusion of the ingrowing membranes (Plate 3.3 C). Entrapped nuclear material and granulose storage granules were sometimes visible within the forespore compartment.

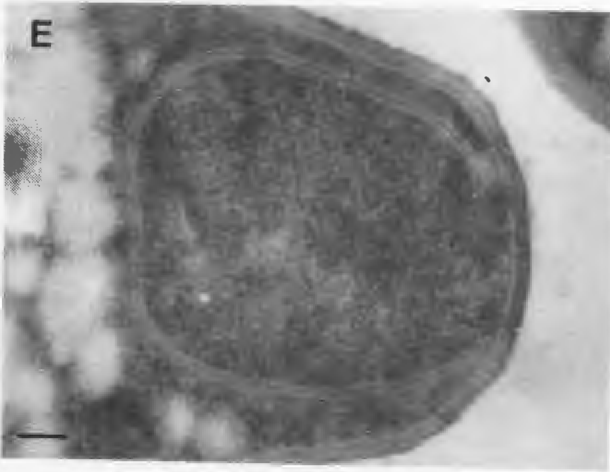
Stage III: The progressive engulfment of the forespore was accomplished through the localised extension of the mother cell membrane between the periphery of the forespore and the cell wall (Plate 3.4 A-H). A small indentation of plasma membrane on the mother cell side of the spore septum was often detected at the start of engulfment (Plate 3.4 B). Mesosome-like structures which may be associated with the developing membranes were sometimes observed during stages II and III (Plate 3.4 G and H).

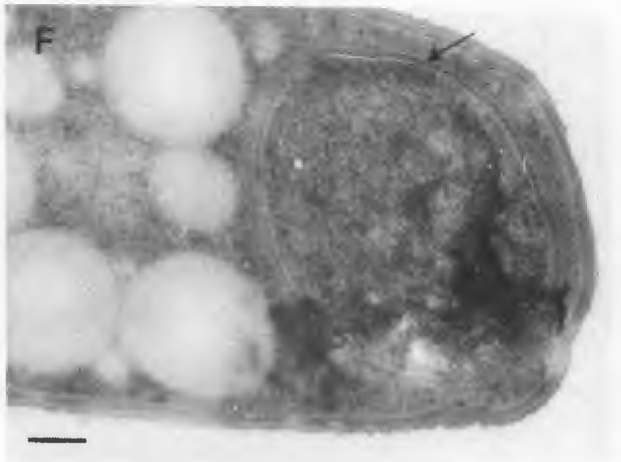
The fully engulfed spore protoplast, embedded within the mother cell cytoplasm (Plate 3.3 D), was enclosed by a distinct inner and outer forespore membrane (Plate 3.4 F). The occluded granulose observed during the earlier developmental stage, did not persist through engulfment.

Stages IV and V: Spore coat material was detected before the onset of cortex formation in *C. acetobutylicum* P262 (Plate 3.5 A-F). The coat was deposited as discrete electron-dense fragments periphral to the double forespore membrane (Plate 3.5 A, B). As more spore coat material was produced, the individual pieces coalesced to yield a continuous, undifferentiated structure which enveloped the spore core (Plate 3.5 C). An electron lucent cytoplasmic zone, as described by Murrell (1967),









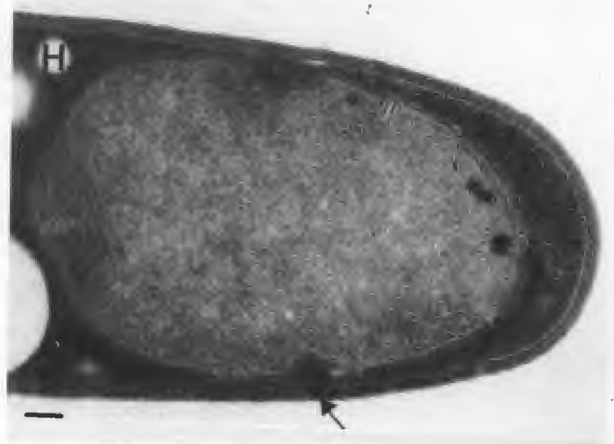


PLATE 3.4

PLATE 3.4 Electron micrographs of *C. acetobutylicum* P262 showing the progressive engulfment of the forespore during stage III of development.

(A) The completion of spore septum formation

(B) Onset of engulfment (arrowed)

(C, D, E) Progressive engulfment of the forespore

(F) Fully engulfed spore protoplast enclosed by an inner and outer forespore membrane (arrowed)

(G, H) Engulfed forespore showing mesosome-like structures often seen in association with the developing spore membranes (arrowed).

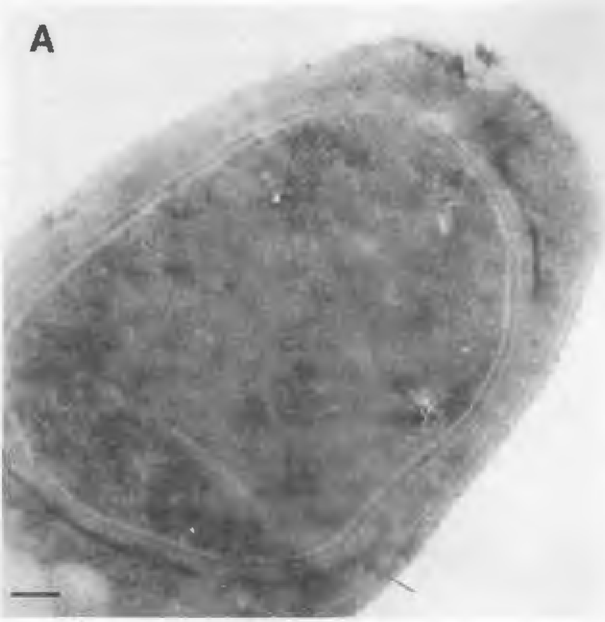
Bars: 0.1 um.

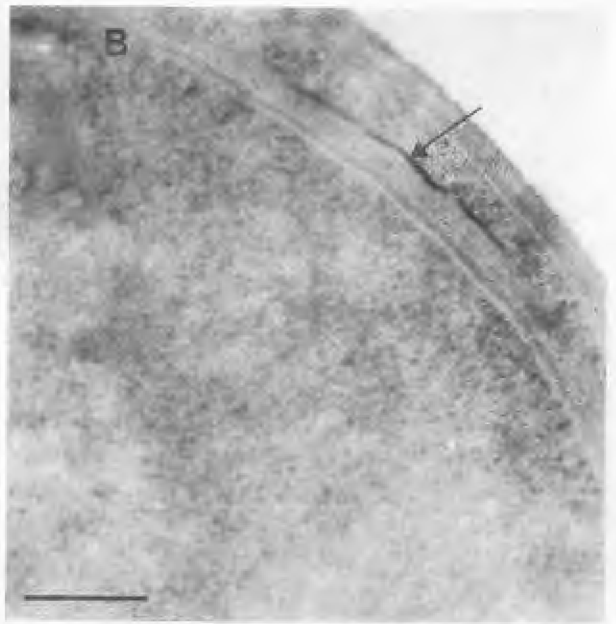
was incorporated between the growing coat fragments and the outer membrane of the forespore. This "space" accommodated cortex formation.

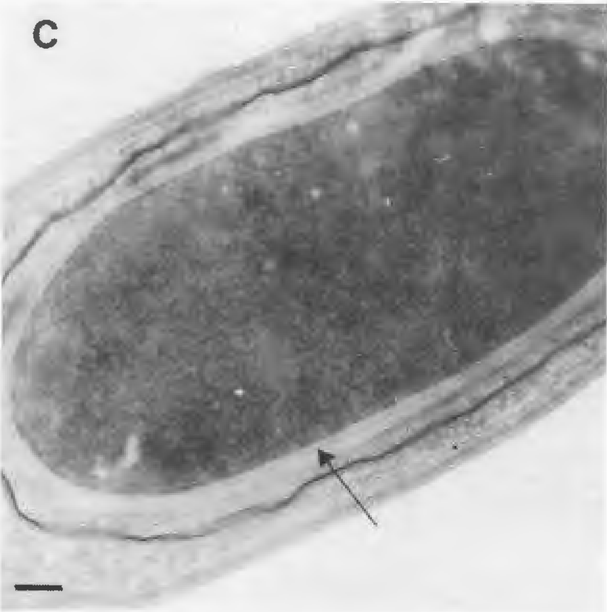
The electron micrographs (Plate 3.5 D and E) depicting coat and cortex development represent an abortive sporulation in which the spore has been released well before maturity (stage VI). These abortive spores are useful for illustrating the spore structure during stages IV and V of sporulation.

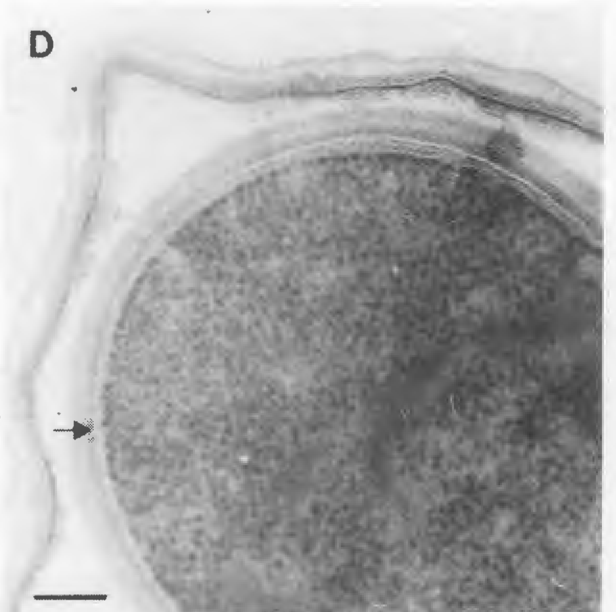
Cortical material was deposited between the inner and outer forespore membranes (Plate 3.5 C). The developing cortex was observed to encroach into the area surrounding the spore core (Plate 3.5 D and E). The spore membranes were difficult to visualise. However, the inner forespore membrane appeared to divide off following the initiation of cortex formation to give the double membrane structure shown in Plte 3.5 D. The exosporium, which was not visible in the intact sporulating cell (see Plate 3.3), appeared to form during stage V and could be distinguished as a loose outer integument covering the premature free spore (Plate 3.5 E).

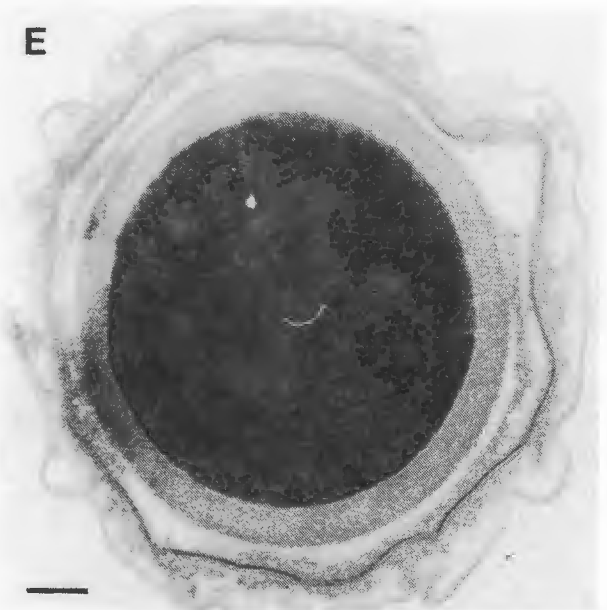
Stage VI: The penultimate stage of spore development is shown in Plate 3.5 F. The fully expanded, electron-transparent cortex is juxtapose to the coat edge. The spore coat is more distinctive. The inner and outer layers are apparent and have assumed the typical corrugated appearance of the mature spore.











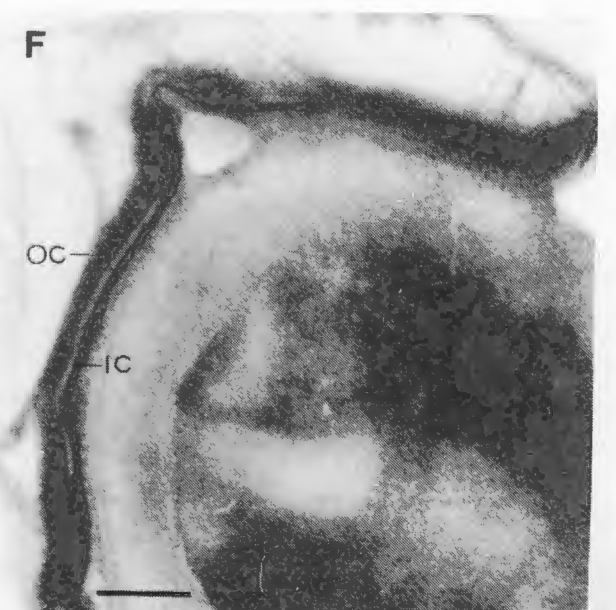


PLATE 3.5

PLATE 3.5 Electron micrographs of C. acetobutylicum P262 showing spore coat and cortex formation during stages IV and V of sporulation.

(A, B) Deposition of electron-dense coat fragment (arrowed) periferal to the double forespore membrane

(C) Completion of coat formation and the start of cortex deposition between the inner and outer membranes of the forespore (arrowed)

(D) Details of cortex development into the electron lucent area surrounding the spore core. The inner forespore membrane appears to have divided off to give a double membrane structure (arrowed)

(E) The completion of exosporium formation (arrowed) during stages IV and V of sporulation

(F) Details of spore maturation showing the fully expanded cortical zone and the inner (IC) and outer (O C) layers of the spore coat.

Bars: 0.1 um.

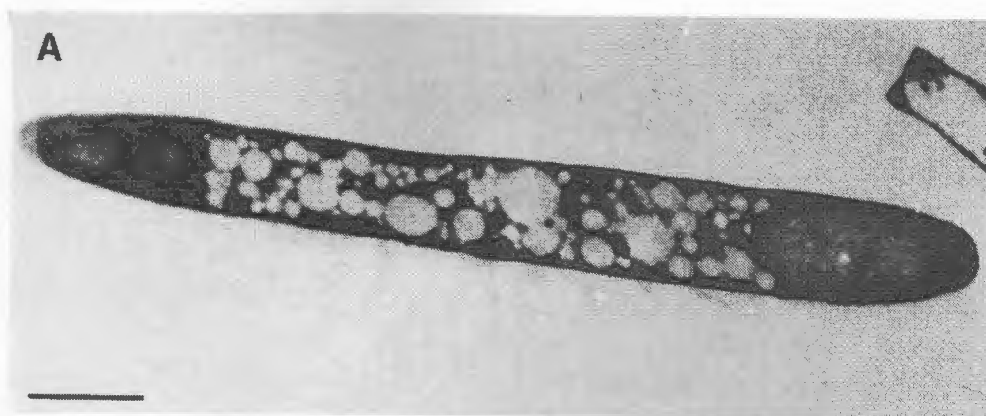


PLATE 3.6 Electron micrographs of C. acetobutylicum P262 showing dipolar sporulation.

(A) Completion of engulfment

(B) Spore maturation

Bars: 0.5 um.

Stage VII: Autolysis of the sporagium with the liberation of the mature spore completed sporulation in *C. acetobutylicum* P262. The exosporium which fitted loosely around the freed spore was triangular in shape and open at one end (Plate 3.3 G and H).

Bipolar sporulation followed the same sequence of events. The various morphological stages occurred in unison at each pole (Plate 3.6).

3.3.2. Solvent production and sporulation in CBM

The growth and developmental changes observed in CAMM were monitored in CBM. The time scale of events were more concise in the complex system. Spore maturity was attained in one third of the time taken to reach the same stage in the defined medium.

3.3.2.1. Growth and physiological changes

The growth and physiological changes which occurred in CBM are illustrated in Fig 3.2a and 3.2b. Growth was measured by total and viable cell counts.

The short lag period (2 h) following the inoculation of CBM was not accompanied by cell lysis, which was a feature of the defined system. The initial cell concentration was *c* 10^6 cells ml^{-1}. During the exponential phase (2 to 6 h), the doubling time was *c* 30 min, three-fold faster than the growth rate in CAMM. The pH fell rapidly during this period. A pH decrease from pH

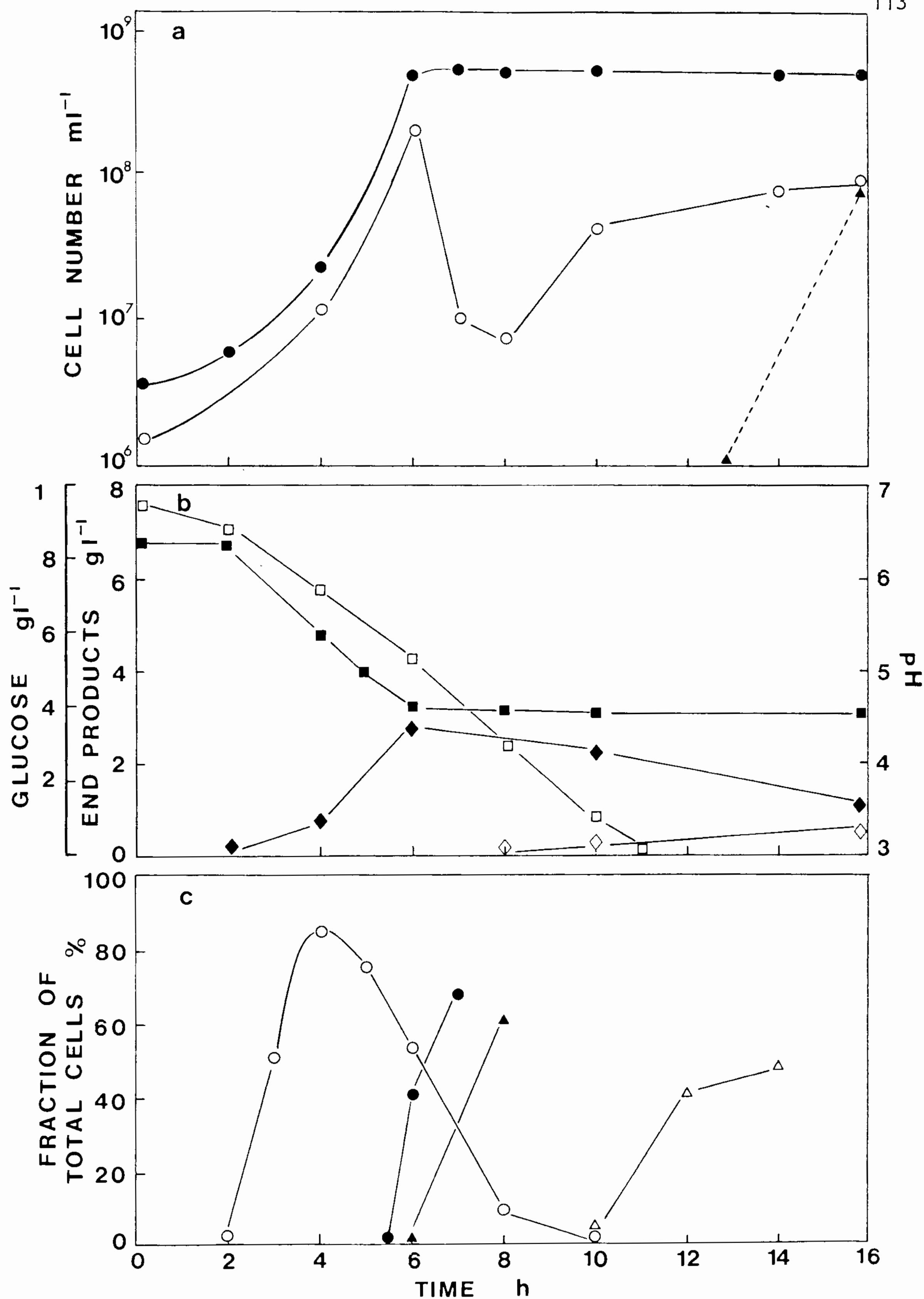


Figure 3.2

Growth (a), physiological (b) and morphological (c) changes in C. acetobutylicum P262 grown in CBM.

(a) (●), total cell count; (○), colony forming units; and (▲), mature endospores.

(b) (□), residual glucose; (■), pH; (◆), total acids; and (◇), total solvents.

(c) (○), motility; (●), granulose; (▲), clostridial forms; and (△), forespore septa.

6.5 to pH 4.5 was associated with the utilisation of 5 g l^{-1} glucose and the production of 3 g l^{-1} acetate and butyrate. The viable count followed the total count during this period.

Granulose accumulation at 5.5 h was associated with a decrease in colony forming units. Cell viability in CBM followed a pattern similar to that observed in CAMM. The number of viable cells increased after 8 h and stabilised at 12 h.

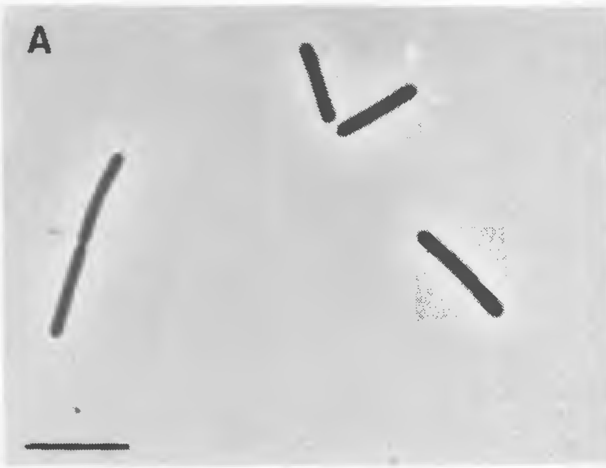
Very low levels of solvents (< 1 g l^{-1}) were detected during the stationary growth phase (after 6 h). A small pH recovery to pH 4.6 and the consumption of the remaining 5 g l^{-1} glucose occurred between 6 and 10 h.

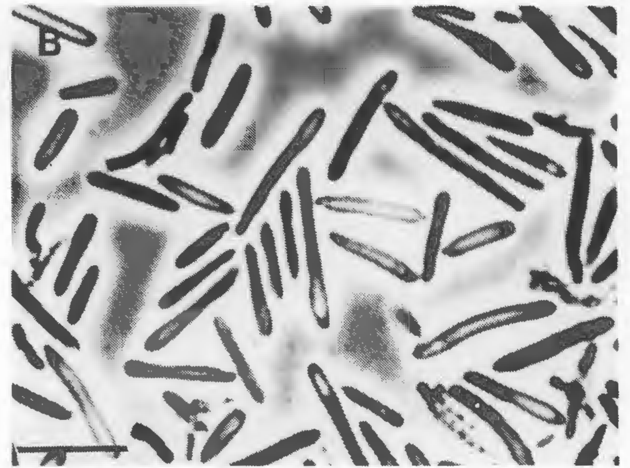
3.3.2.2. Morphological and cytological changes

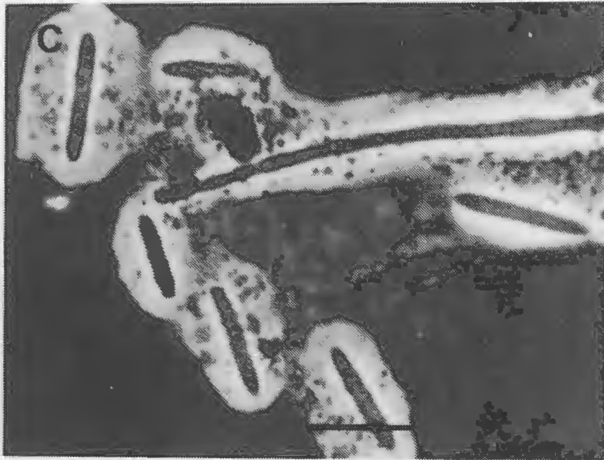
The morphological changes observed immediately following the inoculation of CBM and during the vegetative stage, were very similar to those which occurred in CAMM (Fig. 3.2c, Plate 3.7).

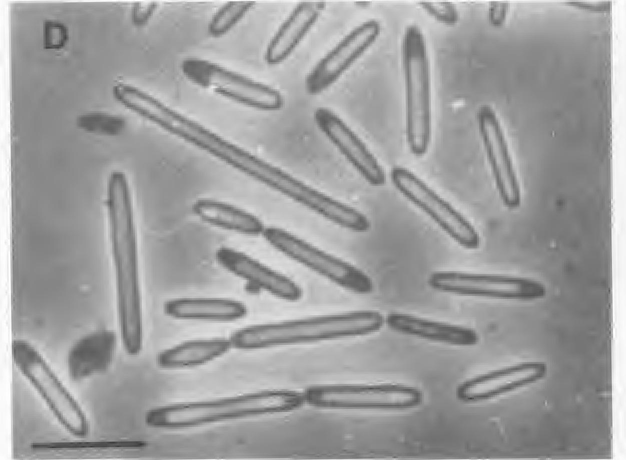
Generally, the CBM grown cells were larger than the vegetative rods isolated from CAMM (Plate 3.7 A). The motile period extended from 2 to 8 h. Approximately 60% of the rods entering the stationary phase at 6 h formed clostridial forms which were Gram-positive. The remaining 40% of the population underwent degenerative changes and became Gram-negative. The clostridial stage cells were longer and thinner than the cigar-shaped clostridial forms typical of CAMM cultures (Plate 3.7 B). Capsule formation was associated with the clostridial stage in the CBM system (Plate 3.7 C).

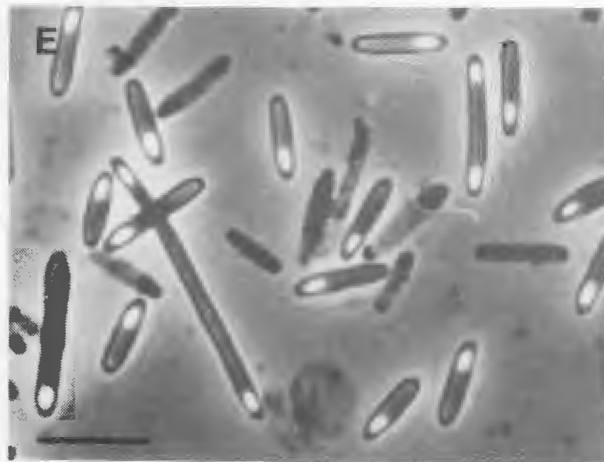
Phase-dark forespore formation occurred between 10 and 12 h (Plate 3.7 D), but only 20 - 30% (c 1 x 10^8 cells ml^{-1}) of the stage II/III cells formed mature spores after 16 h (Plate 3.7 F). The remaining clostridial forms became phase-dark. After 10 h, the population was predominantly (70 - 80%) Gram-negative. A significant proportion of the sporulating cells (8 - 12%) formed double spores (Plate 3.7 E).











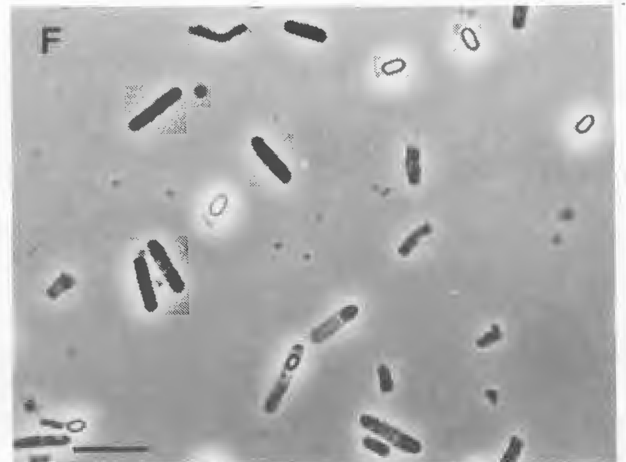


PLATE 3.7: Photomicrographs of cytological changes in C. acetobutylicum during growth in CBM.

A Actively growing phase-dark vegetative rods (2 - 8 h)
B Elongated, thin, clostridial stage cells (8 h)
C Negatively-stained clostridial forms showing voluminous capsule deposition (8 h)
D Clostridial forms with phase-dark forespores (12 h)
E Clostridial forms with phase-bright forespores, and bipolar sporulating cells (16 h)
F Released spores and degenerating cells (24 h)

Bars: 10 um.

3.3.3. Morphological and cytological changes in other clostridia

The five *Clostridium* strains which were used for comparative studies in the previous chapter, were examined for the morphological changes which characterised sporulation in *C. acetobutylicum* P262 (Table 3.1).

Sucrose CBM agar containing 40 g l^{-1} sucrose was used in these studies. The solid medium stimulated granulose accumulation, capsule formation and sporulation in the P262 strain.

All five strains were motile during the vegetative growth period (*c* 15 h incubation) *C. acetobutylicum* P262 and the two *C. butyricum* strains, NRRL 592 and NRRL 593, responded similarly to the various assays. Clostridial stage formation preceeded sporulation, capsule production occurred and the cells stained positively for the presence of granulose. Granulose production occurred in *C. acetobutylicum* NRRL 527, but accumulation was limited and the cells did not stain a uniform brown colour. *C. acetobutylicum* ATCC 824 and ATCC 10132 failed to synthesise granulose under the specific culture conditions used in these studies. The three *C. acetobutylicum* strains did not form the clostridial stage and capsule production was not observed.

TABLE 3.1

Morphological characteristics of *Clostridium* strains grown on 4% sucrose CBM agar.

Characteristic[a]	STRAIN					
	P262	ATCC 824	ATCC 10132	NRRL 527	NRRL 592	NRRL 593
Motility	++	++	++	++	++	++
Clostridial forms	++	-	-	-	++	++
Granulose	++	-	-	+	++	++
Capsule	++	-	-	-	++	++
Sporulation	++	+	++	++	++	++

[a] ++ Level of morphological feature comparable to that in strain P262

\+ Reduced level of morphological feature

\- Morphological feature not observed

3.3.4. Protease Production

Crude cell extracts and culture supernatants from vegetative and sporulating *C. acetobutylicum* P262 cells had insufficient proteinase activity for detection by azocasein, azocol and liquid casein assay procedures. However, cultures grown anaerobically in CAMM and containing vegetative or sporulating organisms, produced extracellular gelatin-hydrolysing activity when assayed by a well plate method.

A liquid assay, which was more quantitative, was used to assay gelatinase activity in CAMM grown cultures (Fig. 3.3). Cell-free supernatant samples (1 ml) harvested during the exponential and ensuing sporulation stages were assayed for proteolytic activity on the gelatin substrate (0.5 mg ml^{-1}; pH 7.0). Protease activity was detected throughout growth and development, but the levels were very low and incubation times of up to 1 h were required for the hydrolysis of the substrate by enzyme samples. A small peak in activity was observed at 30 h and corresponded to the onset of the stationary growth phase.

The exoenzyme activity was reduced by EDTA (10 - 20 mM) and pCMB (0.5 - 1 mM), but was not significantly affected by 5 - 10 mM PMSF (Table 3.2). This observation suggests the presence of more than one protease: a metalloprotease inhibited by the metal-chelating agent (EDTA); and a thioprotease sensitive to the sulfhydryl reagent, pCMB (Morihara, 1974).

No gelatin hydrolysing activity was detected in CBM broth cultures.

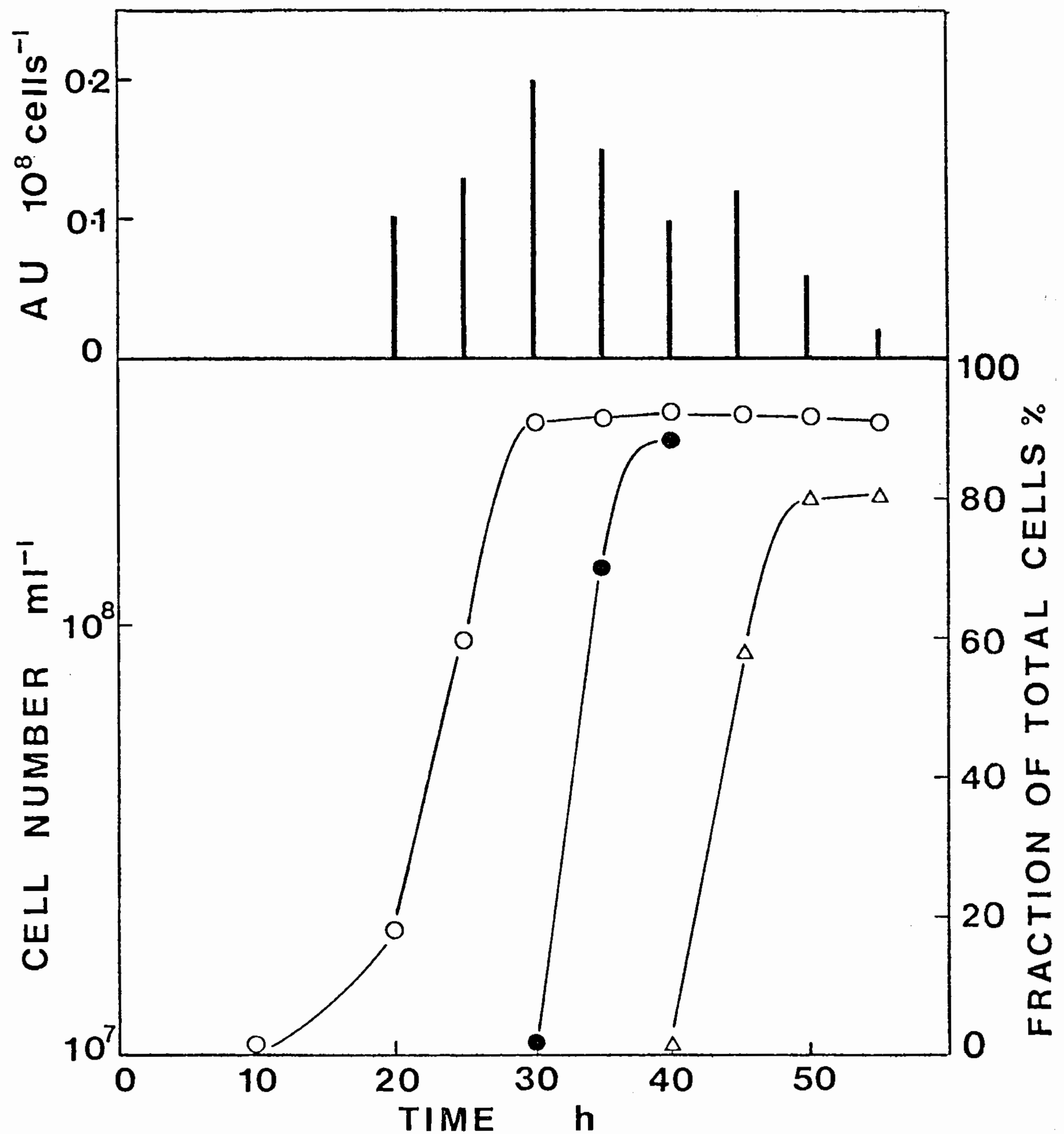


Figure 3.3

Extracellular protease activity of C. acetobutylicum P262 during growth and sporulation in CAMM. Total cell counts (○); clostridial stage formation (●); forespore production (△). The histogram summarises the protease activity in supernatant culture samples.

TABLE 3.2

Effect of protease inhibitors on gelatin hydrolysing activity in CAMM. Activities are expressed as a percentage of the uninhibited control.

Inhibitor	Concentration mM	Activity %
Control		100
pCMB	1	13
	0.5	27
PMSF	10	89
	5	96
EDTA	20	16
	10	36

3.4. DISCUSSION

A complex and a defined culture system were used to determine the relationship between the physiological and morphological events which constitute growth and sporulation in *C. acetobutylicum* P262.

The physiological changes monitored during the production of solvents from glucose correlated well with the physiologial events associated with the industrial fermentation of molasses (Spivey, 1978). The two well defined physiological phases (the acidogenic phase and solventogenic phase) which occur during the ABE fermentation were identified in the laboratory systems.

The acidogenic phase was associated with the exponential growth phase, the accumulation of short chain fatty acids, and a decrease in the pH of the medium. The results obtained confirmed those of Davies and Stephenson (1941) and O'Brien and Morris (1971), who reported that acetate and butyrate, but not acetone and butanol, were produced by *C. acetobutylicum* during exponential growth.

The shift to the solventogenic phase was associated with the inhibition of cell division in *C. acetobutylicum* P262. A pH recovery during the second phase of the fermentation coincided with the production of acetone, butanol and ethanol. The decrease in titratable acidity which occurred during the production of solvents from molasses (Spivey, 1978; Jones *et*

al., 1982) could not be correlated with the variable levels of acetate and butyrate often observed during solvent production in CAMM. It is generally accepted that acetate and butyrate are converted to acetone and butanol during the normal ABE fermentation (Prescott and Dunn, 1959). However, the results obtained in the defined laboratory fermentation medium (CAMM) suggest that the metabolism of the remaining glucose is largely responsible for solvent production after the pH breakpoint. The direct fermentation of substrate to solvents has been reported in other studies involving C. acetobutylicum (Häggström and Molin, 1980; Gottschalk and Bahl, 1981). Elucidation of the fermentation pathways and the fate of acid end-products during the fermentation requires a better understanding of the biochemistry of solvent production. This will be achieved through the use of radioactive labelling techniques and purified enzyme systems.

A third fermentation phase, which is less distinct, is associated with the levelling off of solvent production and a stabilisation of the pH increase in the ABE fermentation (Spivey, 1978). In CAMM and CBM, the final fermentation phase was associated either with the exhaustion of glucose or inhibition of further consumption. The cessation of metabolic activity occurred as the final stages of endospore development were being completed.

The shift in fermentation end-product formation during glucose metabolism by C. acetobutylicum P26 was related to the

morphological changes in the cell population. Comparative studies revealed a correlation between the development of the clostridial stage (and the associated granulose and capsule deposition) and the production of acetone and butanol. This idea was supported by the fact that good clostridial stage formation is associated with high solvent production in the ABE fermentation system (Spivey, 1978). The distinctive morphological and cytological stages of C. acetobutylicum are routinely used to monitor the progress of the industrial fermentation and as an aid in identifying the cause of failed fermentations (Spivey, 1978; Robson and Jones, 1982).

The clostridial stage cells which had an intact Gram-positive cell wall, were associated with forespore formation in C. acetobutylicum P262. Sporulation was never observed in the absence of clostridial stage formation and solvent production always accompanied spore development under the culture conditions tested. The majority of sporulating cells (c 80%) in CAMM reached maturity, however, only 30 - 50% of the phase-dark forespores formed in CBM grown cells developed full refractility. These clostridial stage cells were not typically swollen and may contain less granulose. In this respect, the CBM cells resembled the abortive sporulation mutant of Mackey and Morris (1974b), which accumulate reduced levels of granulose.

The sporulation requirements of C. acetobutylicum P262 are complex. Results obtained from the study aimed at characterising the fermentation process and endospore formation in CAMM and CBM

have suggested that pH and acetate and butyrate concentration are important for the cessation of active cell growth and the successful initiation of the solventogenic phase. In addition, an adequate supply of nutrients appears to be necessary for the full development of the second stage of the fermentation and the completion of endospore formation.

Spore formation in the C. butyricum strains NRRL 592 and NRRL 593 was accompanied by granulose production and the presporulation clostridial stage. Both strains produce solvents (Compere and Griffith, 1979). However, solventogenesis, clostridial stage formation and sporulation do not appear to be linked in other Clostridium strains. The distinctive clostridial stage was not observed during solvent production and sporulation by C. acetobutylicum strains ATCC 824, ATCC 10132 and NRRL 527. Granulose deposition and sporulation could be separated under certain conditions in C. pasteurianum (Mackey and Morris, 1974b). Some butyric acid clostridia (eg. C. pasteurianum and C. butyricum) which form endospores do not produce substantial levels of solvents; and H_2, Co_2, acetate and butyrate are the major fermentation end-products (Wood, 1961; Jungerman et al., 1973; Gottschalk et al., 1981). The circumstances under which solvent production, clostridial stage formation and sporulation might become dissociated in C. acetobutylicum P262 have not been identified.

The utilisation of phase-contrast and Nomanski interference-contrast microscopy allowed delineation of the gross

morphological changes wich occurred during sporulation in _C. acetobutylicum_ P262. Higher resolution obtainable with the electron microscope revealed more detail of the ultrastructural changes which constitute spore formation. Several ditinctive features were observed:

1. Axial filament formation did not appear to be a necessary prelude to sporulation in _C. acetobutylicum_ P262. This would support the notion that filamentation is a non-specific response of DNA to the adverse culture conditions which support sporulation in other endospore-forming bacteria (Fitz-James and Young, 1969; Young and Mandelstam, 1979).

2. The initiation of spore coat formation occurred before the onset of spore cortex formation. _Stages IV and V_ of the classical sporulation sequence were also reversed in _C. pasteurianum_ (Mackey and Morris, 1971) and the sporulation process in these two butyric acid clostridia appear to be very similar.

3. The coat was not deposited as continuous layers as in _C. bifermentans_ (Johnstone and Holland, 1977), but rather by the linking of preformed material, as observed in _C. pasteurianum_ (Mackey and Morris, 1971) and _Clostridium pectinovorum_ (Fitz-James, 1962).

4. The spore coat consisted of a clearly defined inner and outer layer. Single layer, laminated and rigid spore coats have been observed in other Bacillus and Clostridium species (Murrell, 1967; Mackey and Morris, 1971).

5. The spore coat surface bore no appendages.

6. The exosporium did not have the multilamellar structure characteristic of other costridia (Santo et al., 1969; Mackey and Morris, 1971).

7. Bipolar septation is considered as an abnormal phenomenon which occurs in "abortively disporic" mutants of B. subtilis (Young and Mandelstam, 1979). However, cells with fully refractile bipolar endospores were routinely observed in sporulating C. acetobutylicum P262 cultures. Bipolar sporulation was also observed in a putrefactive anaerobe (Santo et al., 1969) and Clostridium oceanicum (Smith, 1970). The symmetry and synchrony in the spore development process which occurred at each end of the sporulating cell would suggest a control directed, at least initially, from a common control point.

Residual proteolytic activity specific for a gelatin substrate was observed in C. acetobutylicum P262 cultures. The very low levels of activity may explain the reason why Holdeman et al., (1977) have classified C. acetobutylicum as a species not capable of gelatin liquifaction. Limited proteolytic activity

has been detected in other butyric acid clostridia (Uchino *et al.*, 1968; Egorov *et al.*, 1972; Mackey and Morris, 1974a). Extracellular protease production was not suitable for use as a sporulation marker in *C. acetobutylicum* P262.

CHAPTER IV

ISOLATION AND CHARACTERISATION OF SPORULATION MUTANTS

Summary

Sporulation mutants resistant to antibiotics directed against RNA polymerase and ribosomes, and mutants unable to synthesise granulose were isolated. Eight of these mutants were used in studies to illucidate the relationship between solvent production and the morphological changes which occur in *C. acetobutylicum* P262. Sporulation mutants which were unable to form the clostridial stage (*cls* mutants) did not produce solvents. Oligosporogenous mutants which showed reduced clostridial stage formation produced intermediate levels of solvents. Sporulation mutants blocked after the clostridial stage, which were unable to form mature spores (*spo* mutants), produced normal levels of solvents. These results support the hypothesis that the clostridial stage is the solvent producing stage in *C. acetobutylicum* P262.

4.1. INTRODUCTION

Considerable knowledge about the process of sporulation in bacteria has been obtained by examining the morphological and biochemical properties of mutants containing genetic lesions which affect or inhibit the formation of endospores.

Studies utilising sporulation mutants have been particularly useful in distinguishing between specific and non-specific sporulation requirements, and the factors involved in the induction and regulation of the differentiation process. B. subtilis mutants in particular, have been used to map spo loci and to determine the way in which the sporulation genome is expressed.

The isolation of sporulation mutants in members of the Bacillus group has proved to be relatively easy. Colony pigmentation on enriched solid media is characteristic of many strains of sporulating B. subtilis cells. Loss of pigmentation in clones of asporogenous mutants has provided a convenient method for the direct selection of sporulation-defective mutants. In species which do not produce a pigment, the colonial morphology of asporogenous mutants is sufficiently distinct from that of the wild-type strain to enable direct mutant selection (Schaeffer, 1969; Avrova, 1979).

A number of mutants that are drug-resistant and concomitantly altered in their sporulation pattern have been isolated. The majority carry mutations which alter either RNA polymerase or the ribosomes. In some cases, the complex phenotype is a result of a single mutation (Doi et al., 1970; Sonenshein et al., 1974). The advantage of drug-resistant mutations over other types of sporulation mutations is that the primary genetic lesion can be identified. The precise manner in which sporulation is affected is more difficult to define. However, the mutation may convey a better understanding of the process.

Rifampicin (rif), streptolydigin (std) and streptovaricin (stv) are known to affect RNA polymerase (Wehrli and Staehelin, 1971). Haworth and Brown (1973) mapped the loci for the RNA polymerase complex by means of mutants resistant to rif, std or stv. All three mutations were closely linked to the cys A14 marker. The gene that sustained rif mutations was identified as that coding for the β subunit of RNA polymerase (Linn et al., 1975). Mutants resistant to antibiotics directed against RNA polymerase are often defective in endospore formation (Leighton, 1973; Sonenshein et al., 1974; Sumida-Yasumoto and Doi, 1977). However, a number of mutants resistant to rif, std or stv and which contain an altered RNA polymerase, sporulate normally (Sonenshein et al., 1974).

Further evidence has been presented to suggest that results obtained from mutant studies should be interpreted with caution.

The inability of a rifampicin-resistant mutant to sporulate was corrected by the addition of amino acids to the sporulation medium (Pun et al., 1975). This would suggest that the asporogeny which resulted from an alteration in the RNA polymerase was an indirect consequence of the mutation (eg. inefficient transcriptional activity for amino acid biosynthetic genes), and was not caused by an altered specificity towards the sporulation genes.

Bhattacharya and Sarkar (1981) have provided evidence to suggest that RNA polymerase is involved in DNA chain elongation in B. brevis. DNA replication was inhibited by streptolydigin, rifampicin and lipiarmycin, all of which specifically inhibited the bacterial RNA polymerase. DNA synthesis was not inhibited by rifampicin in a strain of B. brevis which contained a rifampicin-resistant RNA polymerase. It is well established that DNA replication is essential for the initiation of sporulation in Bacillus (Young and Mandelstam, 1979).

In view of the damaging effects of rifampicin in B. subtilis (Coote et al., 1973), experimentation with conditional drug resistant mutants should also be viewed critically.

A wide range of antibiotics affect sporulation through interaction with the ribosomes.

The spectinomycin-specific locus codes for a ribosomal protein of the 30S subunit (Graham and Bott, 1975; Bott et al., 1978). Mutation and reversion studies in B. subtilis have demonstrated an inseparable association between antibiotic resistance and an altered sporulation phenotype (Bott et al., 1978). Streptomycin-resistant mutants of B. subtilis which were temperature-sensitive for sporulation also carried defects in the 30S ribosomal subunit (Leighton, 1974). Conditional sporulation mutants resistant to erythromycin contained an altered 50S ribosomal protein (Domoto et al., 1975; Tipper et al., 1977).

Some of the criticisms which have been aimed at studies utilising RNA polymerase-directed antibiotics, are also valid in studies utilising aminoglycoside antibiotics and authors have been cautious in their comments about drug-resistant, asporogenous mutants in which the lesion may be ribosomal.

Sporulation mutants of C. acetobutylicum P262 resistant to antibiotics and mutants which are unable to synthesise intracellular reserves of granulose, have been isolated. This chapter describes the characterisation of 8 sporulation mutants which were used to help elucidate the relationship between solvent production and the morphological changes which occur during the fermentation. It was also possible to confirm the relevance of extracellular protease production during mature spore formation.

4.2. MATERIALS AND METHODS

4.2.1. Bacterial Strain

The C. acetobutylicum P262 strain was used. Cultures were incubated at 34°C and all manipulations were performed under stringent anaerobic conditions in an anaerobic glove box.

4.2.2. Culture Media.

Culture media used are listed in Appendix A.

4.2.3. General Methods

Heat shocking and inoculation, fermentation methods, and growth and morphological determinations were outlined in Chapter II. Unless otherwise stated, the same methods have been used.

4.2.4. Physical Measurements

Determination of proteolytic activity, pH and solvent analyses were carried out as described in sections 2.2 and 3.2.

4.2.5. Mutagenesis

4.2.5.1. N-methyl-N'-nitro-N-nitrosoguanidine

Organisms harvested from CBM cultures in the mid-exponential phase of growth (5 x 10^7 cells ml^{-1}) were washed once with sterile distilled water and resuspended in fresh CBM at 37°C containing 10, 20 and 30 ug ml^{-1} N-methyl-N-nitro-N-nitrosoguanidine (NTG). At 5 min time intervals, samples (0.1 ml) were removed, diluted and plated on CBM agar. Plates were scored after 24 h anaerobic incubation at 34°C and a survival curve was plotted.

4.2.5.2. Ethyl methane sulphonate

Exponential phase CBM cultures (5 x 10^7 cells ml^{-1}) harvested and washed once in sterile distilled water, were resuspended in fresh CBM containing 2.5% (v/v) dissolved ethyl methane sulphonate (EMS). The culture was incubated at 37°C. Samples were removed periodically and plated out on CBM agar to determine the number of survivors.

4.2.5.3. UV irradiation

A 10 ml sample of exponential phase CBM cells (5 x 10^7 cells ml^{-1}) in distilled water held under anaerobic conditions was exposed to UV irradiation from an ultraviolet lamp (Mineralight Lamp, model UVSL-58) (254 nm) giving an output of 7.5 - 25 $Jm^{-2}sec^{-1}$, a distance of 28 cm from the lamp. Samples (1 ml) were removed at 7.5 sec intervals. The survivors were allowed to resume anaerobic growth in the dark at 34°C.

4.2.6. Isolation of mutants

Streptolydigin and streptomycin were obtained from the Upjohn Company (Michigan, USA), rifampicin from Ciba-Geigy, and erythromycin was supplied by Sigma. Spontaneous antibiotic-resistant mutants were selected on CBM agar containing rifampicin (1 - 100 ug ml^{-1}), streptolydigin (20 - 50 ug ml^{-1}), streptovaricin (10 - 50 ug ml^{-1}), or erythromycin (1 - 2 ug ml^{-1}).

Antibiotic-resistant colonies which were also defective in sporulation had a distinct morphology and were easily identified. Sporulation mutants grew as greyish-white flat colonies on CBM agar, whereas the sporulating colonies contained a thick central zone and had a creamy-yellow colouration.

Stable sporulation mutants were isolated from the 10% survivors of exponential phase CBM cultures (5 x 10^7 cells ml^{-1}) treated with EMS (2.5% v/v) for 20 min at 37^oC. The washed cells were allowed to "grow out" for 2 - 5 h in fresh CBM before diluting and plating on antibiotic agar plates. The auxotrophic mutants which were also antibiotic-resistant, were selected in the same way following a penicillin counter-selection step in minimal medium containing 0.1 - 1 ug ml^{-1} penicillin. The auxotrophic lesions were determined on minimal agar plates supplemented with amino acids.

Granulose-negative mutants were isolated by the method of Robson et al. (1974). The mutagenised culture was diluted to give discrete colonies when plated on CBM agar and incubated for 24 h. The open dishes were inverted over I_2 crystals for 1 min. The colonies of the granulose-producing strains were coloured a dark blue by the sublimed I_2 vapour, whereas the granulose-negative mutant colonies showed no colour change.

4.2.7. Maintenance of sporulation mutants

Three methods were used for the maintenance of stock cultures of mutants unable to produce heat-resistant spores.

4.2.7.1. Beef liver medium (ATCC catalogue No. 38)

An actively motile exponential phase inoculum of the mutant strain (0.1 ml) was incubated in 15 ml beef liver medium for 24 h at 34°C. The culture was then stored at room temperature. A reduced atmosphere above the medium was not necessary for the maintenance of the stock cultures.

4.2.7.2. Glycerol medium

Mid-exponential phase CBM cells (5 x 10^{7} cells ml^{-1}) were harvested and resuspended in a half volume CBM broth containing 20% glycerol. Samples (0.5 ml) were dispensed into Bijou bottles under anaerobic conditions, sealed tightly and stored in air at -20°C.

4.2.7.3. Freeze dried cultures

A two day old plate culture of the mutant strain was scraped into a sterile skim milk medium (20% w/v) to give a heavy suspension. Blotting paper strips were dipped into the cell suspension and placed into labelled vials stoppered loosely with rubber caps. The vials were packed into trays which were

immediately loaded on to a Labotec Dry-o-Vac Freeze Drier and lyophilised overnight. All manipulations, except the freeze drying step were carried out under stringent anaerobic conditions. Aseptic culture techniques were used throughout.

Resuscitation of the mutant strains was achieved by resuspending the blotting paper strips, or small samples (0.1 - 0.5 ml) of the beef liver and glycerol media in CBM broth (10 ml), which was then incubated at 34°C for 5 - 15 h.

Experiments performed with the sporulation mutants were repeated at least three times. The results reported in this chapter are typical of the experimental data obtained.

4.3. RESULTS

4.3.1. Isolation of sporulation mutants of *C. acetobutylicum* P262

Sporulation mutants of *C. acetobutylicum* P262 were readily identified on solid media. The defective mutants had a distinct colony morphology. Isolated colonies were typically flat, mottled-greyish, and did not have the domed, creamy-yellow appearance exhibited by wild-type clones.

4.3.1.1. Spontaneous antibiotic-resistant mutants

A number of antibiotic-resistant mutants which had altered sporulation properties were isolated directly on antibiotic agar (Table 4.1; Table 4.2). Two groups of developmental stage mutants were obtained. The asporogenous mutants of the first type were blocked before the clostridial developmental stage. These were designated cls mutants and remained as vegetative rods. The second type of mutants which were blocked after the clostridial stage, were totally or partially blocked in the production of mature spores. These were designated spo mutants and were either asporogenous or oligosporogenous, depending on whether their ability to produce spores was completely or partially blocked.

Clones which grew in the presence of rifampicin, streptolydigin and streptovaricin were presumed to contain an altered RNA polymerase (Table 4.1). These mutants showed the same sporulation defects in the presence and absence of the drug concerned. The sporulation block in each case was not temperature-sensitive. The mutant properties remained the same at incubation temperatures of 28 and 34°C (data not shown). Some mutants which were resistant to the RNA polymerase-directed antibiotics, sporulated normally, but have been omitted from this study.

Seven spontaneous erythromycin-resistant mutants were isolated on CBM plates supplemented with 1 - 2 ug ml^{-1} of the

TABLE 4.1

Sporulation mutants of *C. acetobutylicum* P262 isolated by spontaneous resistance to antibiotics which affect transcription.

		Sporulation feature[b] in CBM	
Strain	Resistant Phenotype[a]	Clostridial forms	Mature spores
P262	s	++	++
cls-1	rif	-	-
cls-2	rif	-	-
spo-1	rif	++	-
spo-2	rif	++	-
spo-3	rif	+	+
spo-4	rif	+	+
spo-5	rif	+	+
cls-5	std	-	-
cls-6	std	-	-
cls-7	stv	-	-
spo-6	stv	++	-
spo-7	stv	+	+
spo-8	stv	++	+

[a] s, sensitivity to rifampicin (20 ng ml^{-1}), streptolydigin (15 ug ml^{-1}), and streptovaricin (5 ug ml^{-1}); rif, resistance to rifampicin (100 ug ml^{-1}), std, resistance to streptolydigin (50 ug ml^{-1}); stv, resistance to streptovaricin (50 ug ml^{-1}).

[b] ++, normal wild-type levels of sporulation features; +, reduced levels of sporulation features; -, no sporulation features.

antibiotic (Table 4.2). Three of these mutants were also defective in the sporulation process. The E 1a and E 7 mutants formed the clostridial stage, but did not sporulate, and the E 5 mutant behaved as a cls mutant, producing neither refractile spores nor the clostridial stage. The four remaining erythromycin-resistant mutants did not show altered sporulation properties in CBM. However, when the nutrient medium was supplemented with antibiotic at a concentration which was sub-inhibitory to the growth of the mutant (1 ug ml^{-1}), E 1b and E 4 did not sporulate and E 2 and E 6 formed reduced levels of spores.

4.3.1.2. Mutagenesis

Additional sporulation mutants and mutants defective in granulose production were isolated from the survivors of mutagenic treatment.

Mutants isolated on CBM agar following UV irradiation (10 Jm^{-2} sec^{-1}) were not stable and regained the sporulation properties typical of the wild-type strain when recultured (data not shown). Extraneous UV-absorbing material, such as protein, which protects the irradiated organism, and photo-reversal of the mutagenic effect are among the technical difficulties associated with UV mutagenesis (Meynell and Meynell, 1970).

TABLE 4.2

Erythromycin-resistant mutants of C. acetobutylicum P262 isolated by spontaneous resistance to antibiotic.

	Sporulation feature[a] in CBM		Sporulation feature in CBM + Ery (1 ug ml^{-1})	
Strain	Clostridial forms	Mature spores	Clostridial forms	Mature spores
P262	++	++	NG	NG
E1a	++	-	++	-
E1b	++	++	+	-
E2	++	++	++	+
E4	++	++	++	-
E5	-	-	-	-
E6	++	++	++	+
E7	++	-	+	-

[a] ++, normal wild-type levels of sporulation features; +, reduced levels of sporulation features; -, zero sporulation features. NG, no growth.

The mutagen NTG is highly mutagenic at low killing levels and has been shown to induce multiple mutations in localised regions of the chromosome (Adelberg *et al*., 1965). For this reason, NTG was not suitable for the isolation of sporulation mutants in *C. acetobutylicum* P262. The kill curve for NTG (10 - 20 ug ml^{-1}) mutagenesis is given in Fig. 4.1a. The mutagen EMS, which has a similar mutational effect to that of NTG, but is less likely to yield multiple mutations (Carlton and Brown, 1981), was used in the isolation of mutant strains. A 10% survival rate was obtained when exponential phase CBM cultures (*c* 5 x 10^7 cells ml^{-1}) were treated with 2.5% (v/v) EMS for 20 min at 37°C (Fig. 4.1b).

A number of mutants with altered sporulation properties were isolated following EMS mutagenesis of an exponential phase CBM culture (*c* 5 x 10^7 cells ml^{-1}) (Table 4.3). The sporulation mutants which were selected on rifampicin plates presumably had mutations in the genes coding for RNA polymerase. Some of these mutants also contained auxotrophic lesions (*pro*$^-$; *his*$^-$) or were deficient in the production of a cell-free autolysin (*lyt*) (Allcock, 1981).

The *cls*-3 and *cls*-4 mutants which were isolated as granulose-negative mutants were rifampicin-sensitive. These mutants were altered in some general regulatory function which affected granulose biosynthesis.

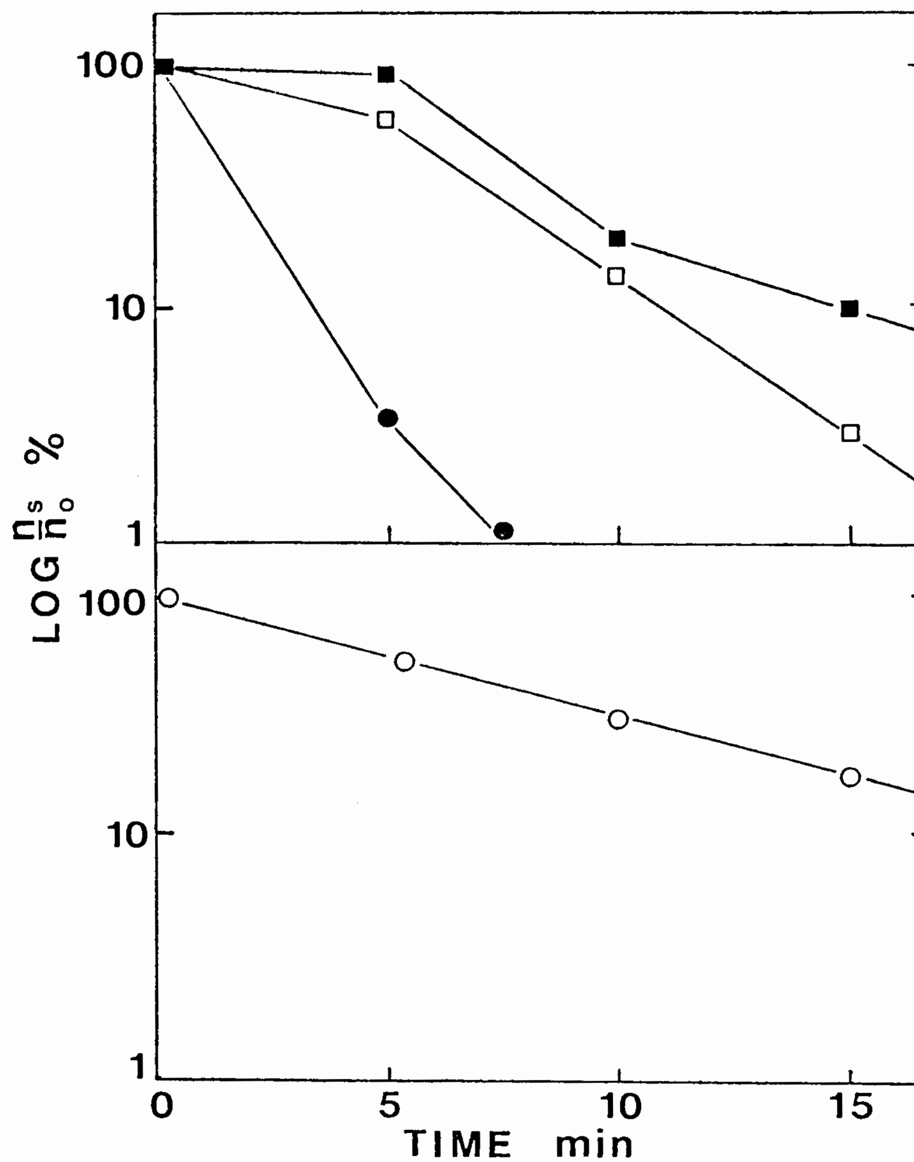


Figure 4.1

Survival by exponential phase C. acetobutylicum P262 cultures after treatment with NTG and EMS.

(a) NTG, 10 ug ml^{-1} (■); 20 ug ml^{-1} (□); 30 ug ml^{-1} (●).

(b) EMS, 2.5% v/v (○).

TABLE 4.3

Sporulation mutants of C. acetobutylicum P262 selected following mutagenesis with EMS (2.5% v/v).

Strain	Resistant[a] Phenotype	Sporulation feature in CBM[b]	
		Clostridial forms	Mature spores
P262	s	++	++
cls-3	s	-	-
cls-4	s	-	-
cls-8	rif	-	-
spo-9	rif	++	+
his^{-} cls-1	std	-	-
pro spo-1	std	++	-
lyt spo-1	stv	++	-

[a] s, sensitive to rifampicin (20 ng ml^{-1}); streptolydigin (15 ug ml^{-1}), and streptovaricin (5 ug ml^{-1}); rif, resistant to rifampicin (100 ug ml^{-1}); std, resistant to streptolydigin (50 ug ml^{-1}); stv, resistant to streptovaricin (50 ug ml^{-1}).

[b] ++, normal wild-type levels of sporulation features; +, reduced levels of sporulation features; -, zero sporulation features.

4.3.2. Characterisation of sporulation mutants of *C. acetobutylicum* P262

Eight stable pleiotrophic sporulation mutants were chosen for further morphological, physiological and biochemical characterisation (Fig. 4.2; Table 4.4). In all but one of the sporulation mutants, vegetative cell development and final cell concentration in CBM an CAMM were similar to those of the wild-type strain (Fig. 4.2).

The *cls*-2 mutant grew poorly in liquid media (final cell concentration, 1.05 x 10^8 cells ml^{-1}). However, this mutant grew normally (final cell concentration *c* 4 x 10^8 cells ml^{-1}) and produced spores in CAMM supplemented with vitamin-free casamino acids (1 - 2 g l^{-1}). This would suggest the presence of some defect which indirectly affects the sporulation process, and is responsible for the behavioural patterns displayed by the *cls*-2 mutant in different media.

The *cls* mutants which remained as vegetative rods, were unable to synthesise granulose and did not produce forespore septa or the capsular material associated with the clostridial stage (Table 4.4). The *spo* mutants which produced typical phase-bright swollen clostridial forms, accumulated granulose and produced an extracellular capsule. The oligosporogenous mutants (*spo*-3 and *spo*-4) produced the clostridial stage, forespore septa and refractile spores at approximately half the frequency observed in the wild-type cultures. The asporogenous mutant,

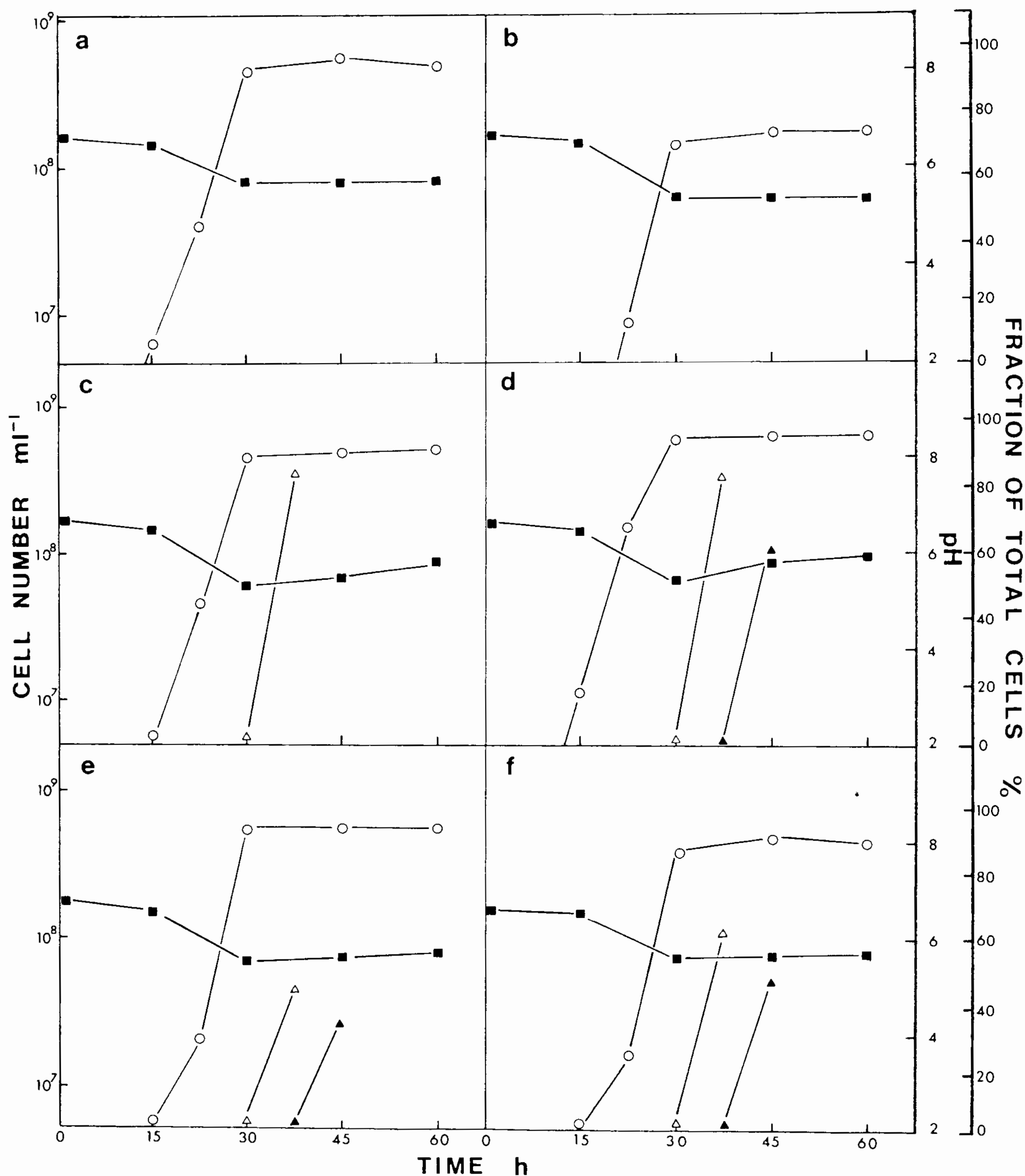


Figure 4.2

Growth and development of C. acetobutylicum P262 sporulation mutants in CAMM. (a) cls-1, cls-3, cls-4; (b) cls-2; (c) spo-1; (d) spo-2; (e) spo-3; (f) spo-4.

Total Cell count (○); pH (■); Clostridial forms (△); Forespore septa (▲).

TABLE 4.4

Characterisation of *C. acetobutylicum* P262 sporulation mutants in liquid media.

Strain	Characteristic[a]							Rifampicin[b]	Solvent (g/l)
	Clostridial forms	Granulose	Capsule	Septum	Forespore	Mature spore	Protease		
P262	++	++	++	++	++	++	++	s	16.864
cls-1	-	-	-	-	-	-	++	r	0.168
cls-2	-	-	-	-	-	-	+	r	0.489
cls-3	-	-	-	-	-	-	ND	s	0.357
cls-4	-	-	-	-	-	-	ND	s	0.196
spo-1	++	++	++	+	-	-	++	r	16.125
spo-2	++	++	++	++	-	-	-	r	15.411
spo-3	+	+	+	+	+	+	++	r	1.077
spo-4	+	+	+	+	+	+	++	r	2.501

[a] ++, normal wild-type levels of sporulation features; +, reduced levels of sporulation features; -, no sporulation features; ND, feature not determined.

[b] s, sensitive to rifampicin (20 ng ml^{-1}); r, resistant to rifampicin (100 ug ml^{-1}).

spo-1, developed the forespore septa associated with stage II of sporulation in a low percentage of the population (1 - 5% in CAMM). The spo-2 mutant produced sporulation septa, but phase-grey and phase-bright forespores were never observed. This mutant was considered to be a stage II mutant (Piggot and Coote, 1976).

A relationship between the level of clostridial stage formation and the amount of solvent produced in a molasses fermentation medium (MFM) (Barber et al., 1979) was observed (Table 4.4). The two asporogenous mutants (spo-1 and spo-2) which produced clostridial forms at the same frequency as the P262 strain, formed solvents in amounts similar to those produced by the wild-type strain. The two oligosporogenous mutants (spo-3 and spo-4) which formed reduced numbers of the clostridial stage, produced intermediate levels of solvents. The cls mutants (cls-1, -2, -3 and -4) which did not form the clostridial stage, failed to produce solvents in MFM.

Five of the sporulation mutants produced an extracellular protease which was able to hydrolyse gelatin. There was no correlation between the presence of proteolytic activity and the ability of the various strains to form clostridial forms and complete stage II of sporulation.

4.3.3. Maintenance of sporulation mutants

Sporulation is used as a convenient method of preserving cultures on a routine basis. Strains of C. acetobutylicum used in the industrial ABE process, are stored as spores transferred to sterile soil and allowed to dry (Spivey, 1978). In the laboratory, spore stocks are usually maintained at $4^{o}C$ in sterile distilled water. The sporulation mutants which formed low levels of heat-resistant spores could also be stored in this way. Spores derived from oligosporogenous mutants, when recultured, sporulated at the same low frequency. The maintenance of asporogenous mutants proved to be difficult and it was necessary to find alternative storage conditions that would make it possible to retain the properties of the mutant cultures over a long period of time.

Methods such as batch reculturing on liquid or solid nutrient media were not suitable. The rapid loss of viability by mutant strains unable to form spores made it necessary to perform weekly sub-culturings. At this frequency of transfer, asporogeny of the cls-type was selected. Sporulation mutants were more effectively stored at room temperature in a beef liver medium. Exponential phase cells stored in a 20% glycerol CBM medium at $-20^{o}C$, and lyophilised samples sealed in ampules under vacuum were suitable alternative storage methods.

C. acetobutylicum mutants have been successfully stored for two years in a lyophilised state and as suspensions in beef liver medium and in glycerol. Yearly sub-culturing of the beef liver stocks did not lead to any significant change in the observed viability, morphology and physiological properties of the stored cultures when revived in CBM or CAMM.

4.4 DISCUSSION

A number of morphological mutants which showed altered sporulation properties were isolated. Preliminary studies revealed mutants blocked prior to septum formation as well as those blocked after stage II of the sporulation process in C. acetobutylicum P262. All the strains appear to be effector mutants altered in one or other of the complex control processes which regulate sporulation. It is possible that the antibiotic-resistant derivatives of the standard strain contain defects in the RNA polymerase or 50S subunit of the ribosome. It has been demonstrated that subtle modifications in the transcriptional and translational enzymes which give rise to antibiotic resistance in B. subtilis, can have severe effects on the temporal expression of the sporulation genome (Sumida-Yasumoto and Doi, 1977; Bott et al., 1978).

The asporogenous phenotype of the mutants selected for their inability to accumulate intracellular reserves of granulose is likely to be a consequence of a regulatory defect which inhibits the pathway to granulose biosynthesis as well as the switch to the solvent-producing clostridial stage. A number of granulose-negative mutants which produce a modified type of clostridial stage cell (neither swollen nor typically phase-bright) have been isolated for C. acetobutylicum P262 (Reysenbach, personal communication). These strains produce solvents, but the levels of endospore formation are low. The defect in this case is likely to be in a structural gene of the

granulose biosynthetic pathway. The granulose-negative mutants of C. acetobutylicum P262 resemble certain Type Culture strains which are unable to synthesise granulose, but which produce solvents and endospores.

The results obtained from mutant studies suggest that the stationary phase events in C. acetobutylicum P262 are not inextricably linked. However, granulose accumulation, solvent production and clostridial stage and endospore formation appear to share common features in the regulation of their individual pathways.

A study of the morphological and physiological changes in CAMM (Chapter III) suggested that the clostridial stage is involved in the conversion of glucose to neutral solvents in C. acetobutylicum P262. This conclusion is supported by the isolation and characterisation of eight sporulation mutants which either failed to form clostridial stages and produced no solvents or formed reduced numbers of clostridial stages and produced intermediate levels of solvents. The identification of a distinct morphological stage associated with solvent production and the isolation of spo mutants which can be maintained as clostridial forms are being investigated further in a continuous culture system.

Sporulation mutants of C. acetobutylicum P262 have proved to be relatively easy to isolate. The isolation and characterisation of additional mutants will provide a useful

basis for future biochemical studies and will contribute to an understanding of the metabolic activity of the developing cell. Future studies using such mutants may also help to elucidate the way in which the various events associated with solventogenic fermentation phase are interlinked.

Information obtained from the study of mutants has been of tremendous value in the study of differentiation in B. subtilis. Mutant strains have been used in complementation studies to map the bacterial chromosome and to define sporulation-specific loci. A genetic transfer system using transformation (Reid et al., 1983) and protoplast fusion (Jones et al., submitted for publication) have been established in C. acetobutylicum P262. Although the genetic studies are still in their infancy, it is hoped that it will become possible to investigate the mode of genetic regulation in solvent production and endospore formation in C. acetobutylicum P262.

CHAPTER V

ENVIRONMENTAL FACTORS AND THE INDUCTION OF SPORULATION

Summary

The environmental factors responsible for the induction of the solventogenic phase of the fermentation and the sporulation process were investigated in liquid media. A complex interaction between acetate, butyrate and pH, limited cell growth and regulated the initiation of solventogenesis, clostridial stage formation, granulose accumulation and extracellular capsule production. Endospore formation which was associated with the solventogenic phase, required the presence of an exogenous supply of nitrogen and carbon and was influenced by the incubation temperature. The maintenance of a high partial pressure of hydrogen did not favour the solventogenic fermentation phase or endospore formation.

The exposure of 6 h complex (CBM) and 8 h minimal medium (GSMM) cultures of C. acetobutylicum P262 to air resulted in a simple method for the induction of sporulation. The frequency of sporulation was increased from 20 to 50% in CBM and zero to 30% in GSMM. The optimum level of sporulation was obtained after a 30 min exposure of CBM culture to aerobic conditions and at a culture pH of pH 4.8 - 5.0. An elevated culture Eh, and the addition of H_2O_2 or the quenchers of toxic oxygen metabolites in

anaerobic cultures did not mimic the effect of air on the induction of sporulation. A rifampicin-resistant asporogenous mutant which produced the clostridial stage under anaerobic conditions, formed mature spores in the presence of air.

5.1. INTRODUCTION

Endospore formation is repressed in actively dividing bacterial cultures. The cessation of growth is a prime condition for the onset of sporulation. The probability that a cell will choose to sporulate in preference to continued vegetative growth, is governed largely by environmental factors. The external factors which are usually considered as likely inducers of endospore formation are:

1. Adverse nutritional conditions which no longer support cell division.
2. The accumulation of some catabolite which becomes toxic to the cell.

A number of comprehensive review articles summarise the role of these environmental conditions in sporulation (Schaeffer *et al*., 1965; Halvorsen, 1962; Vinter, 1969).

The hypothesis that sporulation in bacilli is a response to the depletion of nutrients necessary for growth was proposed by Büchner in 1890. Starvation conditions which trigger sporulation

have been studied most extensively in the aerobic endospore-forming bacteria (Knaysi, 1948; Schaeffer et al., 1965; Dawes and Mandelstam, 1970). The first detailed investigation of the nutritional factors whose limitation may induce sporulation was presented by Grelet (1957). The nutrient-rich medium which was used, supported exponential growth without sporulation, until the supply of glucose became depleted. The limitation of carbon- or nitrogen-containing substrates, and certain growth factors and minerals, which resulted in the cessation of growth, were able to induce sporulation. However, not every type of growth limitation caused sporulation. The author distinguished two sets of conditions for sporogenesis. Firstly, those conditions which appeared at a definite stage in the culture, inhibited vegetative growth and prompted sporulation, and secondly, those conditions continuously realised from the beginning of the culture which were necessary for the completion of spore formation.

Total nutrient starvation is not a necessary requirement for sporulation in Bacillus. The process may be initiated when the culture growth rate is limited by a restriction in the supply of nutrients. This condition may be achieved by the slow mechanical feeding of substrate (Dawes and Mandelstam, 1970) or, in the presence of a carbon or nitrogen source which is metabolised slowly (Schaeffer et al., 1965; Sterlini and Mandelstam, 1969). The likelihood of sporulation taking place in the nutrient-poor environment increases with a decrease in growth rate (Dawes and Mandelstam, 1970).

The temperature of cultivation which affects the bacterial growth rate, also influences endospore production (Vinter, 1969). Schaeffer et al., (1965) demonstrated that growth rate and sporulation frequency in B. subtilis varied independently at different incubation temperatures. However, this conclusion was based on data obtained at only two temperatures (viz. 25 and 37°C). A positive correlation between the chelated form of dipicolinic acid (DPA) and the thermoresistance of the liberated spore at different incubation temperatures has been reported (see Halvorsen, 1962 and Vinter, 1969).

In Bacillus a large proportion (about one third) of the available substrate is converted to biomass during aerobic growth. The inhibition of growth prior to the onset of sporulation is less likely to occur as a consequence of the accumulation of some metabolite which is detrimental to the cell. However, in the Clostridia, the fermentation pathways utilised during growth under anaerobic conditions account for only a small proportion of substrate conversion to biomass, while the bulk is catabolised to end-product. The highly reduced nature of organic acids and alcohols produced during the anaerobic fermentation make these harmful to biological material. Therefore, anaerobic bacteria tend to be strongly inhibited when the end-products of glucose metabolism are present at relatively low concentrations (Leung, 1980; Herrero, 1983).

Endospore formation has been less extensively studied in the anaerobic bacteria. However, it is apparent that the cell

population approaching the stationary growth phase may encounter conditions which are quite different from the nutrient starvation conditions which limit growth and allow sporulation to proceed in Bacillus.

A number of clostridia may be induced to sporulate after the completion of growth and in the presence of relatively high concentrations of nutrients.

When C. butyricum was grown in a medium which contained 5 g l^{-1} glucose, only 15% of the substrate was used during the exponential growth phase (Murrell, 1967). The enhanced glucose consumption (55%) which occurred after the induction of the stationary growth phase, was indicative of inhibition by acid end-product production (Herrero, 1983).

A specific role of end-product accumulation in the induction of sporulation was demonstrated by Strasdine and Melville (1968). The fermentation of various carbohydrates with concurrent acid production was characteristic of those cultures of C. botulinum giving good endospore formation. Bacterial growth in the absence of acid production yielded cells with a reduced ability to form spores. A substantial amount of evidence has been provided to support the conclusion that acid end-product production is directly involved in the initiation of the solventogenic phase in C. acetobutylicum (Häggström and Molin, 1980; Gottschal and Morris, 1981a and 1982; Yu and Saddler, 1983).

Unlike Bacillus, the course of endospore development in many clostridia is not subject to catabolite repression. There is an actual requirement for an exogenous supply of energy in the differentiating cell (Day and Costilow, 1964a and b; Hsu and Ordal, 1969a; Hickey and Johnston, 1981).

A second environmental factor which is capable of limiting growth in anaerobic bacteria is the exposure to oxygen. Molecular oxygen acts as a terminal oxidant, thus disrupting the normal mode of energy generation. It has been postulated that oxygen preferentially consumes the cells "reducing power" (NADH). The interference with the balanced anaerobic cycle of NADH production and consumption may leave the cell with insufficient reserves to maintain normal biosynthesis under aerobic conditions (O'Brien and Morris, 1971). The disruption of other metabolic functions (eg. ATP generation) may occur as a consequence of the altered NADH to NAD^+ ratio. It would appear that oxygen, like acid end-product accumulation, will halt growth with an efficiency determined by the rate at which interaction with the metabolically generated prime electron donors occurs. It has not been established whether these conditions of growth inhibition facilitate the onset of sporogenesis.

During bacterial growth, the culture medium is changing constantly and it is likely that the main influencing factors discussed above are also dependent on other culture conditions. The stringent pH requirements exhibited by many clostridia (discussed in Chapters I and II) are also true of a number of

Bacilli (Dawes and Mandelstam, 1970; Brown and Chesson, 1971; Kudo and Horikoshi, 1979). Nevertheless, it is generally accepted that sporulation will occur under various environmental conditions which have in common the prime effect of stopping or reducing growth.

A number of physical and chemical factors affect the growth of *C. acetobutylicum* P262. Certain of these factors have been used to determine the effect of environmental conditions on the shift to solventogenesis and the induction of sporulation in this bacterium.

5.2 MATERIALS AND METHODS

5.2.1. Bacterial strains

The *C. acetobutylicum* P262 strain and seven rifampicin-resistant sporulation-defective mutants (characterised in Table 4.1) were used.

5.2.2. Culture media

Use was made of the chemically defined media CAMM and GSMM and the complex medium CBM, which are described in Appendix A.

5.2.3. General methods

Heat shocking of spores, culture inoculation and fermentation methods have been described (2.2.3 and 4.2.7). Unless otherwise stated, the same methods have been used.

5.2.4. Physical measurements, growth determination and characterisation of cell morphology

Physical measurements, growth and morphological determinations were outlined in section 2.2 and in 3.2.

5.2.5. Environmental conditions

The effect of the nature and level of nutrients and fermentation end-products, pH, incubation temperature, and anaerobic gas atmosphere on the diphasic fermentation, clostridial stage and endospore formation was determined in CAMM. Details have been outlined in the text.

The effect of aeration on endospore formation in CBM and GSMM was determined by two different experimental systems. In the first system, culture samples (5 ml) were removed at various time intervals during the growth cycle of anaerobic cultures and incubated aerobically without shaking at 34°C in 100 ml volumetric flasks stoppered with cotton wool bungs. Spore counts were made 24 h after the initial exposure to air with a Thoma counting chamber (Webb, England) and a Zeiss photomicroscope fitted with phase-contrast optics. In the second experimental system, cells were grown anaerobically for 6 h in CBM (c % x 10^{8} cells ml^{-1}) before exposure to air for different time intervals. The cells were then reincubated under anaerobic conditions in an anaerobic glove box. Spore counts were made 24 h after the initial exposure to air. Cultures were aerated by shaking on a

Gallenkamp orbital incubator at 100 rev. min^{-1}. The concentration of dissolved oxygen was measured with an oxygen meter (Process Instrumentation, Model 2700) and the Eh was measured with a pH meter (Orion Research model 701/digital pH meter) fitted with platinum and calomel reference electrodes.

5.2.6. Peroxide and oxygen quenchers

Catalase, superoxide dismutase (SOD) and peroxide were all purchased from Sigma. One unit of catalase activity was defined as the amount of enzyme required to decompose 1 μ Mol H_2O_2 per minute at pH 7 and 25^oC. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of redution of cytochrome C by 50% at 25^oC and pH 7.8.

The experimental data presented in this chapter are resuls typical of each experiment. However, experiments were repeated at least three times.

5.3. RESULTS

5.3.1. Effect of the level and nature of nutrients and fermentation end-products on the solventogenic phase and endospore formation in C. acetobutylicum P262

5.3.1.1. Effect of carbon source

C. acetobutylicum is able to utilise a wide variety of carbohydrate substrates including monosaccharides, disaccharides, polysaccharides and some pentose sugars (Holdeman *et al*., 1977). Eight different sugars which supported the growth of *C. acetobutylicum* P262 were tested for their capacity to promote solvent production, clostridial stage formation and sporulation (Table 5.1). The CAMM containing 60 g l^{-1} sugars was used except in the case of raffinose, L-arabinose and D-xylose, where growth was severely retarded at this concentration of substrate. These three sugars were tested at a concentration of 30 g l^{-1}.

The fastest growth rates were obtained with cells grown in the 30 g l^{-1} glucose and 60 g l^{-1} fructose media, both of which gave generation times of 80 min. A significant decrease in generation time (to 110 min) was observed when the concentration of glucose was increased to 60 g l^{-1}. With the exception of galactose which gave a generation time of 130 min, growth was faster in the presence of the monosaccharides. A reduced growth rate was obtained in cultures containing di- or trisaccharides (120 min generation time). The pentose sugars supported the slowest growth rates (130 and 180 min generation time).

The sugars which were tested at a concentration of 60 g l^{-1} exhibited a 70 - 95% conversion to the clostridial stage and produced *c* 11 - 14 g l^{-1} solvents. Media containing fructose yielded the lowest solvent level, produced the highest proportion

TABLE 5.1

Effect of carbon source on growth rate, solvent production, clostridial form formation and endospore formation in C. acetobutylicum P262. The number of cells which formed clostridial forms and endospores is expressed as a percentage of the maximum cell count obtained in CAMM containing 60 g l^{-1} glucose and a maximum cell count of 5 - 6 x 10^8 cells ml^{-1}.

Carbohydrate	Concentration ($g\ l^{-1}$)	Growth Rate (min)	Total Solvents ($g\ l^{-1}$) (60 - 80h)	Clostridial Forms (%)	Endospores (%)
Glucose	60	110	13.83	80-90	70-80
Fructose	60	80	10.99	85-95	80-90
Galactose	60	130	13.34	70-80	5-10
Maltose	60	120	13.29	70-80	1- 5
Sucrose	60	120	13.36	70-80	1- 5
Glucose	30	80	5.28	60-70	< 1
Raffinose	30	120	6.31	70-80	40-50
L-arabinose	30	135	6.23	80-90	40-50
D-xylose	30	180	5.13	70-80	40-50

of clostridial forms and the greatest conversion to mature spores (80 - 90%). The sugars tested at a concentration of 30 g l^{-1}, also exhibited a high proportion of cells in the clostridial stage. Substrate fermentation generated c 5 - 6 g l^{-1} neutral end-products.

A marked effect of sugar source on endospore formation was observed. Fructose and glucose (60 g l^{-1}) which supported high growth rates, also produced the largest quantity of spores. Media containing galactose, sucrose and maltose showed a very much reduced frequency of endospore formation (1 - 10%). Raffinose, L-arabinose and D-xylose at a concentration of 30 g l^{-1} in CAMM produced 40 - 50% endospores, while cells grown in the presence of glucose at this concentration showed less than 1% endospore formation.

Other carbon substrates, viz.: glyceraldehyde, trehalose, glycogen, starch, mannitol, sorbitol, xylitol, D-ribose, D-arabinose and L-xylose, did not support the growth of *C. acetobutylicum* P262 in CAMM.

A more extensive investigation into the effect of limitation of substrate was carried out in CAMM containing different concentrations of glucose. Total cell count (measure of growth), glucose utilisation, end-product production and clostridial stage and endospore formation were monitored in cultures containing 5 - 60 g l^{-1} glucose (Fig. 5.1; Table 5.2).

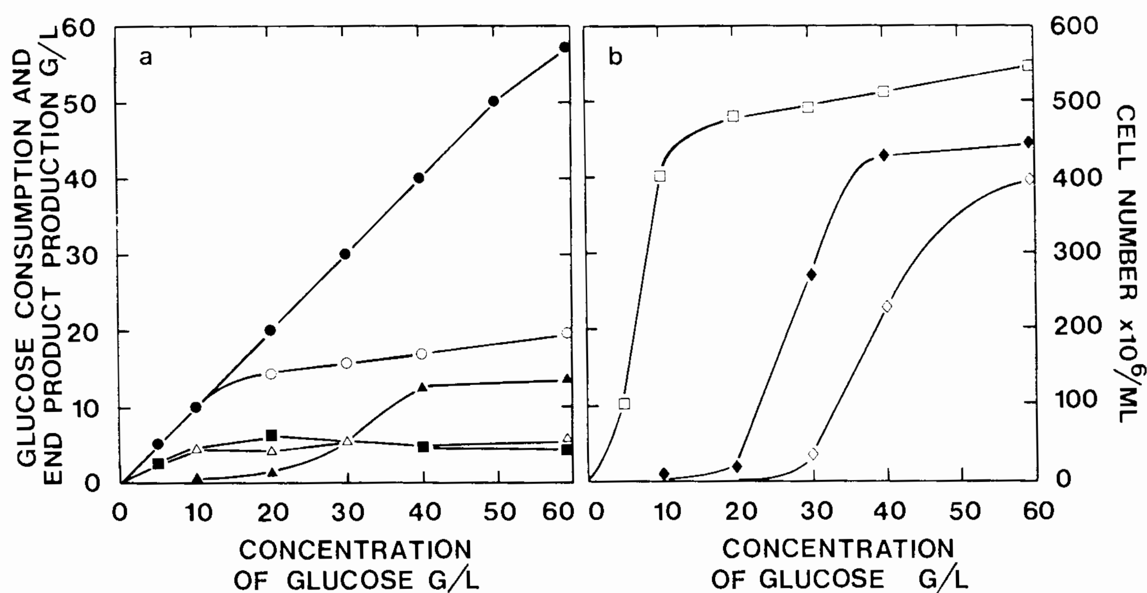


Figure 5.1

Effect of glucose on (a) end-product formation, and (b) growth, clostridial stage and endospore formation in C. acetobutylicum P262. Glucose (5 - 60 g ℓ^{-1}) was added to CAMM cultures and samples were assayed at the end of the acidogenic phase (20 - 30 h) and at the end of the solventogenic phase (60 - 80 h).

(a) glucose consumed after 20-30 h (○);
glucose consumed after 60-80 h (●);
acid end-products after 20-30 h (△);
acid end-products after 60-80 h (■);
total solvents after 60-80 h (▲).

(b) total cell count (□);
clostridial stage count (◆);
endospore count (◇).

TABLE 5.2. Effect of glucose concentration on growth, pH, end-product formation, clostridial form formation and endospore formation in *C. acetobutylicum* P262. Glucose (5 - 60 g ℓ^{-1}) was added to CAMM cultures and samples were assayed at time 0 h (initial), 20 - 30 h (end of acidogenic phase) and after 60 - 80 h (end of solventogenic phase). The number of cells which formed clostridial forms and endospores is expressed as a percentage of the maximum cell count obtained in CAMM (60 g ℓ^{-1} glucose).

Glucose (g ℓ^{-1})			pH	Total Acids (g ℓ^{-1})		Total Solvents (g ℓ^{-1})	Maximum Cell Count x 10^6 ml^{-1}		
0 h	20-30 h	60-80 h	20-30 h	20-30 h	60-80 h	60-80 h	Total Count	Clostridial Forms	Endospores
5	0	0	6.00	2.40	2.76	0.10	100	0	0
10	0.2	0	5.95	4.03	4.60	0.26	400	6 (1%)	0
20	5.5	0	5.90	3.93	6.09	1.10	480	18 (32%)	< 1 (< 1%)
30	14.0	0	5.75	5.33	5.65	5.28	490	270 (49%)	31 (6%)
40	23.0	0	5.40	4.30	4.15	12.06	510	427 (78%)	226 (41%)
58	38.0	< 0.5	5.50	4.03	5.32	13.59	550	466 (81%)	396 (72%)

There was a premature cessation of cell yield in the culture containing the lowest concentration of glucose (5 g l^{-1}). The total cell population at the end of the growth phase was less than 20% of that obtained at higher glucose concentrations. The cell population attained a constant level (4 - 6 x 10^8 cells ml^{-1}) at the higher concentrations of glucose. In media containing less than 20 g l^{-1} glucose (Fig. 5.1), all the substrate was consumed during the initial (acidogenic) fermentation phase. Low levels of acid end-product were produced, resulting in a relatively small drop in the culture pH (Table 5.2). Under these conditions, there was no development of the second fermentation phase and no solvent production, clostridial stage formation or endospore formation occurred.

Above a threshold level of 10 g l^{-1} glucose (Fig. 5.1, Table 5.2), increasing concentrations of residual sugar remained in the medium at the end of the first fermentation phase. The concentration of acid metabolites resulting from the initial fermentation, also reached a threshold level (4 - 5 g l^{-1}) and remained constant as the glucose concentration was increased. The consumption of the residual glucose during the second stage of the fermentation was accompanied by a corresponding increase in the production of solvents. There was little change in the concentration of acid end-products during this stage.

The conversion of vegetative rods to clostridial forms only occurred in cultures which contained concentrations of glucose above the threshold level, residual glucose, and an amount of

acetate and butyrate of 4 - 5 g l^{-1} at the pH breakpoint. The proportion of cells which formed the clostridial stage increased from 1% at 10 g l^{-1} to 80 - 90% in media containing 40 to 58 g l^{-1} glucose. Inreased clostridial stage formation was accompanied by an increased solvent yield from < 1 g l^{-1} to > 12 g l^{-1}.

Endospore formation only occurred in media conaining 30 g l^{-1} or more glucose. A maximum of 70 - 80% mature spores were produced in CAMM incorporating 60 g l^{-1} glucose.

5.3.1.2. Effect of nitrogen source

The effect of different nitrogen sources on the development of the solventogenic phase and sporulation was investigated in CAMM (Table 5.3). The four different ammonium salts produced constant and relatively high levels of solvents (13 - 15 g l^{-1}). A large proportion of the cell population (50 - 90%) converted to the clostridial stage during the second phase of the fermentation. A marked difference was observed in the percentage of endospores which formed in the different cultures. Diammonium hydrogen phosphate (DAP) and ammonium nitrate supported good sporulation (70 - 80%). The CAMM containing ammonium acetate as the sole nitrogen source, produced reduced levels of mature spores. Endospore formation was more variable in these cultures. Cultures containing ammonium sulphate, exhibited the lowest conversion to the clostridial stage (50 - 60%) and also produced the lowest level of endospores (10 - 25%).

TABLE 5.3

Effect of different nitrogen sources on the production of solvents, clostridial forms and endospores in CAMM (60 g l^{-1} glucose). The number of cells which formed clostridial forms and endospores is expressed as a percentage of the maximum cell count obtained in CAMM containing 6 g l^{-1} DAP and a maximum cell count of 5 - 6 x 10^8 cells ml^{-1}.

Nitrogen Source	Concentration (g l^{-1})	Total Solvents (g l^{-1})	Clostridial Forms (%)	Endospores (%)
DAP	6	14.11	80 - 90	70 - 80
NH_4NO_3	7.2	13.07	80 - 90	70 - 80
NH_4 acetate	7.2	14.46	80 - 90	5 - 60
$(NH_4)_2SO_4$	6	14.31	50 - 60	10 - 25

The effect of nitrogen limitation on growth, residual glucose, pH, end-product production, and the formation of clostridial forms and endospores was monitored in CAMM (58 g l^{-1} glucose) containing reduced concentrations of DAP (Table 5.4, Fig. 5.2). Excess phosphate was present in these cultures as potassium phosphate. When DAP was present at a concentration of less than 0.6 g l^{-1}, glucose consumption accounted for only one third of the initial glucose concentration. The total number of cells formed during the fermentation was lower than that observed at higher DAP concentrations (Table 5.4).

No clostridial forms, solvents or endospores were produced in cultures containing less than 0.3 g l^{-1} DAP and in these cultures, acetate and butyrate failed to attain the critical threshold level (4 - 5 gl^{-1}). In cultures containing 0.3 g l^{-1} or more DAP, glucose utilisation resulted in the production of threshold levels of acid end-products and clostridial stage formation and solvent production were initiated. In cultures where glucose consumption was incomplete (0.3 to 1 g l^{-1} DAP), solvent production was proportionately lower. A constant amount of solvent was measured in cultures where glucose consumption was complete (2 to 6 g l^{-1} DAP).

Endospore production only occurred in CAMM media containing concentrations of DAP above 1 g l^{-1}. As the nitrogen concentration was increased, the number of cells which formed endospores showed a uniform increase. Endospore production reached a maximum (70 - 80%) when the concentration of DAP was 3 - 4 g l^{-1} and remained stable at higher concentrations (6 g l^{-1} DAP).

TABLE 5.4 Effect of nitrogen (DAP) on growth, glucose utilisation, pH and the production of end-products, clostridial forms and endospores in C. acetobutylicum P262 grown in CAMM (60 g ℓ^{-1} glucose) containing different concentrations of DAP (0.06 - 6 g ℓ^{-1}). Excess phosphate was present as potassium phosphate. Samples were assayed at time 0 h (initial), 20-30 h (end of acidogenic phase) and after 60-80 h (end of solventogenic phase). The number of cells which formed clostridial forms and endospores is expressed as a percentage of the maximum cell count obtained in CAMM (6 g ℓ^{-1} DAP).

DAP	Glucose (g ℓ^{-1})			pH	Total Acids (g ℓ^{-1})		Total Solvents (g ℓ^{-1})	Maximum Cell Count x $10^6 m\ell^{-1}$		
(g ℓ^{-1})	0 h	20-30 h	60-80 h	20-30 h	20-30 h	60-80 h	60-80 h	Total Count	Clostridial Forms	Endospores
0.06	58	50	50	6.20	2.20	2.96	0.14	75	0	0
0.09	58	46	45	5.75	2.44	3.20	0.14	118	0	0
0.30	58	45	38	5.49	4.01	3.79	0.61	368	46 (9%)	0
0.6	58	42	32	5.45	4.05	3.47	4.49	548	247 (46%)	0
1.0	58	40	10	5.35	4.45	3.60	8.51	575	489 (91%)	< 1 (< 1%)
2.0	58	38	0	5.40	5.05	3.89	12.09	545	455 (85%)	182 (34%)
3.0	58	36	0	5.46	6,40	3.60	12.74	554	471 (88%)	360 (67%)
4.0	58	36	0	5.48	6.02	2.55	13.33	560	476 (88%)	420 (78%)
6.0	58	36	0	5.50	6.11	3.82	13.36	535	455 (85%)	402 (75%)

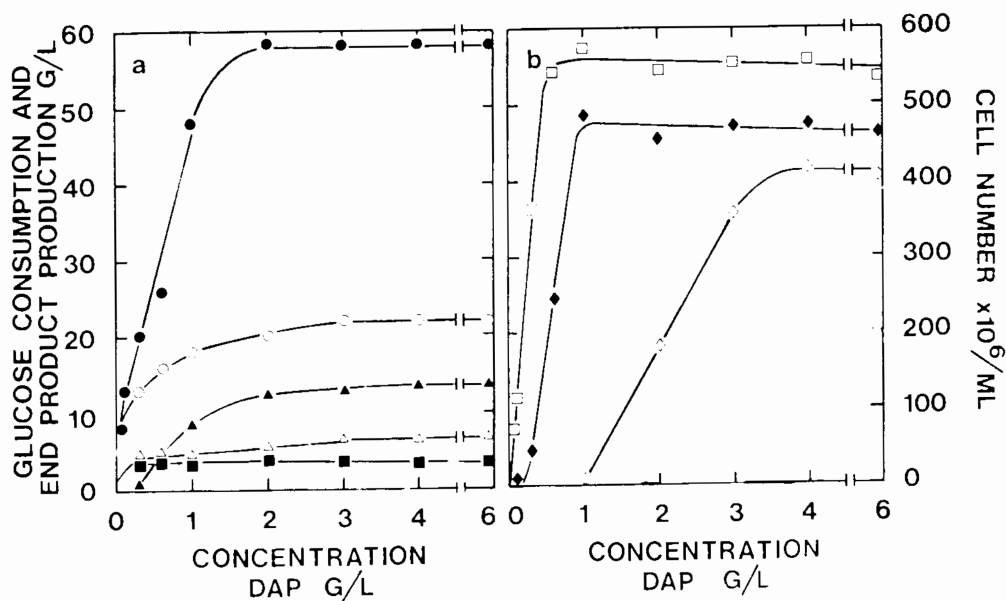


Figure 5.2

Effect of nitrogen on (a) glucose utilisation and end-product formation, and (b) growth, clostridial stage and endospore formation in C. acetobutylicum P262. Cells were grown in CAMM (60 g ℓ^{-1} glucose; 0.06 - 6 g ℓ^{-1} DAP). Excess phosphate was present as potassium phosphate. Samples were assayed at the end of the acidogenic phase (20-30 h) and at the end of the solventogenic phase (60 - 80 h).

(a) glucose consumed after 20-30 h (○);
glucose consumed after 60-80 h (●);
acid end-products after 20-30 h (△);
acid end-products after 60-80 h (■);
total solvents after 60-80 h (▲).

(b) total cell count (□);
clostridial form count (◆);
endospore count (◇).

The effect of adding extra nitrogen in the form of ammonia or DAP was monitored in CAMM containing 1 g l^{-1} DAP (Table 5.5).

Control cultures which contained no extra nitrogen showed an 80 - 90% conversion to the clostridial stage and yielded solvent concentrations of 7 - 8 g l^{-1} with less than 1% of the cells producing endospores. The addition of increased concentrations of ammonia resulted in an enhancement of solvent production and endospore formation. The ammonia was more effective when added initially, than when added at the end of the first fermentation phase. The addition of extra DAP had a similar effect. but the addition of phosphate alone in the form of potassium phosphate buffer did not stimulate solvent production or endospore formation. The addition of acetate and butyrate to cultures containing 1 g l^{-1} DAP also had no enhancing effect on solvent production or endospore formation as the acid threshold had already been attaind in these cultures.

5.3.1.3. <u>Effect of acid end-products</u>

Experimental evidence obtained from the study of the effects of glucose concentration in CAMM indicated that a threshold level of acids and residual glucose at the pH breakpoint, is necessary for the development of the second fermentation phase in *C. acetobutylicum* P262 (Table 5.2). A further investigation was implemented to determine the relationship between the substrate and acid end-products.

TABLE 5.5 Effect of addition of ammonia, DAP, potassium phosphate and acetate/butyrate on the production of solvents, clostridial forms and endospores in CAMM (60 g ℓ^{-1} glucose) containing DAP (1 g ℓ^{-1}). The number of cells which formed clostridial forms and endospores is expressed as a percentage of the total count.

Addition	Concentration (g ℓ^{-1})	Time of addition (h)	Total solvents (g ℓ^{-1})	Clostridial forms (%)	Endospores (%)
None	-	0	7.57	80-90	< 1
NH_3	0.5	0	8.62	80-90	40-50
NH_3	2.5	0	14.74	80-90	60-70
NH_3	2.5	24	11.72	80-90	40-50
DAP	5.0	24	11.25	70-80	50-60
$K_2H\ PO_4$ $KH_2\ PO_4$	7.8	0	7.34	70-80	< 1
Acetate and Butyrate	1 + 1	0	7.46	70-80	< 5

Varying concentrations of acid end-products were added to growing cultures, or to cells resuspended in media containing different concentrations of glucose (Fig. 5.3; Table 5.6).

Cells were grown in CAMM containing 30 g l^{-1} glucose. At this concenration, half of the glucose present is utilised during the acid-producing phase. Between 50 and 70% of the cells enter the clostridial stage producing 5 - 6 g l^{-1} neutral solvents (Fig. 5.3a). When acetate (1 g l^{-1}) and butyrate (1 g l^{-1}) were added at the pH breakpoint (Fig. 5.3b), the elevated level of acetate was maintained, but the level of butyrate decreased during the solventogenic phase. There was an increase in the number of clostridial forms produced (up to 85%) and the solvent yield was increased from 5.1 g l^{-1} in the control culture to 7.5 g l^{-1} in the culture treated with acid. Endospore formation was also enhanced from < 1% in the control culture to 25% after the addition of acids. The total cell counts in the two cultures remained the same.

In a similar experiment, cells pregrown to the mid-exponential phase in a 30 g l^{-1} glucose CAMM, were harvested, washed and resuspended in fresh media containing various concentrations of glucose and acetate and butyrate (Table 5.6).

Cells resuspended in the 5 g l^{-1} glucose CAMM, with and without the addition of acetate and butyrate, failed to form the clostridial stage and sporulate. Clostridial form and endospore formation occurred in media containing higher levels of glucose

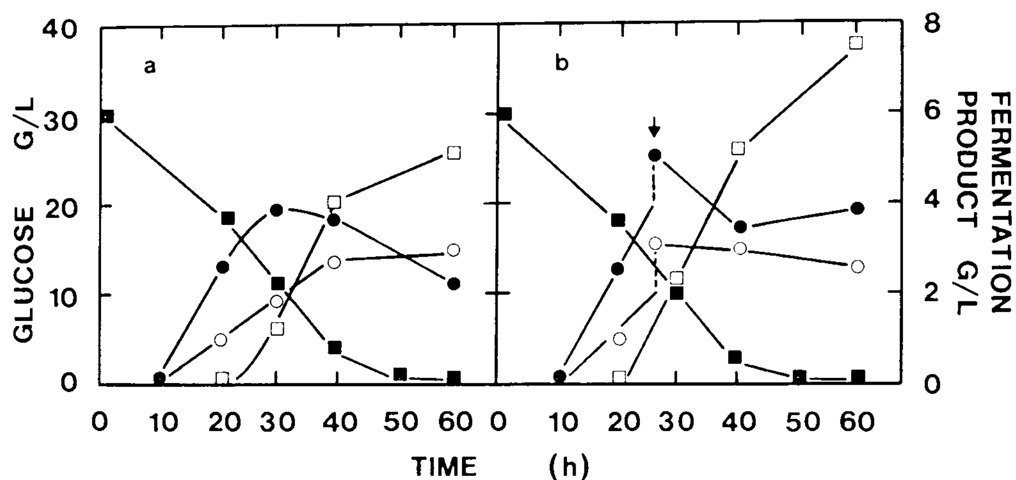


Figure 5.3

Effect of acetate and butyrate on end-product formation in C. acetobutylicum P262 grown in CAMM (30 g ℓ^{-1} glucose).
(a) No addition. (b) Addition of acetate (1 g ℓ^{-1}) and butyrate (1 g ℓ^{-1}) at 26 h (↓).
Residual glucose (■); acetate (○); butyrate + acetate (●); and total solvents (□).

TABLE 5.6 Effect of glucose, acetate and butyrate concentrations on endospore formation in *C. acetobutylicum* P262. Exponential phase cells grown in CAMM (30 g ℓ^{-1}) were harvested, washed and resuspended in CAMM containing different concentrations of glucose, acetate and butyrate. The number of cells which formed endospores is expressed as a percentage of control cultures resuspended in CAMM (60 g ℓ^{-1} glucose). Control cultures contained 1.5 - 2 x 10^8 endospores mℓ^{-1}.

Resuspension Medium			Endospores
Glucose (g ℓ^{-1})	Acetate (g ℓ^{-1})	Butyrate (g ℓ^{-1})	(%)
5	0	0	0
5	1	1	0
5	2	2	0
10	0	0	< 1
10	1	1	< 1
10	2	2	< 1
20	0	0	3
20	1	1	11
20	2	2	30
30	0	0	13
30	1	1	53
30	2	2	100
30	3	3	100
30	4	4	40
60	0	0	100

and acetate and butyrate. The highest proportion of clostridial stages and endospores was obtained in CAMM containing 30 g l^{-1} glucose and to which 2 to 3 g l^{-1} acetate and 2 to 3 g l^{-1} butyrate had been added. Acids added at higher concentrations (4 g l^{-1} of each) inhibited clostridial stage and endospore formation in the presence of 30 g l^{-1} glucose.

Experiments in which acetate and butyrate were substituted by a non-metabolisable acid demonstrated an actual requirement for the fermentation end-products, rather than an indirect effect of the metabolite on the pH of the medium. When the biological acids were replaced by a concentration of $HClO_3$ sufficient to lower the culture pH to the same level, there was no enhancement of clostridial stage formation or endospore production (Table 5.7).

5.3.2. Effect of pH on the solventogenic phase and endospore formation in *C. acetobutylicum* P262

During the industrial ABE fermentation process (Spivey, 1978; Jones *et al*., 1982) and in the chemically defined medium (this study), the production of acid end-products by *C. acetobutylicum* results in a drop in the pH of the fermentation medium to the pH breakpoint. A rise in the culture pH accompanies solvent production during the second stage of the fermentation. A fairly narrow pH range (pH 5.2 - 5.8) at the pH breakpoint is vital for the induction of the solventogenic phase. In CAMM which had a reduced buffering capacity, the prodution of

TABLE 5.7 Effect of $HClO_3$ on endospore formation in C. acetobutylicum P262. Exponential cells grown in CAMM (30 g ℓ^{-1} glucose) were harvested, washed and resuspended in CAMM (30 g ℓ^{-1} glucose) containing either $HClO_3$ (1.4 g ℓ^{-1}) or acetate (2 g ℓ^{-1}) and butyrate (2 g ℓ^{-1}). The number of cells which formed endospores is expressed as a percentage of control cultures resuspended in CAMM (60 g ℓ^{-1} glucose). The control cultures contained 1.5 - 2 x 10^8 endospores $m\ell^{-1}$.

Resuspension Medium	Initial pH	Final pH	Endospores (%)
CAMM	6.90	5.75	16
CAMM + $HClO_3$	6.00	5.45	< 1
CAMM + Acetate and butyrate	5.95	5.65	100

acids during the early part of the fermentation reduced the pH of the medium to pH 4.0 - 4.5. Under these conditions, the majority of the population (c 90%) failed to undergo the shift to the second fermentation phase and endospore formation did not occur. When the pH conditions were maintained above pH 6.0, premature cell lysis occurred before the shift to the second phase of the fermentation was initiated.

5.3.3. Effect of incubation temperature on endospore formation in *C. acetobutylicum* P262

It was established in Chapter II that an incubation temperature of 37°C is optimum for the growth of *C. acetobutylicum* P262 in CBM (Fig 2.2). However, endospore formation was reduced when the incubation temperature was greater than 36°C (Table 2.3). A similar effect of temperature on growth and sporulation was observed in CAMM (Table 5.8). The lower percentage of cells which formed sporulation septa at 37°C (detected by method of Smith and Ellner, 1957) suggested a temperature-sensitive step at the level of forespore formation in *C. acetobutylicum* P262. The total cell count, cell viability and clostridial stage formation was not significantly affected by the incubation temperature.

Proportionately more dispores were observed at 30°C (10 - 15%) than at 34°C (1 - 5%). The observed temperature effect may be a consequence of the reduced growth rate which occurs at the lower incubation temperature.

TABLE 5.8

Effect of incubation temperature on growth, clostridial form formation and endospore formation in CAMM. The number of cells which formed clostridial forms and endospores is expressed as a percentage of the total cell count.

Incubation Temperature (°C)	Maximum cell count x 10^6 ml^{-1}		Clostridial forms (%)	Endospores (%)
	Viability	Total count		
30	6	520	80 - 90	60 - 70
34	465	555	80 - 90	70 - 80
37	488	546	80 - 90	10 - 20

5.3.4. Effect of anaerobic gas atmosphere on solventogenesis and endospore formation in *C. acetobutylicum* P262

A likely candidate for an environmental regulator of the reductive pathway to solvent production, is hydrogen partial pressure (pH_2).

In an attempt to determine a physiological role of H_2 in the *Clostridium* fermentation by strain P262, cultures were grown in the presence of different gas atmospheres (Table 5.9). An increased hydrogen partial pressure did not support good solvent production in CAMM, and the final sporulation level was lower than that obtained in the presence of an N_2, CO_2 and mixed gas atmosphere. In cultures grown under a hydrogen atmosphere, the clostridial stage cells were not typical and only 30 - 50% of the population was observed to undergo morphological changes. There was a corresponding reduction in the number of endospores formed in the presence of H_2.

5.3.5. Effect of aeration on endospore formation in *C. acetobutylicum* P262

Isolated colonies of *C. acetobutylicum* grown anaerobically for 2 days and then incubated aerobically, developed into unique elongated fruiting body-like structures (Jones *et al*., 1980). A high proportion of sporulating cells and free spores were contained within the tough sheath surrounding the macroscopic structure. Aerobic conditions also had a marked effect on sporulation in liquid media.

TABLE 5.9

Effect of anaerobic gas atmosphere on solvent production, clostridial form formation and endospore formation in CAMM. The number of cells which formed clostridial forms and endospores is expressed as a percentage of the total cell count which was 4 - 5 x 10^6 cells ml^{-1}.

Gas Atmosphere	Concentration (% v/v)	Total Solvents ($g\ l^{-1}$)	Clostridial forms (%)	Endospores (%)
$CO_2:N_2:H_2$	70:20:10	10.16	80 - 90	70 - 80
N_2	100	10.10	70 - 80	60 - 70
CO_2	100	11.06	70 - 80	60 - 70
H_2	100	4.02	40 - 50	20 - 30

5.3.5.1. Effect of aeration on endospore formation in liquid media

The complex medium (CBM) and the glucose-mineral salts-biotin medium (GSMM) described previously (see Chapter II) were used. Neither system supported good sporulation under anaerobic conditions. No sporulation occurred in the defined medium and a maximum of 20 - 30% mature spores were produced in CBM. Increased levels of sporulation were obtained when cell samples removed from the CBM and GSMM cultures were incubated aerobically (Figs. 5.4 and 5.5). The highest level of sporulation in CBM occurred when cells approaching the stationary growth phase (6 h), were transferred to aerobic conditions (Fig. 5.4). A 2- to 3-fold increase in the yield of mature spores was obtained. The pH of the culture medium at the time of transfer was c pH 4.8. The stimulatory effect of air on sporulation was less obvious during the vegetative phase (2 - 5 h). When cell samples were removed after 7 h, the continued incubation in the presence of air did not improve the level of sporulaton above that obtained in anaerobic control cultures.

Sporulation was induced in GSMM when mid-exponential phase cells (8 - 12 h) were exposed to air (Fig. 5.5). The largest increase in sporulation, from 0 to 30%, was obtained when an 8 h anaerobic culture was transferred to aerobic conditions. At this stage, the pH of the culture medium was approximately pH 5.0. Sporulation was induced to a lesser extent in culture samples exposed to air at earlier times (4 - 6 h). After 10 h, the

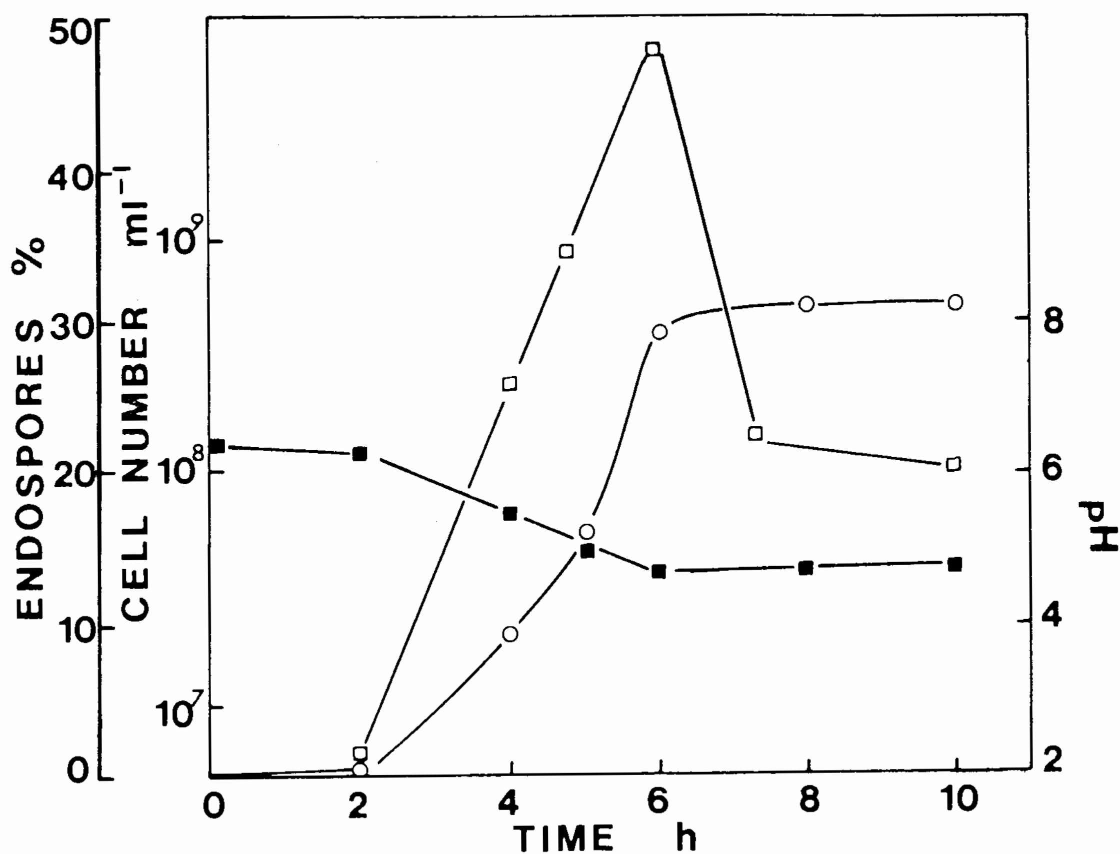


Figure 5.4

Effect of aeration on endospore formation in CBM. Samples (5 ml) of anaerobic culture were removed at different time intervals and incubated aerobically at 34°C. Total cell count (○); pH (■); % endspores (□), (made after 24 h exposure to air and expressed as a percentage of the maximum cell count [4 x 10^8 cells ml^{-1}]).

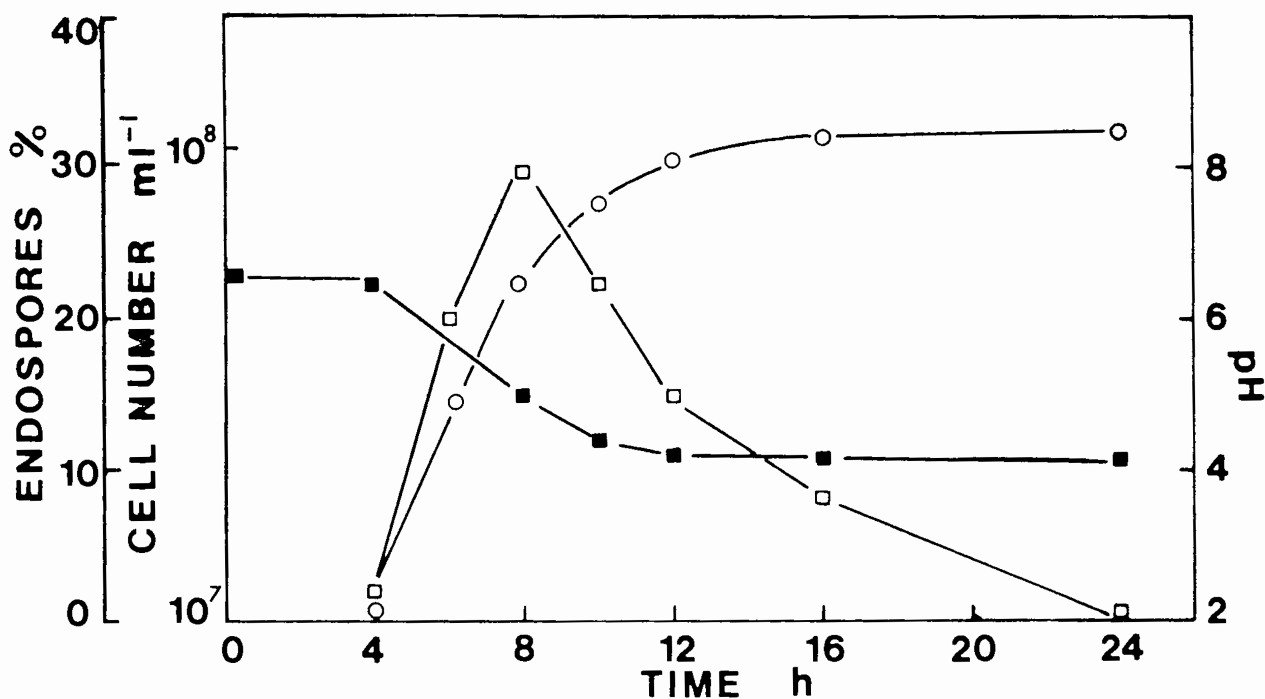


Figure 5.5

Effect of aeration on endospore formation in GSMM. Samples (5 ml) of anaerobic culture were removed at different time intervals and incubated aerobically at 34°C. Total cell count (○); pH (■); % endospores (□) (made after 24 h exposure to air and expressed as a percentage of the maximum cell count [1 x 10^8 cells ml^{-1}]).

reduced sporulation frequency appeared to be a consequence of degenerative changes in the population and the pH of the culture was low (c pH 4.1).

Cells grown in CAMM were more sensitive to inhibition by aerobic conditions and sporulation could not be stimulated above the levels obtained under anaerobic conditions (data not shown).

5.3.5.2. Oxygen and the redox potential (Eh) of the culture

Clostridia generate a low culture Eh due to the organisms' reductive metabolism. The minimum Eh established in growing cultures of clostridia may rane from -100 to -450 mV (Morris and O'Brien, 1971). The redox potential of aerated culture media changes in proportion to the logarithm of the prevailing pO_2, and Eh measurement has been successfully employed as a means of assessing very low levels of dissolved oxygen.

Complex medium (CBM) was used in the studies relating to the regulation of endospore formation by air. Under anaerobic conditions, the oxidation reduction potential of growing P262 cultures is maintained at approximately -300 mV (Fig. 5.6). The Eh value monitored in a late-exponential CBM culture (6 h) exposed to air, showed a rapid increase from Eh -300 mV to Eh +20 mV within 30 min. There was little change in the culture Eh upon further incubation in air.

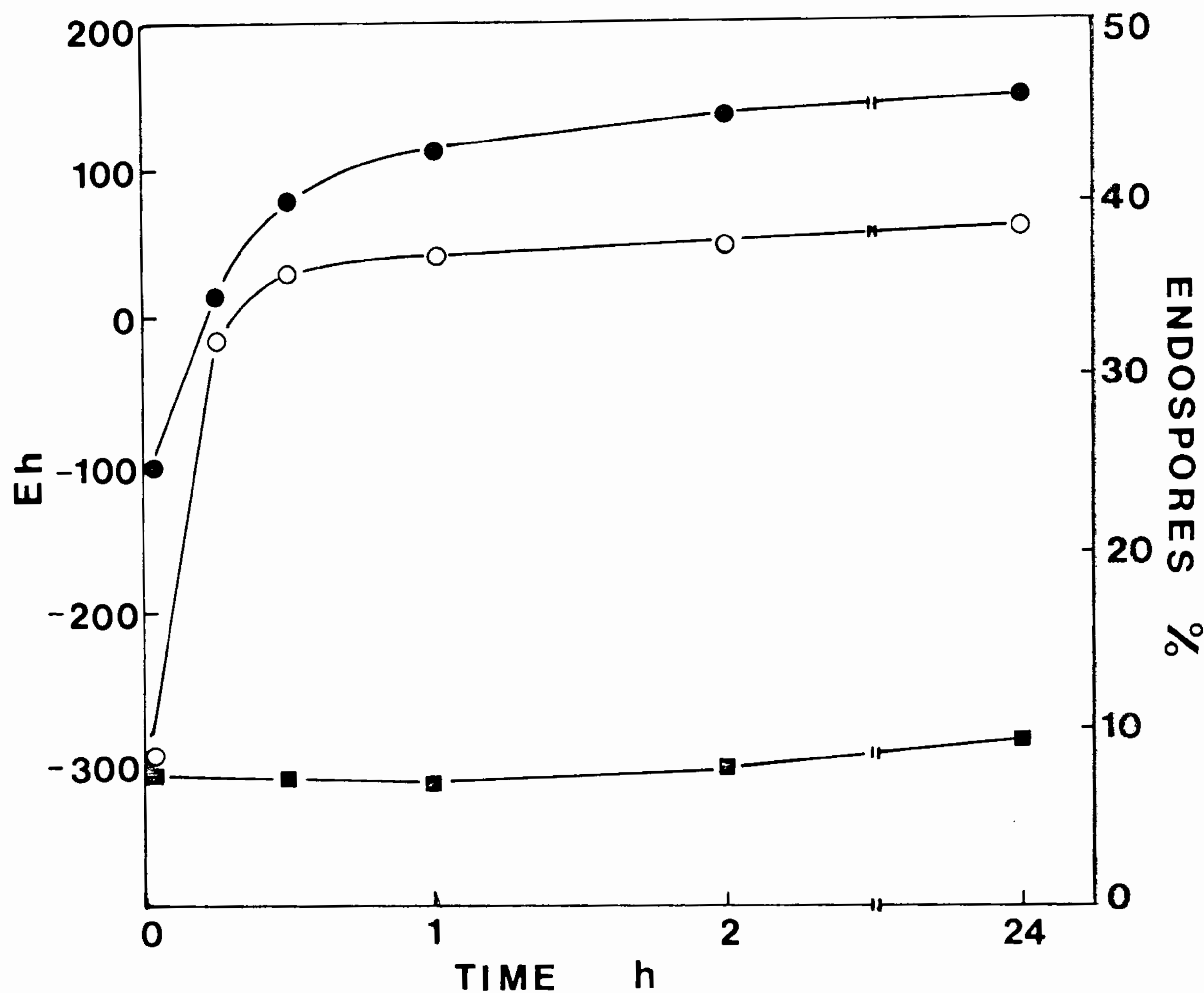


Figure 5.6

Effect of Eh on endospore formation in C. acetobutylicum P262 cultures exposed to oxygen for increasing time intervals before reincubating under anaerobic conditions.
Eh value during anaerobic growth (■); Eh value in an exponential (6 h) anaerobic culture exposed to air for a further 24 h (○); Corresponding spore counts (●) (made after 24 h incubation and expressed as a percentage of maximum cell number in anaerobic control cultures [4 x 10^8 cells ml^{-1}]).

Culture samples taken after 6 h anaerobic growth were used to determine the minimum duration of aerobic conditions required to stimulate sporulation (Fig. 5.6). During the first 30 min exposure, there was a rapid increase in sporulation from 20 to 38%. A further increase from 38 to 45% occurred after prolonged incubation (2 - 24 h) in air.

The amount of dissolved oxygen in all the abovementioned cultures remained below levels which were detectable with an oxygen electrode.

Complex medium cultures (6 h), aerated by shaking for short time intervals prior to reincubation anaerobically, showed decreased levels of sporulation (Table 5.10). Dissolved oxygen (< 1 mg dm^{-3} at 1 atm air) (Morris, 1975), could be measured in samples which were agitated for 120 seconds and in these cultures, sporulation occurred in < 1% of the population.

Anaerobic C. acetobutylicum P262 cultures grew normally at an Eh of +326 mV, maintained by the addition of 0.5 mM potassium ferricyanide. When the poised system was made aerobic at the end of the growth phase (6 h), the Eh remained unchanged. Phase-bright clostridial forms were produced in the aerobic and anaerobic cultures containing potassium ferricyanide, but spores were not observed.

TABLE 5.10

Effect of aeration by shaking on endospore formation in *C. acetobutylicum* P262. Late exponential cultures (6 h) in CBM were shaken in air for short time intervals prior to reincubation under anaerobic conditions. The number of cells which formed mature spores after 24 h is expressed as a percentage of the maximum cell count in anaerobic control cultures (4×10^8 cells ml^{-1}).

Duration of shaking (sec)		Endospores (%)
Controls:	anaerobic	20
	aerobic	40
	5	26
	20	17
	30	11
	120	< 1

5.3.5.3. Effect of peroxide and quenchers of toxic oxygen derivatives

The addition of catalase and superoxide dismutase (SOD), the quenchers of toxic oxygen metabolites in aerobic cultures, did not affect the final levels of sporulation in *C. acetobutylicum* P262 cultures exposed to air (Table 5.11). However, under anaerobic conditions, SOD inhibited sporulation in CBM. Less than 1% mature spores were formed after 24 h.

Hydrogen peroxide (H_2O_2) production in biological systems is the consequence of a two-electron reduction of molecular oxygen. In most aerobic bacteria, H_2O_2 is quickly removed by active catalases and peroxidases. The addition of H_2O_2 did not mimic the "oxygen effect" on sporulation in anaerobic cultures of strain P262 (Table 5.11). The sporulation of aerobically incubated cultures was more sensitive to H_2O_2 and 100 mg ml^{-1} peroxide completely inhibited sporulation in air.

5.3.5.4. Sporulation mutants

The effect of aeration on the sporulation of the pleitrophic rifampicin-resistant sporulation mutants (see Chapter III) was investigated (Table 5.12). An interesting mutant was the *spo*-2 mutant which produced the clostridial stage and forespore septa, but did not sporulate under anaerobic conditions. However, in the presence of air, this mutant was induced to sporulate and 30% mature spores were obtained. The other *spo* mutant (*spo*-1), which

TABLE 5.11

Effect of catalase, SOD and H_2O_2 on endospore formation in CBM cultures. Additions were made to late exponential (6 h) cultures which were then incubated anaerobically or aerobically for 24 h. The number of cells which formed endospores is expressed as a percentage of the maximum cell count obtained in anaerobic control cultures (4 x 10^8 cells ml^{-1}).

Addition	Concentration	Endospores (%)	
		Anaerobic	Aerobic
None		20	40
Catalase	200 U ml^{-1}	16	36
SOD	200 U ml^{-1}	< 1	30
H_2O_2	10 mg ml^{-1}	22	33
H_2O_2	100 mg ml^{-1}	18	0

TABLE 5.12

Endospore formation by sporulation deficient mutants of *C. acetobutylicum* P262 under anaerobic and aerobic conditions. Cultures (6 h) in CBM were incubated anaerobically or aerobically for a further 24 h. The number of cells which formed endospores is expressed as a percentage of the maximum cell count in anaerobic cultures (3 - 4 x 10^8 cells ml^{-1}).

Strain	Endospores (%)	
	Anaerobic	Aerobic
P262	20	50
cls-1	0	0
cls-2	0	0
spo-1	0	0
spo-2	0	30
spo-3	10	30
spo-4	15	20
spo-5	10	10

also produced the clostridial stage, but few sporulation septa (< 5% in CBM), and the two cls mutants (cls-1; cls-2), which did not produce the clostridial stage were unable to sporulate under anaerobic or aerobic conditions. Sporulation was enhanced in two of the oligosporogenous mutants (spo-3; spo-4) exposed to air, but not in the third oligosporogenous mutant (spo-5).

5.4. DICUSSION

Chemically defined and complex liquid media were used to determine the environmental factors which are involved in the shift from the acidogenic phase to the solventogenic phase and in the production of endospores in C. acetobutylicum P262. The shift to the solventogenic phase and the onset of sporulation are directly linked to the cessation of active growth. It is therefore important to distinguish between the factors responsible for the inhibition of cell division and those responsible for the induction of the various physiological and morphological changes.

The growth of a bacterial population in batch culture is usually limited by the amount of available nutrients or by the accumulation of metabolites which are inhibitory to the cell.

In C. acetobutylicum cultures containing low concentrations of glucose (< 10 g l^{-1}), complete utilisation of the substrate during the vegetative growth phase yielded very low levels of acetate and butyrate. Under these conditions, premature growth inhibition was a consequence of carbohydrate depletion rather than end-product inhibition. A similar situation was observed in nitrogen-limited cultures (< 0.3 g l^{-1} DAP). Incomplete glucose utilisation and premature growth inhibition occurred before the concentration of acid end-product had reached an inhibitory level.

Nutrient limitation by carbon or nitrogen did not permit the development of the solventogenic phase and no endospore formation occurred.

Media which contained increased concentrations of glucose and nitrogen supported a total cell population of between 4 and 6 x 10^8 cells ml^{-1}. The accumulation of 4 - 5 g l^{-1} acetate and butyrate represented the growth-limiting threshold in CAMM. Once this threshold level had been reached, the specific growth rate was reduced and cell division was inhibited. The cessation of growth occurred although substrate was still present in excess.

In addition to the inhibitory effect of acid end-product on cell division, recent investigations have provided evidence to support the conclusion that acetate and butyrate are also directly involved in the triggering of solvent production in C. acetobutylicum (Häggström and Molin, 1980; Gottschal and Morris,

1981a and 1982; Yu and Saddler, 1983). The results obtained from resuspension studies as well as the direct addition of acids to strain P262 cultures indicated that these acids are directly involved in the initiation of the whole of the second phase of the fermentation, including clostridial stage formation, solvent production, granulose accumulation and extracellular capsule production in C. acetobutylicum P262. The production of low levels of ethanol in cultures limited by nutrient starvation suggests that the biochemical pathway for ethanol production is either regulated by a lower threshold level of acetate and butyrate, or by some factor other than acid concentration.

The decrease in the culture pH which occurs as a result of acid end-product production also plays an important role in the onset of the second phase of the fermentation. The results of this study as well as those reported by Gottschal and Morris (1981a) in which non-metabolisable acids were used to replace acetate and butyrate, indicated that the attainment of low pH alone is not sufficient to initiate the solventogenic phase. However, the pH of the culture medium is important and must be maintained within a relatively narrow pH range to ensure that the shift to the solventogenic phase is achieved. The pH range appears to differ between C. acetobutylicum strains and the threshold of acid end-products required to inhibit growth and trigger the metabolic shift, is likely to vary depending on the pH of the culture. The degree of acid ionisation is determined by the pH conditions of the medium. Since it is only the uncharged form of the acid which is able to cross the cell

membrane, the pH of the medium may influence both the external and internal concentrations of acetate and butyrate (Gottschal and Morris, 1981a). The pH conditions which were unfavourable to the induction of the second phase of the fermentation, also inhibited the development of mature spores in C. acetobutylicum P262.

In addition to the threshold concentration of acid end-products, and the correct pH level, there was also a requirement for the presence of residual glucose at the end of the acidogenic phase. The additional glucose was necessary for the successful development of the solventogenic phase and endospore formation. The proportion of cells which formed clostridial forms and the solvent yields were dependent on the amount of residual glucose and maximum production occurred when the concentration of remaining glucose was in excess of 20 - 30 g l^{-1}. A threshold concentration of nitrogen (1 g l^{-1} DAP), which maintained culture viability, was also required for the development of the solventogenic phase.

A requirement for a minimum level of glucose and nitrogen during solvent production has been reported in other strains of C. acetobutylicum (Gottschal and Morris, 1981b; Monot et al., 1982).

Mature endospore formation in C. acetobutylicum P262 required an exogenous supply of carbon and nitrogen (but possibly not phosphate). The number of endospores produced showed a

direct relationship to the initial concentration of glucose and ammonia. Maximum endospore production occurred at 60 g l^{-1} glucose and 4 g l^{-1} DAP. It was also apparent that the endogenous carbon source (granulose) which accumulated during the clostridial stage, could not be used as the sole energy supply for differentiation.

The initiation of sporulation in C. acetobutylicum P262 is complex and although the cessation of growth and the onset of the solventogenic phase may be brought about by a threshold concentration of acetate and butyrate, these acid end-products may not be directly involved in the initiation of the sporulation process.

Solvent production and the conversion of cells to the clostridial stage were not significantly affected by the type of carbohydrate or ammonium salt used to support growth. However, there was an effect on the number of endospores produced during the fermentation. The addition of extra nitrogen to CAMM containing low concentrations of DAP (1 g l^{-1}) enhanced solvent production and endospore formation, whereas the addition of excess phosphate or acetate and butyrate to nitrogen depleted cultures was without effect.

The production of mature spores was not related to the growth rate of the CAMM culture. This is in contrast to the situation which exists in C. thermosaccharolyticum (Hsu and Ordal, 1969b). In C. thermosaccharolyticum, sporulation was

obtained in the presence of various carbon sources which had in common the effect of reducing the growth rate. The altered metabolic pattern which was associated with the sporulating population, did not occur in media in which glucose or a similar readily metabolisable carbohydrate was present in excess (Hsu and Ordal, 1970). A poorly utilisable carbon source such as starch or raffinose was also essential for good sporulation in C. perfringens (Labbe and Rey, 1979).

An incubation temperature above 36°C increased the culture growth rate and severely inhibited sporulation in C. acetobutylicum P262. Solvent yields were consistently higher at 34°C than at 37°C, however the difference was less than 1 g l^{-1} (van der Westhuizen, 1982). This observation was supported by the fact that there was no significant difference in the proportion of cells which formed the clostridial stage in CAMM at the two different temperatures.

The temperature-sensitive sporulation observed in the P262 strain resembles that of B. subtilis ts-5 (Leighton et al., 1972), a temperature-sensitive mutant, which failed to produce early sporulation markers at the restrictive temperature, and was blocked at the first morphological stage of development. This phenomenon provides a novel physiological tool for manipulative studies in the wild-type Clostridium strain.

The maintenance of a high partial pressure of hydrogen in CAMM did not encourage clostridial stage formation, the

conversion of acid end-products to neutral solvents, or the development of mature spores in C. acetobutylicum P262. A mixed gas atmosphere containing N_2, CO_2 and H_2 (70:20:10 [v/v/v]) or N_2 or CO_2 alone provided a more suitable gaseous atmosphere for the shift to the solventogenic phase and the production of endospores. Rogers and Hansen (1983) found that an H_2:CO_2 (95:5 [v/v]) gas phase during incubation did not enhance butanol formation, nor the activity of two dehydrogenases involved in the solvent producing fermentation pathway in C. acetobutylicum.

The stringency of the an aerobic atmospheric conditions during growth had a significant effect on the induction of sporulation in C. acetobutylicum P262.

The exposure of complete and minimal medium cultures to air provided an easily manipulatable system for the induction of sporulation in C. acetobutylicum P262. Sporulation in cultues exposed to air was influenced by the nature of the culture medium, the age of the exposed cells and the degree of exposure. Anaerobic CBM cultures approaching the end of the exponential phase and mid-logarithmic phase GSMM cells achieved maximum sporulation after exposure to aerobic conditions. At this stage, the C. acetobutylicum P262 cells appeared to be in the most suitable physiological state to respond to the aerobic sporulation trigger. It is interesting to note that the highest sporulation was induced in GSMM before the pH of the medium dropped below pH 4.8. A corresponding pH condition supported the highest level of sporulation in aerobic CBM cultures.

A relationship between culture Eh and the degree of sporulation in CBM was observed. However, Eh was not the only requirement for improved endospore formation. Anaerobic cultures poised at an Eh value of +326 mV with potassium ferricyanide grew normally, but failed to sporulate. A similar effect of Eh on growth of C. acetobutylicum was observed by O'Brien and Morris (1971). They concluded that oxygen inhibition of growth occurs by some mechanism other than the non-specific elevation of culture Eh.

The induction of sporulation in air did not appear to be a consequence of H_2O_2 or the generation of other toxic oxygen derivatives by C. acetobutylicum P262. Hydrogen peroxide did not enhance sporulation under anaerobic conditions. It has been suggested that H_2O_2 is destroyed by interaction with pyruvate an other reducing components of the medium (O'Brien and Morris, 1971). A concentration of 100 mg ml^{-1} H_2O_2 prevented the production of spores in cultures of C. acetobutylicum P262 exposed to air. Catalase and SOD did not inhibit sporulation in aerobic cultures. However, SOD inhibited sporulation under anaerobic conditions. The reason for this phenomenon is not known. Hewitt and Morris (1975) have reported the production of SOD by a C. acetobutylicum strain which is able to survive transient exposure to low concentrations of oxygen.

The reductive detoxification of oxygen by anaerobic bacteria at the expense of reducing power is reflected in a specific increase in NADH oxidase activity and a rapid decrease in the

intracellular ATP levels (O'Brien and Morris, 1971). The way in which these conditions, regulated by the amount of contaminating oxygen, inhibit growth and provide the trigger which initiates sporulation in C. acetobutylicum P262 cultures is complex. It would also seem unlikely that any one unifying hypotheses would adequately account for the oxygen sensitivity displayed by all clostridia in different media.

The environmental factors responsible for the initiation of sporulation in C. acetobutylicum P262 differed markedly from those in B. subtilis, where sporulation is initiated by nutrient starvation (Young and Mandelstam, 1979). In C. acetobutylicum P262, there was a requirement for an exogenous supply of carbon and nitrogen and sporulation occurred in cultures limited rather by acid end-product accumulation (or by the presence of air). Under the experimental conditions used in this study, mature endospore production did not occur in the absence of the shift to the second fermentation phase. However, acetate and butyrate, which inhibit growth and trigger the solventogenic phase, may not be directly involved in the initiation of forespore septum formation. This will be a topic of future research.

CHAPTER VI

THE USE OF INHIBITORS FOR STUDYING SPORULATION

Summary

A number of chemical inhibitors which affect cellular functions in *B. subtilis* were used to study differentiation in *C. acetobutylicum* P262. Inhibitors of DNA replication inhibited forespore septum formation. However, clostridial stage formation was not inhibited, suggesting that this stage is an essential but independent event, distinguishable from sporulation-specific events which require chromosomal replication for induction. Two physiological inhibitors, fluoracetic acid and diethyl malonate, also blocked the sporulation process after clostridial stage formation. Inosine, caffeine and decoynine stimulated sporulation at concentrations which retarded growth in the P262 strain. Two other purine analogs, 6-mercaptopurine and 6-thioguanine which reduced the exponential growth rate did not promote sporulation.

6.1. INTRODUCTION

The fundamental question of the regulation of differentiation remains unsolved, but nevertheless, important advances have been made.

Experimental evidence provided by a number of workers has indicated that the initiation of sporulation is related to the cell cycle and to chromosomal replication in B. subtilis (Dawes et al., 1971; Mandelstam et al., 1971; Mandelstam and Higgs, 1974; Dunn et al., 1978; Young and Jeffs, 1978). Induction of sporulation occurs as the replication fork passes a restricted region of the bacterial chromosome, about 15 - 20 min from the replication origin.

The requirement for DNA replication during the initiation of sporulation clearly distinguishes sporulation from other catabolite repressible functions in B. subtilis. The inhibition of DNA replication has been used as a criterion for determining events which are unique to sporulation.

Clarke and Mandelstam (1980) used a temperature-sensitive chromosomal initiation mutant to obtain a cell population which was synchronised in replication. Samples exposed to step-down conditions at intervals during the replication cycle were optimally receptive to the induction of sporulation approximately 15 min after the beginning of chromosomal duplication. However, the induction of serine protease and RNAase production, two early sporulation marker events, were not related to the state of chromosomal replication. Specific inhibitors of DNA replication have been used to illustrate the same point. Acridine orange, which inhibits DNA template function by intercalation between DNA base pairs and a structurally similar compound, promethazine, blocked spore formation in B. sutilis at concentrations which

were sub-inhibitory to growth (Burke and Spizizen, 1977). Serine protease production, antibiotic synthesis and the catabolite repressed enzymes of the tricarboxylic acid cycle were not sensitive to sporulation inhibitory concentrations of the drug. Acridine orange also reduced the frequency of spores in a number of catabolite resistant mutants (Takahashi and MacKenzie, 1982). Spore development proceeded as far as stage IV in the presence of the inhibitor. Rogolsky and Nakamura (1974) reported the preferential inhibition of sporulation by another intercalating agent, ethidium bromide. Post logarithmic proteolytic and antibiotic activity were not inhibited by the dye. Cells escaped inhibition when ethidium bromide was added at the end of the first sporulation stage and prior to the synthesis of germ cell wall peptidoglycan.

It is apparent that although the repression of sporulation and certain other catabolite sensitive functions may involve some common catabolite repressor, a fundamental difference exists between the processes with regard to chromosomal replication.

A variety of compounds which interfere with purine biosynthesis stimulate sporulation. The effectiveness of these compounds varied from one strain to another and with the carbon source. Decoynine, a specific inhibitor of GMP synthetase in *B. subtilis*, induced sporulation when added to exponential cells at a concentration which partially inhibited growth (Mitani *et al*., 1977; Freese *et al*., 1978). A specific decrease in GTP (and GDP) which occurred in the presence of the inhibitor has also

been observed under conditions which initiate sporulation by nutrient starvation (Lopez et al., 1979). Freese et al., (1978) demonstrated that a number of purine analogs which caused partial purine starvation will induce sporulation in B. subtilis, whereas the inhibition of pyrimidine nucleotide synthesis had no effect. Methylxanthines increased the sporulation frequency of C. perfringens in certain media and promoted enterotoxin biosynthesis (Sacks and Thompson, 1977; Labbe and Nolan, 1981). These compounds, which interfere with the incorporation of purines into nucleotides, had a weak effect on the induction of sporulation in B. subtilis (Freese et al., 1978). Papaverine, which is a benzyisoquinoline, rather than a purine, also induced sporulation (Sacks, 1982) and enterotoxin production (Craven and Blankenskip, 1982) in certain strains of C. perfringens. The partial reversal of caffeine induced sporulation by exogenous nucleotides and the demonstration that papaverine was able to inhibit nucleoside uptake, suggested that alterations in nucleotide synthesis (possibly also GTP and GDP) may promote sporulation in C. perfringens.

The purine nucleotide, inosine, reversed the selective inhibition of sporulation by m-aminobenzeneboronic acid in B. subtilis (Sekar et al., 1981). Other nucleosides were found to be less effective. A direct stimulation of sporulation by inosine was observed in cells transferred to a minimal salts medium. Inosine also increased the incidence of spore formation in C. perfringens (Sacks and Thompson, 1975). Inosine showed chemical and physical characteristics very similar to those reported for an endogenous factor, "sporogen", which appeared in sporulating Bacillus cells (Srinivasan, 1965).

A decrease in GTP and GDP has been implicated in the initiation of sporulation (Lopez et al., 1979). Under such conditions, there will at least be a transient increase in the pool size for inosine metabolism. An inosine-specific purine nucleoside phosphorylase increases dramatically at the end of logarithmic growth in B. cereus (Gardner and Kornberg, 1967). These observations suggest that inosine represents a positive effector which induces sporulation.

The evidence that different sporulation conditions (including starvation and partial purine limitation) will promote an indirect stringent response (i.e. a decrease of GTP and GDP levels), has prompted the hypothesis that prespore septation per se may not necessarily require the synthesis of any particular effector molecule, but rather that differentiation may be initiated by the establishment of a new balance of polymer synthesis (RNA, protein and membrane) through the control of substrates available to the cell (Freese et al., 1978).

Changes in the enzymes involved in energy production are known to occur at the start of sporulation. Foremost among these are the increases in activity of the enzymes of the tricarboxylic acid cycle, which supply energy and carbon skeletons for amino acid biosynthesis during sporulation in Bacillus. The importance of the tricarboxylic acid cycle for sporulation has been amply demonstrated (Hanson and MacKenchie, 1969; Vinter, 1969). Mutants lacking enzymes of the tricarboxylic acid cycle are blocked at stage 0 or I of sporulation (Szulmajster and Hanson,

1965). Such mutants apparently cannot maintain the concentration of ATP and NADH necessary for spore formation, although in certain circumstances the block may be overcome (Yousten and Hanson, 1972).

The tricarboxylic acid cycle enzymes are not sporulation specific and are expressed at low levels in growing cells. Nonetheless, the coordinate induction of the tricarboxylic acid cycle and sporulation upon nutrient limitation, suggests that whatever causes induction of the metabolic cycle is likely to regulate in parallel the initiation of sporulation.

A number of chemical inhibitors prevent acetate utilisation and block the tricarboxylic acid cycle in sporulating bacilli and other biological systems. Fluoroacetic acid and α-picolinic acid have been found to inhibit the tricarboxylic acid cycle in the aconitase enzyme. The site of malonate inhibition is the succinate dehydrogenase enzyme complex. Fluoroacetic acid reduced glucose assimilation and inhibited refractile spore formation in sporulating C. thermosaccharolyticum cultures (Campbell and Ordal, 1968). However, metabolic inhibition occurred at some site other than the aconitase enzyme.

The environmental conditions responsible for the initiation of sporulation in C. acetobutylicum P262 are different from those in B. subtilis where sporulation is triggered by nutrient deprivation. However, some aspects of the internal effector system may be similar in the two bacteria. An attempt has been

made to determine whether DNA replication and purine limitation are required for the induction of sporulation in _C. acetobutylicum_ P262. The effect of fluoroacetic acid and diethyl malonate on sporulation was also investigated. The effect of the inhibition of DNA synthesis on the induction of sporulation was used as a criterion for determining the sporulation-specific nature of solventogenesis and clostridial stage formation in _C. acetobutylicum_ P262.

6.2. MATERIALS AND METHODS

6.2.1. Bacterial strain

The _C. acetobutylicum_ P262 strain was used.

6.2.2. Culture media

As described in Appendix A.

6.2.3. General methods

Heat shocking procedures, culture inoculation and fermentation methods were described previously (2.2).

6.2.4. Physical measurements, growth determination and the characterisation of cell morphology

Physical measurements, growth and morphological determinations were outlined in section 2.2 and 3.2.

6.2.5. Inhibitor studies

The effect of the following inhibitors on clostridial stage formation and sporulation was studied:

Inhibitors of DNA replication: ethidium bromide, (Merck); acridine orange, (BDH); novobiocin (Boehringer); naladixic acid, (Sigma) and 6-(p-hydroxyphenylazo)-uracil (HPUra), kindly supplied by B. Langley of Imperical Chemical Industries, PLC, London.

Purine analogs: Decoynine was a gift from G. Whitfield of The Upjohn Company, Kalamazoo, Michigan; 6-thioguanine, 6-mercaptopurine and inosine were obtained from Sigma and caffeine was purchased from Fluka Chemical suppliers.

Physiological inhibitors: Diethylmalonate (EDH) and fluoroacetic acid (Merck) were used in this study.

Freshly prepared solutions of each inhibitor were added to mid-exponential phase CBM and CAMM cultures (0.5 - 1 x 10^8 cells ml^{-1}). All manipulations were carried out under stringent anaerobic conditions in an anaerobic glove box (Forma Scientific). All cultures were incubated at 34^oC.

The experimental data presented in this chapter are results typical of each experiment. However, experiments were repeated at least three times.

6.3. RESULTS

6.3.1. Effect of inhibitions of DNA replication on sporulation in *C. acetobutylicum* P262

Different concentrations of inhibitors of DNA synthesis were added to exponential phase cells (*c* 5 x 10^7 cells ml^{-1}) in CBM broth in order to determine the concentration of inhibitor required to obtain complete inhibition of sporulation (Table 6.1). The complex medium was used initially because it allowed the use of optical density as a convenient measure of growth and endospore formation occurred rapidly (24 h) in this medium.

It was found that 10 ug ml^{-1} ethidium bromide, 20 ug ml^{-1} HPUra and novobiocin, and 100 ug ml^{-1} naladixic acid inhibited sporulation. A residual level of endospore formation (10% and less) occurred in these cultures.

Acridine orange (100 ug ml^{-1}) did not affect the growth or sporulation of C. acetobutylicum cultures (data not shown), and it is concluded that this reagent is ineffective at the low pH levels obtained in CBM broth.

Ethidium bromide, HPUra and novobiocin were used in further experiments to determine the effect of each inhibitor on clostridial stage and endospore formation. The defined medium (CAMM) wich produced high levels of mature spores (70 - 80%) in untreated cultures, was used in this study (Table 6.1).

Inhibitors were added to CAMM cultures before the onset of granulose accumulation, when the cell concentration was c 1 x 10^8 cells ml^{-1}. Minimal medium grown cells were more sensitive to inhibition by ethidium bromide than were the complex medium cultures. Ethidium bromide (5 ug ml^{-1}) HPUra (70 ug ml^{-1}) and novobiocin (20 ug ml^{-1}) markedly inhibited sporulation. Approximately 10% of the treated cells escaped inhibition and produced refractile spores.

The production of solvents by C. acetobutylicum P262 cultures treated with different concentrations of ethidium bromide was investigated in CAMM (Table 6.2). The inhibition of the sporulation programme after the clostridial stage did not interfere with solvent production. The highest solvent yield was obtained in the culture treated with 1.0 ug ml^{-1} ethidium bromide. This culture produced the lowest level of sporulation and the majority of the cells remained as clostridial forms.

TABLE 6.1

Effect of inhibitors of DNA replication on clostridial stage and endospore formation in C. acetobutylicum P262. Different concentrations of the inhibitors were added to mid-exponential phase complete and minimal medium cultures. The number of clostridial forms and cells which formed endospores is expressed as a percentage of control cultures without inhibitors. Control cultures produced c 120 x 10^6 endospores ml^{-1} in CBM; and c 500 x 10^6 clostridial stage cells ml^{-1} and c 400 x 10^6 endospores ml^{-1} in CAMM.

Inhibitor	Concentration ($ug\ ml^{-1}$)	CBM	CAMM	
		Endospores (%)	Clostridial forms (%)	Endospores (%)
Control	0	100	100	100
Ethidium	0.5	-	94	10
bromide	10	8	-	-
HPUra	20	10	94	8
Novobiocin	20	0.4	88	3
Naladixic acid	100	4	-	-

TABLE 6.2

Effect of ethidium bromide on clostridial stage and endospore formation and solvent production by *C. acetobutylicum* P262. Increasing concentrations of ethidium bromide were added to mid-exponential phase CAMM cultures. The number of clostridial forms and cells which formed endospores is expressed as a percentage of the control culture without ethidium bromide. Control cultures produced *c* 500 x 10^6 clostridial stage cells ml^{-1} and *c* 400 x 10^6 endospores ml^{-1}.

Ethidium bromide ($\mu g\ ml^{-1}$)	Clostridial forms (%)	Endospores (%)	Solvents ($g\ l^{-1}$)
0	100	100	13.89
0.4	100	50	13.74
1.0	100	10	16.01

6.3.2. Effect of various purine analogs on sporulation in *C. acetobutylicum* P262

Five prurine analogs were tested for their ability to promote sporulation in *C. acetobutylicum* P262. The compounds were added to mid-exponential phase cells (c 5 x 10^7 cells ml^{-1}) grown in CBM.

The concentration of purine analog yielding the highest level of sporulation is reported (Table 6.3). At these concentrations, the inhibition of growth varied from 12% (6-mercaptopurine) to 56% (inosine) of the growth rate obtained in control cultures without any additions. Inosine (0.75 - 1 mg ml^{-1}) promoted sporulation in CBM and a spore titre double that obtained in untreated control cultures was observed. Caffeine (50 ug ml^{-1}) and decoynine (50 ug ml^{-1}) stimulated sporulation to a lesser degree (140% and 115%, respectively). The two mercaptopurines inhibited growth and sporulation at a concentration of 50 ug ml^{-1}. Sporulation was not enhanced in CAMM cultures containing purine analogs.

Inosine (1 mg ml^{-1}) did not overcome the sporulation inhibition by ethidium bromide in complex or minimal media.

TABLE 6.3

Effect of purine analogs on growth and endospore formation in *C. acetobutylicum* P262. Additions were made to mid-exponential phase CBM cultures. The number of cells which formed endospores is expressed as a percentage of the control culture without added purine. Control cultures produced c 120 x 10^6 endospores ml^{-1}.

Addition	Concentration ($ug\ ml^{-1}$)	Generation time (min)	Endospores (%)
None	-	30	100
6-mercaptopurine	50	34	54
6-thioguanine	50	36	38
Inosine	750	39	176
	1 000	47	196
Caffeine	50	37	140
Decoynine	50	35	115

6.3.3. Effect of physiological inhibitors on sporulation in *C. acetobutylicum* P262

Fluoroacetic acid added to mid-exponential phase P262 cells (0.5 - 1 x 10^8 cells ml^{-1}) had a minimum inhibitory concentration of 10 mM in CBM and 50 mM in CAMM. Sub-inhibitory concentrations of fluoroacetic acid (5 mM in the complex medium and 25 mM in the defined medium) inhibited sporulation, but did not affect clostridial stage formation (Table 6.4).

Solvents (13.25 g l^{-1}) were produced in CAMM supplemented with 25 mM fluoroacetic acid. The final solvent ratio [7 : 2 : < 1; *n*-butanol (10.12 g l^{-1}) : acetone (2.77 g l^{-1}) : ethanol (0.369 g l^{-1})] was not affected by this concentration of inhibitor.

Diethyl malonate (20 - 33 mM) also inhibited sporulation at concentrations which were sub-inhibitory for growth in CBM (Table 6.4). Clostridial stage formation was not sensitive to inhibition by diethyl malonate. The effect of this inhibitor on solvent production in CAMM was not determined.

TABLE 6.4

Effect of physiological inhibitors on clostridial stage and endospore formation in C. acetobutylicum P262. Inhibitors were added to mid-exponential phase complete and minimal medium cultures. The number of clostridial forms and cells which formed endospores is expressed as a percentage of control cultures without inhibitors. Control cultures produced c 300 x 10^6 clostridial stage cells ml^{-1} and c 120 x 10^6 endospores ml^{-1} in CBM; and c 500 x 10^6 clostridial stage cells ml^{-1} and c 400 x 10^6 endospores ml^{-1} were produced in CAMM.

Inhibitor	Concentration (mM)	CBM		CAMM		
		Clostridial forms (%)	Endospores (%)	Clostridial forms (%)	Endospores (%)	Solvents ($g\ l^{-1}$)
None	-	100	100	100	100	13.89
Fluoroacetic acid	5	100	15	-	-	-
	25	-	-	90	< 1	13.25
Diethyl malanate	20	100	26	-	-	-
	33	90	4	-	-	-

6.4. DISCUSSION

Ethidium bromide, HPUra, novobiocin and naladixic acid which inhibit sporulation in B. subtilis (Rogolsky and Nakamura, 1974; Dunn et al., 1978; Vazquez-Ramos and Mandelstam, 1981; Takahashi and MacKenzie, 1982), affected sporulation in C. acetobutylicum P262. Forespore septum initiation in stationary phase cultures did not occur in the presence of ethidium bromide, HPUra or novobiocin. High concentrations of naladixic acid (100 ug ml^{-1}) were required to inhibit sporulation in CBM. The residual level of sporulation which was observed in the cultures containing inhibitors suggests that a small proportion of the population was committed to the sporulation process at the time of addition of the inhibitor, and may reflect the degree of asynchrony in the culture. Our results suggest a requirement for DNA replication during sporulation. However, a measurement of DNA synthesise in treated cultures will be necessary to confirm this statement.

It has been shown that the inhibition of sporulation by DNA gyrase inhibitors was not correlated with the inhibition of DNA synthesis in B. subtilis (Vazquez-Ramos and Mandelstam, 1981). It was demonstrated that the inhibition of sporulation resulted rather from an effect of gyrase on RNA synthesis. It is not known whether the concentrations of ethidium bromide (Rogolsky and Nakamura, 1974) and acridine orange (Burke and Spizizen, 1977) which selectively inhibited sporulation in B. subtilis also blocked DNA replication. Both groups suggested an effect of the

inhibitor at the level of transcription which was related to the binding affinity of the intercalating dye to certain promotor sites.

The requirement for DNA replication during the initiation of sporulation in B. subtilis has enabled a clear distinction to be made between sporulation-specific events and those events which may be essential for sporulation, but which are not sporulation-specific (Young and Mandelstam, 1979). The inhibition of sporulation by inhibitors of DNA replication occurred after the clostridial stage in C. acetobutylicum P262. In ethidium bromide treated cultures, solvents were produced. This observation suggests that the solvent-producing clostridial stage is an essential but independent stage which accompanies the initiation of sporulation. It is not a sporulation-specific event as it is not dependent on chromosomal replication. In C. acetobutylicum P262 and B. subtilis, the first sporulation-specific stage which is triggered during DNA replication is the production of a forespore septum. Day and Costilow (1964a) also demonstrated that a small amount of DNA was synthesised in sporulating cultures of C. botulinum. Nucleic acid synthesis ceased after the completion of stage II of sporulation.

Decoynine, a specific inhibitor of GMP synthesis, which induced sporulation in B. subtilis (Mitani et al., 1977), and the purine analog, caffeine, which induced sporulation in C. perfringens (Sacks and Thompson, 1975; Labbe and Nolan, 1981),

were able to stimulate sporulation in C. acetobutylicum P262. Two mercaptopurines which induced sporulation in B. subtilis (Freese et al., 1978), did not promote sporulation in C. perfringens (Labbe and Nolan, 1981) or in C. acetobutylicum P262. Inosine, a precursor of purine nucleotide synthesis, and a sporogen under certain conditions in B. subtilis (Sekar et al., 1981) and C. perfringens (Sacks and Thompson, 1975), stimulated sporulation most effectively in C. acetobutylicum P262. There was a stringent requirement for DNA replication at the time of spore induction, which could not be overcome in the presence of inosine.

The molecular basis for the effect of purine analogs on the induction of sporulation in C. acetobutylicum P262 is not known. Future studies will be aimed at a quantitative measure of the intracellular levels of nucleotides and other cellular pool components in cultures of C. acetobutylicum P262. Apart from GTP and GDP, it is possible that ATP and cyclic nucleotides may play effector roles in the induction of sporulation in C. acetobutylicum P262.

Fluoroacetic acid and diethylmalonate inhibited sporulation in C. acetobutylicum P262. Sporulation was blocked after the clostridial stage and there was no effect of the inhibitor on the amount of acetone, butanol and ethanol formed during the fermentation. In addition, acetate and butyrate production which serve to limit growth and trigger the second fermentation phase in strain P262, were not inhibited. It was not established

whether carbon incorporation into cellular material was the process inhibited by fluoroacetic acid and diethylmalonate. Campbell and Ordal (1968) found that fluoroacetic acid reduced the amount of ^{14}C incorporation into end-products and cellular fractions by 50% in vegetative and sporulating cultures of C. thermosaccharolyticum.

The use of metabolic inhibitors in studies of this nature is subject to the criticism that a detailed knowledge of the mechanism of inhibitory action is not known. It can be argued that C. acetobutylicum P262 is unable to sporulate because of a general metabolic disturbance created by the inhibitor, rather than a more specific relationship between sporulation and the inhibited function.

A very brief investigation of the internal effectors which trigger sporulation in C. acetobutylicum P262 has revealed a complex induction system which is similar in some respects to that which operates in B. subtilis. Forespore septum formation, the first sporulation-specific stage in both organisms, appears to be dependent on chromosomal replication and is stimulated by conditions of purine limitation and in the presence of inosine. The results obtained from studies using inhibitors of DNA replication indicated that the internal effector of clostridial stage formation and of spore induction are different. Certain physiological inhibitors which have been shown to affect the tricarboxylic acid cycle in other endospore-forming bacteria, also inhibited sporulation in C. acetobutylicum P262. However, the mechanism of inhibition was not established.

CHAPTER VII

CONCLUSION

Bacillus subtilis has achieved prominence among the endospore formers as a model system for the study of sporulation in other bacteria.

Sporulation commences with the end of exponential growth. In Bacillus the process is usually initiated when the cell encounters certain adverse nutritional conditions and asymmetric prespore septation occurs. The step-down conditions necessary for spore induction must exist before DNA synthesis has completed a round of replication. Sporulation is initiated as the replication fork passes a point 15 - 20 min from the replication origin (Young and Mandelstam, 1979).

The inhibition of guanine nucleotide synthesis which induces sporulation in the presence of carbon, nitrogen and phosphate, and the reduced levels of GTP and GDP which occur under nutrient starvation conditions, suggest that the purine nucleotides may influence the effector system which triggers sporulation in B. subtilis (Freese, 1981). On the other hand, Yousten and Hanson (1972) propose that a decrease in the energy level of the cell, mediated through changes in ATP concentration or adenylate

charge, is necessary for the onset of endospore formation. It is apparent that the complex biochemical mechanism by which the adverse nutritional conditions induce a stringent-type response which limits growth and provides the stimulus to initiate sporulation, remains unresolved.

Extensive temporal control of transcription is responsible for specific RNA synthesis during the course of sporulation. The expression of sporulation-specific genes appears to be mediated by the sequential modification of RNA polymerase (Losick and Pero, 1981). An increasing body of evidence suggests that there is a supplementary control of differentiation at the level of translation.

The knowledge gained from the study of sporulation in B. subtilis provided a guideline for the investigation of endospore formation in C. acetobutylicum P262. Chemically defined media have facilitated the study of sporulation in other endospore-forming bacteria and a chemically defined medium (CAMM) which supported extensive and relatively synchronous sporulation in C. acetobutylicum P262 was developed. The improved buffering capacity of CAMM was the most important factor responsible for the increased level of mature spores. Cells grown in CAMM produced high yields of solvents (10 - 14 g l^{-1}) and exhibited two physiological fermentation phases which were similar to those observed in the industrial ABE fermentation (Spivey, 1978). A major advantage of the defined medium was that it provided a suitable system for the study of the relationship between solvent

production and endospore formation in the P262 strain. The defined medium also facilitated a correlative physiological and morphological study and an investigation of the chemical and physical factors involved in the induction of solvent production and endospore formation.

The shift to the solventogenic phase was linked both to the cessation of cell division and the triggering of various physiological and morphological changes. A distinction could be made between the factors responsible for the inhibition of cell division and those responsible for the initiation of the solventogenic phase and endospore formation. In cultures which contained > 20 g l^{-1} glucose and > 0.6 g l^{-1} DAP, cell division was inhibited when 4 - 5 g l^{-1} acid end-products were present and neither carbon nor glucose were limiting. The enhanced glucose consumption which occurred following the cessation of growth was indicative of inhibition by acid end-product accumulation (Herrero, 1983). This study has shown that in addition to their effect on cell proliferation, acetate and butyrate also play an important role in the triggering of the shift to solvent production as well as other activities associated with the second phase of the fermentation (viz. clostridial stage formation, granulose accumulation and extracellular capsule production). Other studies have shown that the acid end-products are involved in the induction of solvent production (Gottschal and Morris, 1981a and 1982; Bahl et al., 1982a; Yu and Saddler, 1983; Martin et al., 1983).

The isolation and characterisation of pleiotrophic, sporulation-defective mutants supported the conclusion that the swollen clostridial stage cells represent the morphological stage associated with solvent production and granulose and capsule biosynthesis. Oligosporogenous mutants which formed reduced numbers of clostridial forms, produced intermediate levels of solvents and asporogenous mutants which failed to form the clostridial stage did not produce solvents, capsules or granulose. Although the results obtained from studies utilising mutants indicates that the various stationary phase events are interlinked, it is not known whether the relationship observed is due to some common regulatory mechanism shared by the individual pathways. The recent isolation of granulose-negative mutants which produce solvents and the clostridial stage, would suggest that there are some common regulatory controls, but that the pathways are able to operate independently

The environmental factors responsible for the initiation of sporulation in stationary phase C. acetobutylicum P262 cultures differed markedly from the nutrient starvation conditions which inhibited growth and initiated sporulation in Bacillus. In C. acetobutylicum P262 there was a requirement for an exogenous supply of carbon and nitrogen. Endospores were not produced in media in which the cessation of cell division occurred as a consequence of nutrient deprivation.

Site-specific inhibitors and antibiotics have been used to probe the molecular basis of spore induction in B. subtilis.

Similar studies were performed in C. acetobutylicum P262. In CAMM, endospore formation did not occur in the absence of clostridial stage formation, granulose accumulation or solvent production. However, the events associated with the solventogenic phase are not specific to the sporulation process as they were induced in the absence of chromosomal replication. The use of inhibitors of DNA replication indicated that, as in B. subtilis, septum formation is the first visible sporulation-specific event in C. acetobutylicum P262. The morphological sequence of events leading from septation to mature spore formation are also essentially the same in the two bacteria, the reversal of stages IV and V being the major difference.

The isolation of drug-resistant sporulation mutants suggested a control of sporulation at the level of transcription and translation in C. acetobutylicum P262. In addition, the stimulatory effect of decoynine, caffeine and inosine suggest that purine nucleotides may play a role in the induction of sporulation. These results provide some evidence to indicate that the internal effector system which regulates differentiation in C. acetobutylicum P262 shows some similarities to that which controls sporulation in B. subtilis.

The investigation of stationary phase differentiation in C. acetobutylicum has faciliated the proposal of a model which attempts to describe the mechanisms controlling the transition from the acidogenic phase to the solventogenic phase and endospore formation (Fig. 7.1). Three key features are considered:

1. Factors which are related to the limitation of growth.
2. Physiological and morphological changes related to the initiation of the solventogenic fermentation phase.
3. Initiation of sporulation-specific events leading to endospore formation.

During the initial fermentation phase, glucose is metabolised to acid end-products by the growing culture. The accumulation of acetate and butyrate results in a decrease in the pH which approaches a critical threshold level. A combination of the acid concentration and the culture pH results in the inhibition of cell division and no further increase in cell number occurs. The vegetative rods enter the stationary growth phase while carbon- and nitrogen-containing nutrients are still in excess. The cessation of cell division is accompanied by the inhibition of cell elongation. In Bacillus, the biosynthesis of vegetative cell wall constituents, RNA and protein decreases during growth limitaton by nutrient starvation. It seems likely that a similar reduction in the biosynthetic processes may occur in C. acetobutylicum P262 cells following the cessation of cell division. Experimental evidence indicates a requirement for DNA replication which must continue for at least one round of duplication during the initiation of spore septum formation. It remains to be established in what way catabolism is affected after the inhibition of cell division in cultures containing the threshold level of acetate and butyrate. The enhanced glucose consumption which accompanies the onset of solvent production and granulose and capsule biosynthesis indicates that active catabolism continues in the stationary phase cells throughout the solventogenic phase of the fermentation.

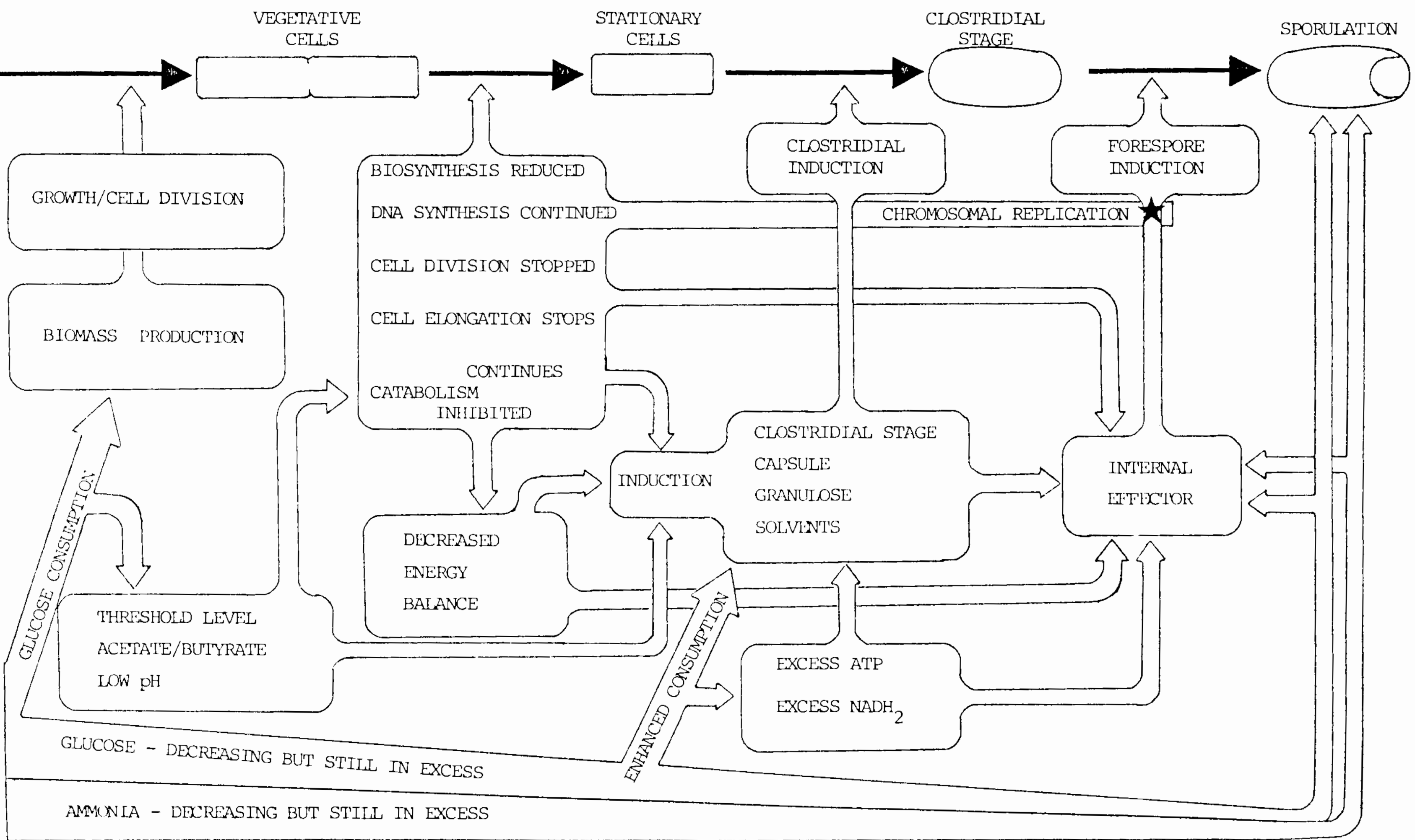


Figure 7.1: Model proposing a mechanism of regulation of solventogenesis and endospore formation in C. acetobutylicum P262.

Since the anaerobic degradation of glucose produces only a small number of ATP molecules compared with the yield of ATP produced during aerobic respiration, anaerobic growth is primarily limited by the rate of energy-producing reactions (Thauer et al., 1977). O'Brien and Morris (1971) observed a one-third decrease in the level of ATP when growth was inhibited in C. acetobutylicum. It is possible that a similar low level in ATP content accompanies the inhibition of growth by acid end-products in C. acetobutylicum P262.

The metabolic shift from acid to solvent production provides a pathway for the continued generation of ATP via an alternative reductive pathway resulting in NAD^+ regeneration. An imbalance in energy level or possibly reducing power at the end of growth may be important in the triggering of solvent production by acetate and butyrate. However, results obtained from nitrogen-limited cultures (which contained sufficient substrate for solvent production) would suggest that the energy levels alone are not sufficient to induce the shift to the solventogenic phase.

Once the cessation of cell division and the switch to the second fermentation phase occurs, the reduced demand for ATP for biosynthesis may result in an increase in the level of the energy-rich compound. Glycogen biosynthesis in procaryotes and starch accumulation in algae and higher plants is regulated by ATP and the available glucose in non-growing cells (Stainer et al., 1976). Growth limitation in the presence of excess glucose

and ATP provides the ideal conditions for the deposition of granulose and the production of an extracellular polysaccharide capsule in C. acetobutylicum P262. In this way, the energy-producing solventogenic phase may be incidentally coupled to granulose and capsule production and the formation of clostridial structure in C. acetobutylicum P262.

ATP levels in the cell at the end of the exponential phase may also provide the trigger which initiates sporulation in non-growing cells. Evidence obtained from inhibitor studies using purine analogs also suggests that a stringent-type control system (low ATP, low GTP) influences endospore formation. The toxic effects of oxygen which inhibited cell division and prompted sporulation also suggests that forespore septum initiation occurs in cells deprived of energy (see O'Brien and Morris, 1971).

It is not known whether the production and utilisation of ATP increases or decreases at the end of the acidogenic phase. However, a measure of ATP levels at the fermentation breakpoint will help to determine whether high or low ATP concentrations play a role in the regulation of the developmental system.

The role of the clostridial stage in endospore formation is still not clear. Although the results presented in this thesis would suggest a requirement for the clostridial stage, it is possible that sporulation bypasses this morphological stage under certain experimental conditions. The clostridial stage is not required for endospore formation in a number of other Clostridium strains.

In *C. acetobutylicum* P262 granulose mobilisation does not appear to be vital as an endogenous supply of energy during differentiation. ATP generation through continued catabolism during the solventogenic fermentation phase appears to be the preferential mechanism of energy production during the development of the mature spore. However, granulose may be utilised under certain conditions when glucose is exhausted or when the sporulating cells are no longer able to transport the substrate.

The sporulation-specific effector responsible for the initiation of spore septum formation in *C. acetobutylicum* P262 appears to have the following essential requirements:

1. The inhibition of cell division by central septum formation.

2. An exogenous supply of carbon and nitrogen.

3. Chromosomal replication to continue for at least one round of synthesis.

Results from this study provide evidence for the first two points. The requirement for chromosomal replication is obviously subject to the criticism which has been aimed at studies using inhibitors in *B. subtilis* (Hanson and MacKenchie, 1969; Freese *et al.*, 1978).

The model which has been proposed for the regulation of the stationary phase events in C. acetobutylicum P262 is largely speculative. Additional and alternative regulatory pathways may well operate in this complex system. Nevertheless, the proposals provide a framework for further research in this field.

An important question which remains unanswered, is the way in which acetate and butyrate are involved in the crucial metabolic change in the fermentation. The acids may act to dissipate the transmembrane pH gradient creating a low intracellular pH condition required to promote solvent production. Alternatively, internal concentrations of acetate and butyrate may play a more specific role in determining the orientation or the metabolic pathway to acid or solvent production.

Direct ATP, NADH, GTP and ppGpp measurements in the wild-type and developmental mutant strains may illustrate the way in which these compounds are involved in the initiation of granulose accumulation, capsule formation, solvent production and sporulation. The results of such studies will also be useful in evaluating the relationships between the various stationary phase events. It is still not known whether these events are regulated by a common effector or whether the association observed is an indirect consequence of some other common requirement.

This study has identified parts of the complex regulatory system which controls solventogenesis and endospore formation in

C. acetobutylicum P262. These are important basic discoveries which will assist future developments aimed at improving the ABE fermentation and acquiring a better understanding of sporulation in the anaerobic bacteria.

APPENDIX A

GENERAL METHODS

Preparation of anaerobic media and solutions

The methods of Moore (1966) and Hungate (1969) were used to prepare pre-reduced, oxygen-free media. Most of the oxygen was driven off by gentle boiling of media for 15 - 20 min. Aliquots (10 ml) of the warm medium were dispensed into Hungate tubes and perfused with H_2 and CO_2 prior to sterilisation by autoclaving. Large volume culture media were not perfused with H_2 and CO_2, but rather autoclaved immediately and allowed to stand for 15 h under stringent anaerobic conditions in an anaerobic glove box (Forma Scientific Inc., Marietta, Ohio; atmosphere 70% N_2 : 20% CO_2 : 10% H_2 [v/v/v]). Media containing agar (1.5% [w/v]) (Difco) was autoclaved, poured into petri dishes and stored in anaerobic jars (flushed with H_2 and CO_2 for 20 min), with silica gel in muslin bags. Cysteine hydrochloride, added to all culture media, scavenged any residual oxygen.

All media and solutions were sterilised by autoclaving at $121^{o}C$ and 105 kPa for 20 min.

Complex culture media

Clostridium Basal Medium (CBM) (O'Brien and Morris, 1971)

Glucose		10.0 g
Casein hydrolysate		4.0 g
Yeast extract (Difco)		4.0 g
$MgSO_4.7H_2O$	Stock solution	1.0 ml
$MnSO_4.4H_2O$	" "	1.0 ml
$FeSO_4.7H_2O$	" "	1.0 ml
p-Aminobenzoic acid	" "	1.0 ml
Biotin	" "	1.0 ml
Thiamine HCl	" "	1.0 ml
$NaHCO_3$		1.0 g
Cysteine HCl		0.5 g
Resazurin stock solution		10.0 ml
Distilled water		1.0 l

Clostridium Basal Medium agar

The required percentage agar (1.5% w/v) was added to CBM medium made as above. Cysteine HCl (10.0 ml) and $NaHCO_3$ (20.0 ml) were added as stock solutions after the medium had been autoclaved and prior to pouring. The solid medium did not contain the anaerobic indicator.

Reinforced Clostridium Medium (RCM)

The reinforced *Clostridium* Medium which was commercially available (Biolab Chemicals, Pretoria, South Africa), contained the following:

Dextrose	5.0 g
Soluble starch	1.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Lab-lemco powder	10.0 g
Na-acetate	3.0 g
NaCl	5.0 g
Cysteine HCl	0.5 g
Distilled water	1.0 l

Molasses Fermentation Medium (MFM) (Barber *et al*., 1979)

Molasses	134.0 g (or amount required to give 6.5% total invert sugar)
$(NH_4)_2SO_4$	2.0 g
$CaCO_3$	1.0 g
Steepwater concentrate	1.5 ml
Distilled water	1.0 l

The pH of the medium was adjusted to pH 7.0 with IN NaOH before autoclaving.

Defined culture media

Glucose-Mineral Salts-Biotin Medium (GSMM) (Holdeman *et al.*, 1977).

Glucose	1.0 g
$(NH_4)_2SO_4$	0.1 g
Salts stock solution	4.0 ml
Biotin " " (x 100)	25.0 ul
Resazurin " "	1.0 ml
Cysteine HCl	0.05 g
$NaHCO_3$	2.0 g
Distilled water	95.0 ml

The modified GSMM medium contained 2 g l^{-1} NH_4 acetate in place of $(NH_4)_2SO_4$.

Clostridium pasteurianum Minimal Medium (CPMM) (Mackey and Morris, 1971)

Glucose	40.0 g
NH_4Cl	3.0 g
$CaCo_3$	20.0 g
NaCl	0.1 g
Ferric sodium sequestrate	0.275 g
$MgSO_4.7H_2O$	0.1 g
$MnSO_4.4H_2O$	15.0 mg

$Na_2MoO_4.2H_2O$	10.0 mg
$CaCl_2$	10.0 mg
Biotin	0.12 mg
p-Aminobenzoic acid	2.0 mg
Cysteine HCl	0.5 g
Distilled water	1.0 l

Sterile potassium phosphate buffer (1.0 M), pH 7.0, was added to the autoclaved broth at a final concentration of 0.05 M.

Clostridium acetobutylicum Minimal Medium (CAMM)

Glucose		6.0 g
Diammonium hydrogen phosphate		0.6 g
$CaCO_3$		2.0 g
Salts	Stock solution	4.0 ml
$MgSO_4.7H_2O$	" "	0.1 ml
$MnSO_4.4H_2O$	" "	0.1 ml
$FeSO_4.7H_2O$	" "	0.1 ml
$ZnSO_4.7H_2O$	" "	0.1 ml
$NaMoO_4.2H_2O$	" "	0.1 ml
p-Aminobenzoic acid	" "	0.1 ml
Thiamine HCl	" "	0.1 ml
Biotin	" " (x 100)	25.0 ul
Cysteine HCl		0.05 g
Distilled water		95.0 ml

Diammonium hydrogen phosphate was autoclaved separately and added to the sterile culture medium. Sterile potassium phosphate buffer (1.0 M) was added to a final concentration of 0.05 M at pH 7.0.

Semi-defined fermentation medium (SFM)

Sucrose		65.0 g
Ca lactate		12.0 g
Yeast extract		2.0 g
Diammonium hydrogen phosphate		1.7 g
$CaCO_3$		2.0 g
$MgSO_4.7H_2O$	Stock solution	1.0 ml
$MnSO_4.4H_2O$	" "	1.0 ml
$FeSO_4.7H_2O$	" "	1.0 ml
$ZnSO_4.7H_2O$	" "	1.0 ml
p-Amino benzoic acid	" "	1.0 ml
Biotin	" "	1.0 ml
Thiamine HCl	" "	1.0 ml
Resazurin	" "	10.0 ml
Cystein HCl		0.5 g
KH_2PO_4		5.0 g
K_2HPO_4		5.0 g
Distilled water (made up to)		1.0 l

The pH of the medium was adjusted to pH 6.5 with a 25% NH_3 solution prior to sterilisation by autoclaving.

<u>Mutant Stock Culture Media</u>

<u>Beef liver medium for anaerobes</u>

(from the American Type Culture Collection Catalogue [13th edn.], 1978, p. 433)

Beef liver (diced)	500,0 g
Tap water	1.0 l

The beef liver was soaked overnight at 4°C. Fat was skimmed off the surface and the medium was autoclaved at 121°C for 10 min. The meat particles were separated from the supernatant by filtration through cheese cloth. The meat was retained and the following were added to the filtrate:

Peptone	10.0 g
K_2HPO_4	1.0 g

The pH was adjusted to pH 7.0 with IN NaOH and the volume made up to 1 l with tap water. Small amounts of $CaCO_3$ (c 0.05 g) and meat to a depth of about 2.0 cm were placed in standard containers and covered with filtrate to a depth of 4 - 5 cm. The containers were capped loosely and autoclaved at 121°C for 15 min.

Stock Solutions

All stock solutions were stored at 4°C unless otherwise indicated.

Salts stock solution

$CaCl_2$ (anhydrous)	0.2 g
$MgSO_4.7H_2O$	0.2 g
K_2HPO_4	1.0 g
KH_2PO_4	1.0 g
$NaHCO_3$	10.0 g
NaCl	2.0 g
Distilled water	1.0 l

The $CaCl_2$ and $MgSO_4$ were first dissolved in 300 ml of the distilled water. The remaining salts were added slowly together with the rest of the water.

$MgSO_4.7H_2O$ Stock Solution

$MgSO_4.7H_2O$	20.0 g
Distilled water	100.0 ml

$MnSO_4.4H_2O$ Stock Solution

$MnSO_4.4H_2O$	1.0 g

Distilled water	100.0 ml

$FeSO_4.7H_2O$ Stock Solution

$FeSO_4.7H_2O$	1.0 g
Distilled water	100.0 ml
Stored at -20^oC.	

$ZnSO_4.7H_2O$ Stock Solution

$ZnSO_4.7H_2O$	5.0 g
Distilled water	100.0 ml

$NaMoO_4.2H_2O$ Stock Solution

$NaMoO_4.2H_2O$	1.0 g
Distilled water	100.0 ml

p-Aminobenzoic acid Stock Solution

p-Aminobenzoic acid	0.1 g
Distilled water	100.0 ml

Thiamine hydrochloride Stock Solution

Thiamine HCl	0.1 g
Distilled water	100.0 ml

Biotin Stock Solution

Biotin	20.0 mg
Distilled water	100.0 ml

The working stock solution was a 1/100 dilution of the above solution.

Cysteine hydrochloride Stock Solution

Cysteine HCl	5.0 g
Distilled water	100.0 ml

Autoclaved.

Sodium bicarbonate Stock Solution

$NaHCO_3$	10.0 g
Distilled water	100.0 ml

Autoclaved.

Resazurin Stock Solution

Resazurin	20.0 mg
Distilled water	100.0 ml

End-Product Determinations

Acetate, butyrate, acetone, butanol and ethanol were measured using a Hewlett Packard 5880A gas chromatograph with a flame ionisation detector. A glass column (1.86 m x 4 mm) packed with 10% diethylene glycol adipate on 80 - 100 mesh chromosorb-W-HP (Supelco) was used with propanol as an internal standard.

The conditions used for end-product determinations were as follows:

Column temperature:-	60 to 180°C progressive at 20°C min^{-1}.
Detector temperature:-	300°C.
Carrier gas (N_2) flow rate:-	30 ml min^{-1}.

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