Metabolic and morphologic differentiation in Escherichia coli and Clostridium beijerinckii

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Metabolic and morphologic differentiation in *Escherichia coli* and *Clostridium beijerinckii*

Ross, Robin Ann, Ph.D.

University of New Hampshire, 1991
METABOLIC AND MORPHOLOGIC DIFFERENTIATION

IN *Escherichia coli* AND *Clostridium beijerinckii*.

BY

ROBIN ANN ROSS

B.S. Biology, Syracuse University, 1984

DISSERTATION

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the Requirements for the Degree of

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in

Microbiology

December, 1991
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I dedicate my dissertation to George and Doreen Ross in appreciation of their excellent parenting skills. Mom and Dad, this would not have been possible without you and your genes.
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"It was the best of times, it was the worst of times."
- A Tale of Two Cities, Charles Dickens 1859

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The second system was *Clostridium beijerinckii*. Differentiation from acidogenesis to solventogenesis and its link to sporulation were investigated using a recycling fermentor. This unique growth system with its 100% cell recycle allows for the study of bacteria as their growth-rate slows. We determined that the shift from acidogenesis to solventogenesis did not occur at a single mass doubling time, but as a series of events. The
rate of acetic acid production decreased immediately after the fermentor was switched from chemostat to recycle mode, whereas the rate of butyric acid production did not change. Propanol was detected 4.5 h after the switch, followed 44.5 h later by an increase in the rates of ethanol and propanol production and the detection of propionic acid. An increase in the rate of butanol production occurred 9 h later, as also did the appearance of phase-dark forespores. This sequence of events did not require elevated levels of guanosine tetraphosphate to occur. An acidogenic recycling fermentor culture stimulated to sporulate and monitored using both phase contrast and electron microscopy produced only butanol as phase-dark forespores were formed. This would suggest a link between sporulation stage III and butanol production in C. beijerinckii.
PROLOGUE

This research program found its initiation in a paper by George and Chen (37) on solventogenic metabolism. The authors compared the production of fermentation products with biomass in batch cultures of Clostridium beijerinckii. From their published figures, we calculated that the shift from acidogenesis to solventogenesis occurred at a mass doubling time (td) of approximately 20 h. It has been shown in a wide variety of prokaryotes that at this td cultures enter a domain of slow growth. This growth phase is distinct from both the domain of fast or exponential growth and the domain of ultraslow growth that occurs in stationary phase cultures. Thus, it was thought possible that a metabolic event responsible for the shift from acidogenic to solventogenic metabolism occurs as the culture enters the second domain of growth. If this is so, could the regulatory nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp), which increases throughout the second growth domain, be the unknown trigger of differentiation?

At the same time studies had begun in this laboratory involving an Escherichia coli cell division mutant that forms transverse walls at cell poles, producing a small chromosome-less cell or minicell. This mutant, strain B/r H266, was first described by Woldringh et al. (130), who noted that minicells were present in old cultures and not in fast growing cultures. Further investigation revealed that E. coli B/r H266 forms minicells only
after the growth rate has slowed to 20 h, whereas all other reported *E. coli* minicell producing strains do so at all growth rates. Thus, the morphologic differentiation of this unique minicell producing strain was growth-rate dependent and occurred in the same growth rate domain as the metabolic differentiation of *C. beijerinckii*. Was ppGpp a trigger here also?
INTRODUCTION

This introduction contains 3 sections: the first section deals with the effects of the stringent response and ppGpp on metabolism, morphology, and growth rate in prokaryotes. The second section discusses minicell formation in *E. coli* and the third section reviews solvent production and sporulation in the solventogenic clostridia, and more generally, sporulation in *Bacillus* and *Clostridium*.

Section I. The Stringent Response, ppGpp, and the Growth of Prokaryotes

Prokaryotes have evolved global regulatory networks to maintain coordination among growth processes in a changing environment. Global regulatory networks alter the pattern of expression of unlinked genes in response to stress and allow the expression of these genes to return to their original state when the stress stimulus is removed (40). Examples of global regulatory networks include the heat-shock response, SOS, oxidation-shock, and stringent response. Only the stringent response is discussed here.

The stringent response functions to control protein synthesis and other macromolecular syntheses during periods of either amino acid starvation or energy limitation. The response is achieved via a family of regulatory nucleotides centered around ppGpp.

ppGpp synthesis in response to amino acid starvation is catalyzed by
(p)ppGpp synthetase I (14) encoded by relA (60 min on the E. coli chromosome) (19, 93). This pyrophosphotransferase is bound to the L11 ribosomal protein of the 50S ribosomal subunit (91) and may also depend on the presence of the L10 ribosomal protein of the same subunit (53).

The relA gene product catalyzes the formation of ppGpp as follows: when the rate of aminoacylation of tRNA falls below the rate of protein synthesis during mRNA translation, codon-specific uncharged tRNA becomes bound to the acceptor site, or A site, of the ribosome (14, 47, 84) causing the (p)ppGpp synthetase I catalyzed transfer of a phosphoryl group from ATP to the 3' hydroxyl of GTP to form pppGpp (19, 112, 113). This reaction is thought to trigger the release of the uncharged tRNA from the A site on the ribosome (99). pppGpp is then dephosphorylated to form ppGpp by the gpp-encoded enzyme (107) and other enzymes including the translational factors EF-Tu and EF-G (45).

The level of ppGpp in the cell is also affected by (p)ppGpp synthetase II which responds to some aspect of energy metabolism, but is not ribosome associated (89). This route produces ppGpp directly, without pppGpp formation (36). The enzyme believed to catalyze the reaction is encoded by relX (60 min on the E. coli chromosome) and is genetically linked to relA (89). A mutation in relX prevents ppGpp from accumulating when the cell is starved for carbon and/or energy (89). ppGpp will accumulate as the growth rate slows in either a relA* or relA background due to ppGpp production via the relX gene product (73, 129). Both the relA and relX pathways are believed to contribute to the basal level of ppGpp present in the
unstressed cell (89).

The degradation of ppGpp and pppGpp is carried out by (p)ppGpp 3′-pyrophosphohydrolase which catalyzes the Mn²⁺ dependent removal of the 3′-pyrophosphate residue from ppGpp and pppGpp (48, 111). Encoded by spoT (82 min on the E. coli chromosome), (p)ppGpp 3′-pyrophosphohydrolase is found in the membrane fractions of cell extracts (48, 111, 112). A spoT mutant has a higher than wild-type basal concentration of ppGpp in the cell (111).

Regulation by ppGpp can occur at either the transcriptional or gene product level. As the concentration of ppGpp in the cell rises, there is a 10-20 fold decrease in the accumulation of stable RNA (tRNA and rRNA) caused by a specific reduction in transcription of stable RNA genes (62). ppGpp affects the binding of RNA polymerase to stable RNA gene promoters (46, 69) by binding RNA polymerase and rendering it unable to initiate transcription (11, 103). Ribosomal protein transcription and translation is slowed, the latter as a secondary response to the negative transcriptional regulation of rRNA (28, 29, 74). Since tRNA is not needed, its synthesis is also slowed (58). Transport and synthesis of RNA precursors is inhibited, but to varying degrees depending on the component (15).

Along with decreased synthesis of the structural components of protein synthesis, phospholipid, fatty acid (86), and peptidoglycan syntheses (60, 59, 123) also decrease. ppGpp is known to control the rate of peptidoglycan synthesis by regulating enzymes in the early (92) and late
(123) stages of peptidoglycan synthesis. The degree of inhibition is correlated to the cellular concentration of ppGpp; as the ppGpp level in the cell rises, incorporation of diaminopimelic acid falls (92, 123). It is felt the activity of penicillin binding protein 1B is the target of ppGpp's regulation of late stages in the synthesis of peptidoglycan (123).

Some amino acid biosynthetic enzymes are under positive control; transcription of the his operon is under positive control of ppGpp (109). Branched chain amino acid transport is increased (97), and some heat shock proteins are produced during the stringent response (44, 124). Stimulation of intracellular proteolysis, indicated by no net protein synthesis even though production continues at a reduced rate, is believed to function as an attempt to replace the missing amino acid (124). Lon product synthesis is stimulated during the stringent response and may account for the proteolysis (124).

The rate of cell division may also be under direct ppGpp control, since changes in the rate of division occur at sub-stringent levels of ppGpp, before protein synthesis is inhibited (16, 18). ppGpp has been proposed in the regulation of the D period of the cell cycle (16). The main evidence for this supposition is that the rel status of a cell affects mean cell length as the growth rate slows: rel mutant have shorter cells compared to wild-types, and spoT have longer cells than wild-types. It seems that ppGpp regulates division negatively and allows for more cell elongation before division, by lengthening the D period. This is the period in the bacterial cell cycle between the completion of chromosome replication and cell separation.
Information concerning ppGpp and the growth rate has been obtained through physiological studies of various prokaryotes in nutrient limitation. When *E. coli*, *Paracoccus denitrificans*, *Bacillus polymyxa* (121), *C. beijerinckii* (2, 100), or *Clostridium C7* (100) are grown in a cell recycle fermentor, three clearly distinguishable domains are observed. These domains are defined by growth rate boundaries and are characterized by different metabolic and morphologic behaviors (3, 4, 16, 18, 100, 121, 122).

Domain 1 is defined by a $t_d$ of 14 h and faster and is characterized by basal levels of ppGpp. When the $t_d$ lengthens beyond approximately 14 h, an abrupt drop in the growth rate occurs due to the diversion of approximately 50% of the cell's energy from biosynthetic costs to growth maintenance costs. This event also coincides with the culture's entry into domain 2 with a $t_d$ of 20 h. The growth rate of domain 2 ranges from $t_d$ of 20 h to approximately 60-70 h, and the ppGpp concentration in the cell rises. When the concentration of ppGpp in the cell reaches a level that triggers the stringent response, the culture enters domain 3, begins growing at a $t_d$ of 100 h, and has a maximum stringent ppGpp concentration (3, 16, 121, 122).

In summary, ppGpp is a regulatory nucleotide that functions not only at a full, stringent concentration to inhibit protein synthesis at both the transcriptional and gene product level, but is also active at intermediate levels, between basal and full stringent, to control cell division and metabolism.
Section II. Morphologic Differentiation in E. coli B/r H266

Cell division is an ordered event, consisting of initiation of septum formation, actual septum formation, and finally cell separation. To date it has not been established how the septum, or transverse wall, is placed with such high fidelity in the central portion of a cell. When this accuracy is lost, the transverse wall can be formed at the pole of the cell, leading to production of a small chromosome-less cell or minicell. All reported strains of E. coli that produce minicells do so at all growth rates. The strain of E. coli that is the subject of this study begins to form minicells only after the growth rate of the culture has slowed to a $t_d$ of 20 h. This raises two questions about regulation of septum formation. First, what is the genetic origin of growth-rate dependent minicell formation in this strain? Second, does ppGpp play a role in the localization of the transverse wall in normal cell division? (at a $t_d$ of 20 h the concentration of ppGpp begins to rise in the cell).

All genetically characterized minicell producing strains of E. coli are caused by mutations at one of three loci: $ftsZ$, $cfcA$, or the $minB$ operon, at 2.2 [110], 79.2 [85], and 26.2 min [24] on the E. coli chromosome, respectively.

$FtsZ$ is believed to be a transmembrane protein whose N-terminal portion extends into the cytoplasm and prefixes components of the cell division machinery to the membrane at preseptal sites [6]. In addition $FtsZ$ has been proposed to be involved in the control of cell division, either as an activator or as an inhibitor of division, depending on its level of $FtsZ$ in the
cell (95). The rate of cell division may be dependent on the formation of a complex of interacting Fts proteins whose activity is influenced by FtsZ molecules (30, 95). When FtsZ is overproduced, polar in addition to midcell septum placement occurs resulting in the formation of minicells and short rods (127).

The cfcAl mutation causes an increase in the number of divisions per cell cycle, resulting in minicell production (85), as similar to the minicell forming ftsZ strain. CfcA controls the frequency of division in relation to DNA replication, by regulating the production of FtsZ (85). cfcAl could cause overproduction of FtsZ, which would explain cells that are shorter than the wild-types, caused by the higher frequency of cell division. The reduced colony forming ability (85) would also be accounted for, as due to minicell formation.

Of the three minicell producing genotypes, most information is available about the minB operon. It was originally felt that mutations in two loci, minA and minB, were necessary for the improper placement of the transverse wall at a position close to the cell pole (34). Later it was found that only minB (26.2 min on the E. coli chromosome [24]) was necessary (21). A minB genotype allows cell poles to be possible septation sites; the phenotype is minicell formation (1). MinB+ was proposed to be an inactivator of old division sites and, when the gene product was mutated, allowed for division near the cell poles, resulting in filaments and minicells (114). The striking difference between the division phenotype in a minB versus a ftsZ or cfcAl strain is that the former places the transverse wall at
the cell pole it is in lieu of placement at a central position, producing minicells and filaments, whereas the latter places a polar transverse wall in addition to a central division, producing minicells and short rods.

Further investigation of minB has resulted in the discovery that it is an operon encoding three peptides. The order is: MinC (25 kD), MinD (30 kD), and MinE (10 kD) (26). Plasmids and phages containing chromosomal inserts of various sections of the minB locus placed downstream of the lac promoter have revealed the phenotypic interaction between the three proteins.

When the chromosomal inserts were induced with IPTG in a ΔminB strain, expression of the three genes independently or in combinations of minCE or minDE did not affect the minicell phenotype, while physiological levels of MinCD caused filamentation (no division), indicating that MinC and MinD together block division (26). Sub-physiological levels of MinCD expression did not affect minicell formation (26). Thus there is a critical concentration of MinCD that is necessary for inhibition of division. When present in multiple copies and induced in a minB+ strain, either minC or minD caused filament formation.

The MinCD division inhibition system is sensitive to high levels of FtsZ (27). When division at all possible sites is prevented by deletion of minE from the minCDE system, filaments form (27). Overproduction of FtsZ can convert this division phenotype to minicell forming, completely inactivating the division-inhibition system (27). It has been suggested that MinCD
interacts directly with FtsZ (12).

When MinE is not produced, division is blocked not only at the poles, but also in the middle of the cell, causing filament formation (26). In a $minB^+$ strain, induction of $minE$ in multiple copies, a single copy, or combined with $minC$, $minD$, or both caused minicell formation (26). In a $\Delta minB$ strain, symmetric division was restored when a single copy of $minCDE$ was induced and minicells were produced when multiple copies of $minCDE$ were induced (26), indicating that merely an increase in the level of MinE is hindering MinCD from preventing division at the cell poles even if MinCD is increased. Minicell formation can be caused by the loss of either MinC or MinD or the overproduction of MinE (27).

To explain the observation that a $minB$ strain produces nucleated rods, minicells, and filaments, Teather et al. (114) proposed that at each division a $minB$ strain forms a septum with equal probability at either an old division site (polar) or a new division site (internal). $minB^+$ has strict rules for accurate placement of the septum in the middle portion of the cell (61) and only one division can occur per unit mass increase.

de Boer et al. (26) modified the model to say that at physiological levels, MinC and MinD act together to inhibit division, while MinE prevents MinCD from blocking division at internal sites. MinD confers MinE sensitivity to the complex, while MinC interacts with septum components or plays a role in the formation of another molecule which, in turn, inhibits division (27). The cellular target of MinCD is not known, but most likely is
FtsZ. Nor is it understood whether MinCD inhibits division directly or is a secondary participant required for the direct inhibitor of division to function. How MinE carries out its function is also unknown. de Boer et al (24) feel that MinE prevents division at internal sites by blocking MinCD function, but what localizes MinE activity to these sites is not known. How the topological specificity is overcome by the overproduction of MinE is also not known.

To extend the explanation of the role of the minB operon to the growth-rate dependent formation of minicells by E. coli B/r H266 requires that a defective interaction between the unknown topological cell component and MinE occurs only at the growth rates in which cellular ppGpp is increasing, since minicells are formed only in this range of growth rates. Is this defective interaction due to a mutation in minE or in the topologic regulator of MinE activity? I investigated the mutation in B/r H266 to determine if it was in minE or in the unidentified topologic regulator of MinE activity. By determining this, it could provide insight as to how division is regulated normally.

Section III. Metabolic and Morphologic Differentiation in

Clostridium beijerinckii NRRL B593

Clostridium beijerinckii is an endospore-forming obligate anaerobe and a member of the solventogenic subgroup of the saccharolytic clostridia, whose best studied member is Clostridium acetobutylicum. Both species
readily differentiate from acidogenic metabolism to solventogenic metabolism. The most striking difference between the two species is \textit{C. beijerinckii}'s ability to differentiate at a neutral pH (37). Minor variations between the two species include fermentation products and their ratios.

The two stage fermentative metabolism of solventogenic clostridia is of interest because the established industrial practice of chemical production by fermentation (96) may become an important alternative to the petroleum-based synthetic route (39, 82). Ethanol, isopropanol, and \textit{n}-butanol are feedstocks for a major fraction of the chemical industry (88) and octane enhancers in alcohol-gasoline blends (51).

\textit{C. beijerinckii} produces acetic acid, butyric acid, and ethanol during acidogenic metabolism; ethanol, butanol, and either acetone or isopropanol or propanol during solventogenic metabolism. The pathways for the production of these acids and solvents are shown in Fig. 1. The exceptions are the propionic acid and propanol paths which are not known, as yet, but have been proposed (33).

Propanol production in \textit{C. butylicum (beijerinckii)} NRRL B593 was first reported to be produced from the fermentation of methylpentoses, but not during growth on glucose (33). \textit{C. beijerinckii} grown with rhamnose as the sole carbon source produced 1,2-propanediol and propanol, but no propionic acid. The route of conversion of 1,2-propanediol to propanol was suggested to be similar to the branched pathway of \textit{Klebsiella pneumoniae} and \textit{Propionibacterium freudenreichii}, even though this pathway results in the formation of equal molar amounts of \textit{n}-propanol and propionic acid.
FIG. 1. Paths of acidogenic and solventogenic metabolism in the solventogenic clostridia. The bold-faced compounds are the emphasized products of fermentation.
It was hypothesized that additional enzymes exist for conversion of propionic acid to n-propanol (33). Later it was shown that C. beijerinckii NRRL B593 can produce propanol and propionic acid in a sucrose-based complex medium without 1,2-propanediol accumulation (2). It would seem more likely that propionic acid is formed without 1,2-propanediol as an intermediate, via a randomizing (succinate) pathway, as in Propionibacterium (108), or by a nonrandomizing (acrylate) pathway, as in Clostridium propionicum (108).

C. acetobutylicum can produce n-propanol only in propionate-supplemented batch cultures, suggesting the conversion of propionate by nonspecific enzymes (52, 56). Propionic acid can be converted via CoA-transferase-acetoacetate decarboxylase or the reversed kinase-phosphotransbutylase path to propionyl-CoA (90), which can then be converted by nonspecific aldehyde and alcohol dehydrogenases to form n-propanol (56). This could also be the case in C. beijerinckii.

The trigger for metabolic differentiation in solventogenic clostridia is still unknown, but a variety of conditions are associated with differentiation. These include increased H₂ pressure (71, 135), CO inhibition of dehydrogenase (20, 68), viologen dyes (98), addition of undissociated weak acids (41, 87), iron limitation (94), sporulation (64), and decreasing the pH to below 5.0 (8). It has been suggested the diffusion of undissociated organic acids into the cell, leading to a drop in intracellular pH, ultimately causes the collapse of ΔpH and ΔΨ, is associated with
differentiation (10). What follows is a discussion of these various treatments.

In *C. acetobutylicum* batch culture without pH control, fermentative metabolism causes the pH to decrease, resulting in cessation of growth at pH 5.0. As the pH decreases, the equilibrium between the ionized and unionized form of the fatty acids produced by the cell shifts to favor the unionized form (106, 126). The unionized form then acts as a protonophore and rapidly fluxes in and out of the cell (67). When inside the cell, the unionized form exists in equilibrium with the ionized form resulting in the dissipation of the ΔpH (79, 102). In the neutrophile *C. thermoaceticum*, which maintains its internal pH at a more alkaline level than the exterior (87), production of acetic acid in batch culture disrupts the ΔpH, but not the ΔΨ, indicating the drop in internal pH inhibits reactions leading to cessation of growth and the fermentation (10). *C. acetobutylicum* lowers its internal pH as the external pH decreases in batch fermentations so that a constant ΔpH of 0.9 to 1.3 is maintained (43, 54, 115). The concentration of undissociated acids in the cell is correlated with the differentiation event; >42 mM acetic and butyric acids and 13-14 mM of butyric acid (115). It has been hypothesized (43) that the intracellular undissociated butyric acid is ionized and converted into butyryl phosphate and butyryl CoA which in turn depletes the cell’s phosphate and CoA pools and it is this situation that is associated with the activation or synthesis of solventogenic enzymes.
Phosphate limited chemostat cultures do produce a higher amount of solvents than other limitations (9).

At neutral pH, *C. acetobutylicum* is able to differentiate to solvent production when supplemented with concentrations of acetate and butyrate higher than necessary to provoke solventogenesis at pH 5.0 (100 mM verses 10 mM each) (52). Addition of weak organic acids other than butyric did not trigger differentiation to solvent production at neutral pH, but did enhance final solvent yields in uncontrolled batch fermentations (56, 57). In unchallenged and 14 h batch cultures challenged with acetic or propionic acid, differentiation occurred when undissociated butyric acid reached 9 mM (56).

Any treatment that decreases hydrogenase activity favors differentiation to solventogenic metabolism (98). Methyl and benzyl viologen are artificial electron carriers which compete with FdH\textsubscript{2} for oxidation by hydrogenase (137). Methyl and benzyl viologen exposure of chemostat cultures at pH 6.3 caused a decrease in hydrogen evolution, shifting the reducing equivalents released through hydrogen evolution to NAD\textsuperscript{+} via NAD:ferredoxin oxidoreductase (98). Rapid removal of NADH is necessary because the oxidoreductase is negatively regulated by low NADH concentrations (94). Increased NADH caused differentiation to solvent production at a neutral pH (6.3) in *C. acetobutylicum* (98).

Increased hydrogen partial pressure, which inhibits hydrogenase, also causes the differentiation to solvent production (135). In *C. saccharoperbutylacetonicum* batch fermentations without pH control,
butanol and acetone were not produced when H₂ was allowed to escape (13).

A treatment affecting the redox environment of the membrane bound, redox-sensitive hydrogenase affects differentiation to solventogenesis (98). Decreased membrane potential and increased transmembrane pH gradient observed prior to differentiation (43, 55, 115) may cause a decrease in hydrogenase activity (98), as would the uncoupling effect of weak organic acids (98).

Iron limited C. acetobutylicum cultures had increased solvent production. In iron-limited chemostat cultures of C. acetobutylicum at pH 4.8, butanol production increased 10% and the specific activity of hydrogenase decreased by 40% and acetoacetate decarboxylase by 25% (65).

The early stages of sporulation are temporally associated with solvent production in batch cultures of C. acetobutylicum (64), indicating they may share regulation (37). In batch cultures of C. beijerinckii at neutral pH, clostridial forms could not be linked with metabolic differentiation (37), but in chemostat cultures at acid pH, solvents and clostridial forms were produced (2). Complete sporulation is not necessary for solvent production, as long as the solvent producing population has undergone the initial stages of sporulation (49, 64).

**Sporulation Stages**

The sequence of morphological changes that occur during sporulation in Clostridium species are usually considered to be essentially the same as those described for Bacillus species (66, 83, 125, 136). The changes have been arbitrarily separated into six or seven stages (stage 0 -
VII and are mostly descriptions of sporulation in *Bacillus* species (32, 83, 136).

Vegetative cells are in stage 0 of sporulation (32, 136). Stage I is characterized by the condensation of the chromosome into an "axial filament" (32). The assignment of a stage to this event has been argued (134) for two reasons: no mutants are known that can not form axial filaments (79a) and similar filaments can be formed by *Escherichia coli* and other non-sporulating bacteria indicating the event is not unique to sporulation (136).

The first morphological change indicating sporulation is forespore division, stage II of the sporulation sequence, occurring after chromosome replication and migration of one copy to a cell pole (32, 136). Mesosomes have been noted at sub-polar positions (79a), but the pole at which the spore septation occurs is indistinguishable from the other (32). The asymmetric division results in two membrane bound cells contained within a continuous cell wall (136). As in normal cell division the wall invaginates, but at a sub-polar position (136). Wall material is present in these annular invaginations, but none is detected in the spore septum (136). Peptidoglycan synthesis is necessary to direct membrane growth for spore septation (35); if blocking agents are used to arrest its synthesis, spore septation will not occur (50, 72).

Stage III is the movement of the mother cell membrane around both sides of the forespore towards the pole resulting in "engulfment" of the forespore (32). The engulfing membranes meet at the cell's pole and fuse to
produce a forespore in the mother cell cytoplasm that has a double
membrane system of reversed polarity (32, 35, 128). In Clostridium, the
event results in the formation of the phase-dark clostridial form (131) which
has also been referred to as a phase-bright clostridial form with a phase-
dark forespore (64).

During stage IV modified peptidoglycan is laid down between the
opposing forespore membranes to form the cortex (32). In Bacillus, this
wall-like material contains meso-diaminopimelic acid instead of lysine, as
in the vegetative cell wall (117). A separate peptidoglycan layer, the
"primordial cell wall", is located closer to the inner membrane of the spore
and will become the germinated spore's cell wall (77). The enzymes for
producing spore-specific peptidoglycan linkages are found only in the
mother cell (116), suggesting that the production of the outer layer of the
cortex occurs at the outer forespore membrane and is directed by the
mother cell (118). The synthesis of the germ cell wall is believed to be
directed by the forespore which contains all the enzymes necessary for
peptidoglycan synthesis to take place on the inner membrane of the
forespore (136). During this stage, the developing endospore becomes visible
by phase-contrast microscopy as a phase-bright body within the cell (77).

During stage V, structural proteins made by the mother cell are laid
down in layers around the outer membrane of the forespore producing a
laminar structure (136). These spore coats make the tough outer shell of
the forespore (5). The forespore matures in stage VI, acquiring its
characteristic resistant properties (136). In stage VII, the mother cell lyses
and the mature spore is released (136). Again, lysis of a cell is an event that is not unique to sporulation and thus some authors feel should not be included as a stage in sporulation (136).

Differences in sporulation among *Clostridium* species and between the two genera do exist. In some *Clostridium* species the process of cortex and coat formation is reversed and/or the resultant spore may have appendages arising from the outer spore coat (131).

The most striking difference in sporulation between *Bacillus* and *Clostridium* species is the production of the clostridial form. During an early sporulation stage in *Clostridium* species, the clostridial cells become swollen in the area of the forming spore (42). Viewed by phase contrast microscopy, the cells are phase-bright with a phase-dark body at their swollen end and are called "phase-bright clostridial forms" by some researchers (64, 131).

Sporulation in *Bacillus* species can be triggered by nutrient deprivation (23, 104), whereas sporulation in *C. butyricum* [reclassified as *C. beijerinckii* (42)] can not (131). *Clostridium* species are able to sporulate in the presence of high glucose concentrations (131); *C. acetobutylicum* requires glucose and ammonia to initiate and complete sporulation (75, 76). Exposure of *C. acetobutylicum* P262 vegetative cells to air can trigger sporulation, and to a greater extent in complex versus minimal medium (131). This induction is not dependent on Eh, the generation of H$_2$O$_2$, or other toxic oxygen derivatives (131).

Solvent production, sporulation (formation of phase-bright clostridial
forms), and granulose formation can be simultaneously lost and returned, after spontaneous reversion, indicating a single mutation in some global regulatory gene (132). An asporogenous (can not form mature free spores), solvent producing *C. acetobutylicum* mutant unable to make granulose was obtained in a phosphate-limited chemostat culture at pH 4.3 (132) demonstrating that if these differentiation events are linked, they are not inseparable.

Meinecke *et al.* felt sporulation is not a prerequisite for solvent production in an asporogenous *C. acetobutylicum* generated in a phosphate-limited chemostat culture, yet sporulation was scored on the ability to produce free spores (80) rather than the production of earlier sporulation stages. It was noted that the asporogenous strain could be blocked in a stage of sporulation before free spore production, when metabolic differentiation could still occur (80). When *C. acetobutylicum* differentiates to solventogenesis at neutral pH due to the addition of acetate and butyrate, frequent but variable sporulation was observed (52).

Jones *et al.* (64) found ethylmethysulfonate mutagenized isolates of *C. acetobutylicum* P262 blocked before forespore septum formation produced low total solvents compared to the parent (97-99% decrease) in 48 h batch cultures (64). Isolates producing granulose and clostridial forms with phase-dark forespores, but not phase-bright forespores, produced wild-type levels of total solvents (64). Another isolate characterized by reduced levels of all sporulation characteristics, including production of free spores, produced low total solvents (6 and 15% of wild-type) (64). It would seem that
the link between metabolic and morphologic differentiation in solventogenic Clostridium is after forespore division and before phase-dark forespore formation.

There is a variety of mechanisms associated with the differentiation to solventogenesis, depending on the growth conditions. After an examination of data reported in the literature, it was apparent an additional trigger was the slowing of a culture's growth rate as it reached the end of the growth cycle (2). The metabolic differentiation event of shifting from acidogenic to solventogenic metabolism could be occurring as the culture enters the second domain of growth and ppGpp could be the unknown trigger of this metabolic differentiation. By using the cell recycle fermentor to culture C. beijerinckii, the point in the growth cycle at which metabolic differentiation occurs and how morphologic differentiation is involved can be determined.
MATERIALS AND METHODS

Section I. Morphologic Differentiation in *Escherichia coli* B/r H298

**Bacterial strains, bacteriophage, and growth conditions.** Table 1 lists the *Escherichia coli* strains and bacteriophage used. If not otherwise noted, overnight cultures were prepared in tryptic soy (TS) broth (Difco Laboratories, Detroit, MI) seeded with the appropriate strain from frozen stock, incubations were at 37°C with shaking, culture dilutions were done in 1% tryptone, and centrifugations were either for 10 min at approximately 10,000 x g, or if in a microfuge (Microfuge 11; Beckman Instruments, Inc., Berkeley, CA), for 5 min at the maximum speed. When making agar overlays, 2.5 mL of the appropriate soft agar medium is added to 1 mL of bacterial culture. Lambda YM (70) was modified to include 10 mM MgCl₂ and Luria-Bertani broth (81) (LB) was modified to include 5 mM CaCl₂. Amino acids and tetracycline (Tc) were used at 25 μg·mL⁻¹. Agar was added at a concentration of 1.5% for plates and 0.8% for soft agar overlays.

**Biphasic growth system.** This growth system (120) consists of an agar-broth bilayer which allows nutrients to diffuse from the agar to the broth layer, forming a substrate open, biomass closed system. Davis-Mingioli minimal medium (22) (DM) was used at a broth-agar ratio of 40:60.

**Nucleoid condensation.** A mix of 23% polyvinylpyrrolidone (PVP), 1.5% Noble agar, and 200 μg·mL⁻¹ Cm (31) was spread on warm slides and
### TABLE 1. *Escherichia coli* strains and Bacteriophage

<table>
<thead>
<tr>
<th>Strain/phage</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1&lt;sub&gt;vir&lt;/sub&gt;</td>
<td>prototroph</td>
<td>P. Kuempel</td>
</tr>
<tr>
<td>12435</td>
<td>min&lt;sup&gt;+&lt;/sup&gt;&lt;sup&gt;*2&lt;/sup&gt;, spoT</td>
<td>ATCC</td>
</tr>
<tr>
<td>B/r H266</td>
<td>min&lt;sup&gt;*2&lt;/sup&gt;, spoT</td>
<td>C. Woldringh</td>
</tr>
<tr>
<td>F375</td>
<td>minB</td>
<td>B. Oudega</td>
</tr>
<tr>
<td>PLK1943</td>
<td>minB, zcf-236::Tn 10</td>
<td>P. Kuempel</td>
</tr>
<tr>
<td></td>
<td>(cotransduces 80% with minB)</td>
<td></td>
</tr>
</tbody>
</table>

1. all strains are K12, except where otherwise noted.
2. growth rate dependent minicell formation.
allowed to harden. Wet mounts were prepared and viewed in phase contrast using an Olympus BH-2 (Marcon Instruments Co., Norwood, MA). Photomicrographs were taken using 35 mm Panatomic-X film (Eastman Kodak Co., Rochester, NY). Samples were scored positive for minicell production if a small cell still attached to a pole of the parent cell had no phase bright nucleoid present.

Transduction. P1vir mediated transduction (81) was modified by the addition of 1 mL of LB broth to the transducing phage-cell mix and 1 h incubation before plating on TS agar with Tc.

Section II. Metabolic and Morphologic Differentiation in

*Clostridium beijerinckii* NRRL B593

**Bacterial strains, maintenance, and growth conditions.** *Clostridium beijerinckii* NRRL B-593 ("Clostridium butylicum" VPI 13437) was provided by Dr. Nakamura from the collection of the Northern Regional Research Laboratories and maintained as a spore stock in a sterile 1:1:1 mixture of garden soil, sand and CaCO3. To produce active cultures, 10 mL of thioglycollate broth was seeded from the spore stock, heat shocked for 10 min at 80°C and held at 30°C until growth was obtained. Transfers and growth on solid medium and as batch cultures were carried out at 30°C in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, MI) containing a mixture of 85% N2, 10% H2, and 5% CO2.

**Fermentor growth studies.** The recycling fermentor growth system (18)
can be used as either a chemostat or a biomass recycle fermentor by switching the continuous outflow from directly out of the growth vessel (chemostat mode) to first passing over a 0.2 μm polycarbonate membrane filter (Nucleopore Corp.) in a cell recycle loop. In the latter case, spent medium is separated to outflow while the cells are returned to the growth vessel. The fermentor was first operated as a chemostat to achieve a steady state domain 1 growth rate (ppGpp levels at basal), and held at this domain 1 growth rate for 5 mass doublings, at which time it was switched to cell recycle mode to begin the progressive nutrient limitation and lengthening mass doubling time.

For both chemostat and recycling operations, the fermentor volume was held at 500 mL using modified George and Chen medium (MGC) (2) with sucrose at the limiting concentration of 3.54 g·L⁻¹ and at a temperature of 35°C. The pH was maintained at 6.8-7.0 with the addition of 1 M NaOH, except where otherwise noted. The broth surface was continuously swept with O₂-free N₂. To seed the recycling fermentor, 10 mL of an active culture was added to 300 mL of MGC. After incubation for 24-36 h, it was used to charge the anaerobic fermentor.

For induction of sporulation, a pulse of air was introduced to the nitrogen flow.

**Bacterial analyses and microscopy.** With all growth systems, cell samples were preserved in 1% formaldehyde, if not otherwise noted. Optical density determinations and phase contrast microscopy have been previously described (2).
For identification and quantitation of fermentation solvents, a Perkin-Elmer 3920 gas chromatograph (Norwalk, CN) with glass columns, carrier gas (nitrogen) flow rate of 24 mL·min⁻¹, and injector and detector temperature of 200°C was used. For solvent detection, a 80/120 Carbopack B / 3% SP-1500 (Supelco Inc.) column was operated with a temperature gradient of 70 to 170°C at 8°C·min⁻¹ and held for 2 min. The injection volume was 2 μL. Organic acids were identified and quantitated using a glass column packed with 80/120 Carbopack B-DA / 4% CARBOWAX 20M (Supelco Inc.) and operated isothermally with an injection volume of 1 μL. Fermentation samples were clarified by centrifugation before injection.

Samples for electron microscopy were fixed in 3% (v/v) glutaraldehyde in 0.1M cacodylate buffer (CB), pH 7.2, post-fixed in 1% (w/v) osmium tetroxide in CB for 2 h and dehydrated in a graded ethanol series. Samples were embedded in an epon-araldite resin mixture and polymerized for 24 h at 60°C. Thin sections were stained with 3% (w/v) uranyl acetate and 0.4% (w/v) lead citrate and examined by transmission electron microscopy using a Hitachi H600 at 80 kV.
RESULTS

Section I. Morphologic Differentiation in *Escherichia coli* B/r H266

**Confirmation of *E. coli* minicell production.** The observation that *E. coli* B/r H266 (B/r H266) produces small daughter cells devoid of chromosomal material was verified by viewing a biphasic culture on chloroform-PVP agar coated slides with phase contrast (Fig. 2). Phase-bright, condensed nucleoids were present in the parent cells and not in the attached minicells. Thus B/r H266 produces true minicells.

**Effect of carbon-energy source on minicell production.** The biphasic growth system was used to determine if the carbon-energy source altered the growth rate dependent minicell formation in B/r H266. Glucose, succinate, lactose, erythritol, and L-alanine grown cultures all produced minicells only after the growth rate had reached 20 h (data not shown). This indicates that minicell production in B/r H266 is not dependent on a particular carbon energy source. Growth rate dependent minicell production in B/r H266 thus appears to be genetic and not physiologic in origin.

**Transduction mapping of the min locus in *E. coli* B/r H266.** To determine the gene mutation causing the unique minicell forming phenotype in B/r H266, transductions using minB and minB+ cotransduced with Tn10 were performed. The locus associated with minicell production in B/r H266 will
FIG. 2. Phase contrast photomicrographs showing minicells devoid of nuclear material, verifying *E. coli* B/r H266's ability to produce minicells. (A) Agar with chloramphenicol added to condense the nucleoid. (B) Agar alone. Cells were grown in a biphasic growth system in Davis-Mingioli medium. Bars represent 1μM.
be referred to as min* in distinguishing it from the minB locus in E. coli K12 (K12) strains. Though the usual practice is followed in referring to the K12 locus as minB, this is actually an operon containing at least 3 genes (25). The particular mutation used in these studies was minD1 (24).

To construct a Tn10 cotransduced with minB+ from a K12 strain, zcf-236::Tn10 minB was transferred from E. coli K12 PLK1943 to E. coli K12 12435 (K12 12435) via P1-mediated cotransduction (81). Tc resistant (Tcr) transductants were selected and screened for their division phenotype. Fifty % of the strain 12435 Tc+ cotransductants formed minicells and 50% divided symmetrically (cross 1, Table 2), mapping zcf-236::Tn10 to about 0.4 min from minB. The two phenotypes, MinB+ and MinB, were cotransduced separately to B/r H266 (crosses 2 and 3, respectively, Table 2) and the Tc+ cotransductants were screened for division phenotype (with the proviso that the min* phenotype cannot be discerned in a minB strain). The transfer of minB+ corrected the Min* phenotype in 100% of the B/r H266 recipients (cross 2), indicating that min* is likely in the same region of the chromosome as minB, i.e. within 2 map minutes, the limit of P1 transduction (7), and that min* is very close to zcf-236::Tn10.

Ninety-six percent of the B/r H266 recipients from these 2 crosses did not produce minicells at any growth rate, while 4% still produced minicells at slower growth rates (cross 3). To confirm that a functional minB+ operon was present in normally dividing B/r H266 recipients, B/r H266 recipients of both minB+ and minB crosses, were transduced to the K12 strain F375 (minB) (crosses 4 and 5, respectively) and Tc+ transductants were selected.
TABLE 2. Frequency of P1vir-mediated transduction of transposon labeled division genes in various strains of *Escherichia coli*.

<table>
<thead>
<tr>
<th>STRAINS</th>
<th>FREQUENCIES OF DIVISION PHENOTYPES (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor</td>
</tr>
<tr>
<td>1.</td>
<td>PLK 1943 <em>minB</em></td>
</tr>
<tr>
<td>2.</td>
<td>K12 12435 <em>minB</em></td>
</tr>
<tr>
<td>3.</td>
<td>K12 12435 <em>minB</em></td>
</tr>
<tr>
<td>4.</td>
<td>B/r H266 <em>minB</em> b</td>
</tr>
<tr>
<td>5.</td>
<td>B/r H266 <em>minB</em> c</td>
</tr>
</tbody>
</table>

* *mutation that causes growth rate dependent minicellulation.

b constructed in cross 2.

c constructed in cross 3.
Forty-four and 50%, respectively, of the K12 recipients were unexpectedly corrected for minicell formation. This demonstrates that the B/r H266 contained a minB+ whether they were the outcome of minB or minB+ crosses. That is, no B/r H266 minB transductants were recovered in any cross, raising the question of whether this mutant locus had been transferred. Recent publication of a new E. coli chromosomal map (7) suggests a 3rd and more likely possibility, that an excisable section in the zcf-236::Tn10 - minB region of the K12 chromosome was interfering in the recovery of the minB locus in B/r H266. Treatment of this point is deferred to the Discussion.

However, the crosses did establish that the min* locus was not in minB, but was within 0.4 min of it. Furthermore, the cotransduction frequencies for correction of Min* in B/r H266 (crosses 2 and 3, Table 2) showed that min* is closely linked to zcf-236::Tn10, probably within 0.1 map min.

In light of the tight linkage between min* and zcf-236::Tn10, a further puzzling outcome was obtained in attempting a back transduction from one of the B/r H266 (zcf-236::Tn10, min*, minB+) isolates. No Tcr transductants of the recipient, K12 12435 minB+, were recovered, suggesting that min* is lethal in the K12 12435 genetic background. This was not pursued however.
Section II. Metabolic and Morphologic Differentiation in

Clostridium beijerinckii NRRL B593

Determination of the Growth Rate Domain of Metabolic Differentiation

The domain in which the differentiation from acidogenic to solventogenic metabolism occurs was determined by using the cell recycle fermentor. This growth system slows the rate at which the culture passes through domains 2 and 3. Thus events occurring in these domains are more clearly defined than in batch growth systems. The experiments were initiated in chemostat mode using MGC with sucrose at a growth limiting concentration of 10mM. The chemostat mode had a dilution rate (D) of 0.12 h\(^{-1}\) (\(t_d = 5.8\)h) and the substrate provision rate (SPR) was 624 µmol·h\(^{-1}\). After a steady state was reached in chemostat mode, the fermentor was switched to 100% cell recycle.

Upon switching from chemostat to recycle mode, the growth curve displayed a linear increase in mass (Fig. 3), producing a continuous increase in \(t_d\) until approximately 25 h in the recycle mode. Then a decrease in the growth rate was seen, from a \(t_d\) 43 to 109 h, resulting in an abrupt inflection in the growth curve. It appears that \(C.\ beijerinckii\) entered domain 2 immediately after the switch to recycle mode and that the inflection at 25 h in the recycle mode marks the culture’s entry into domain 3. The apparent molar yield on sucrose was 48 g biomass·mol in the chemostat mode, 58 g·mol in the first interval of growth after the switch to recycle mode, and 13 g·mol after the inflection at 25 h in recycle mode.
FIG. 3. Clostridium beijerinckii NRRL B-593 grown in sucrose limiting medium at 35°C and a constant pH of 6.8. The system began in chemostat mode ($D = 0.12 \text{ h}^{-1}$) and then was switched to recycle mode at the time indicated by dashed line: (♦) Klett units, (●) acetic acid and ethanol, (□) butyric acid and butanol, (▲) propionic acid and propanol. Isopropanol was not detected.
While in the chemostat mode, the culture was in the acidogenic stage, producing acetic and butyric acid and low levels of ethanol (Fig. 3). After the switch to recycling mode, the rate of acetic acid production fell immediately below a level necessary to prevent washout, stabilizing at a low rate of production, equal to about 10% that of chemostat mode. Butyric acid and ethanol production remained at their chemostat rates. Small but measurable amounts of n-propanol were detected 4.5 h after the switch. Approximately 24 h after the culture entered this new range of slower growth rates (49 h in recycle) an increase in ethanol and n-propanol production occurred and propionic acid was detected; this was followed 9 h later by an increase in butanol production (Fig. 3). There was no uniform increase in all three solvents at any one growth rate.

**Solventogenesis and Sporulation in Domain 3**

When cultures of *C. beijerinckii* are allowed to traverse the three domains of growth rates, sporulation occurs in domain 3. In the fermentation experiment depicted in Fig. 3, phase-dark forespores were observed 12 h after the culture entered domain 3 (37 h in recycle) (Fig. 4A). Fifty-nine h after the inflection (84 h in recycle) phase-bright forespores were detected, and 9 h later the culture turbidity dropped by 50%. The time from the first detection of phase-dark forespores to detection of phase-bright forespores was 50 h. The rate of butanol production increased as the proportion of phase-dark forespores increased. The production of phase-dark forespores and their conversion to phase-bright forespores paralleled approximately the increase and decrease in the rate of butanol
FIG. 4. Comparison of the frequency of *Clostridium beijerinckii* NRRL B593 cell forms present at different times with butanol formation (A) when a domain 3 culture sporulates spontaneously and (B) when a domain 2 culture is exposed to an air pulse. Arrow indicates time of air pulse: (□) phase dark forespores, (△) phase bright forespores, (○) free spores, and (■) butanol.
To determine if sporulation occurs because of an association with solventogenesis or because both are triggered by the slowing of the growth rate, solventogenesis was induced in a fast growing culture. Two chemostat experiments were conducted at a rapid growth rate ($D = 0.12 \, h^{-1}$ \quad [\tau_d = 5.8 \, h]$. At pH 6.8, only butyric and acetic acids were produced in quantity (Table 3). When the pH was lowered to 4.8 to induce solventogenesis (41, 87), acetic acid, butyric acid, butanol, and isopropanol were detected. Low levels of ethanol were present at both pH values. Phase-dark forespores were detected in the chemostat culture only at pH 4.8. At pH 6.8 the culture consisted of regular bacillus-shaped cells. Thus, sporulation and solventogenesis were associated at the domain 1 growth rate.

Solventogenesis and Sporulation in Domain 2

The chelating agent, nitrilotriacetic acid, was added to MGC in a fermentor experiment to prevent the formation of a hazy dark precipitate, which is likely to be insoluble salts formed during autoclaving (63). Under these conditions sporulation occurred in domain 2 in the absence of significant solvent production (Fig. 5). After the switch to recycle mode, the growth curve increased linearly for the duration of the experiment. The apparent molar growth yield on sucrose was $67 \, g \, biomass\cdot mol$ in chemostat and $76 \, g \, biomass\cdot mol$ in recycle mode. Phase-dark forespores constituted 1% of the culture after 14.3 h in recycle mode, and reached a production.
TABLE 3. Product formation by *C. beijerinckii* NRRL B-593 in sucrose-limited chemostat growth at $D = 0.12 \text{ h}^{-1}$, 30°C, under neutral and acidic conditions.

<table>
<thead>
<tr>
<th>pH</th>
<th>Acetic (mM)</th>
<th>Butyric (mM)</th>
<th>Solvents (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8</td>
<td>13</td>
<td>8</td>
<td>0.4 2.5 0.6</td>
</tr>
<tr>
<td>6.8</td>
<td>28</td>
<td>8</td>
<td>0.7 0 0</td>
</tr>
</tbody>
</table>
FIG. 5. *Clostridium beijerinckii* NRRL B-593 grown in sucrose limiting medium with 0.1 g L⁻¹ nitriloacetic acid at 35°C and a constant pH of 6.8. The system began in chemostat mode (D = 0.12 h⁻¹) and then was switched to recycle mode at the time indicated by dashed line: (♦) Klett units, (●) acetic acid and ethanol, (□) butyric acid and butanol, (△) propionic acid and propanol, and (▼) isopropanol.
maximum of 81% 17.2 h later. The culture was 10% free spores approximately 43 h after switching to recycle mode.

Solvents were produced at low levels throughout this experiment, with no significant increase in their production rate. Propionic acid was detected in amounts of 0.2 mM or less. As seen in Fig. 3, the acetic acid production rate fell to a rate lower than in chemostat steady-states (Fig. 5); this resulted in washout. The rate of butyric acid production remained essentially stable. This event occurred in all fermentor runs when the growth system was switched from chemostat to recycle mode, both in defined medium (2) (data not shown) and in MGC, an undefined medium.

The detection of phase-dark forespores in the absence of significant solvent formation is due most likely to the speed with which the culture sporulated. Time spent by the culture in the stage of sporulation associated with solvent production was too short for significant solvent accumulation to occur.

To study the association between morphologic and metabolic differentiation in greater detail, sporulation was induced in a domain 2 culture of C. beijerinckii, when solvents are not formed (cf., Fig. 3). The stages in the sporulation of C. beijerinckii were followed using both phase contrast and electron microscopy, for the following reasons. After analysis of the literature, it is unclear which of the early stages of sporulation is associated with solventogenesis in C. acetobutylicum. This is caused by the inconsistency of the nomenclature used to describe Clostridium sporulation stages 0-IV. This is especially a problem in describing stage III (Fig. 6A
and Fig. 7D). The same authors use the phrase "phase-dark clostridial form" (132) to describe stage III of sporulation and in a different publication use "phase-bright clostridial forms with phase-dark forespores" (64, 131) to describe the same stage. Others use the term phase-bright clostridial form to describe stage IV of sporulation (132). Thus the confusion. Figure 8 reviews the stages of C. beijerinckii as interpreted from phase contrast microscopy and lists the nomenclature used in this study to describe the sporulation stages of interest.

Electron microscopy was used in conjunction with phase contrast to obtain more information concerning the stages of sporulation of C. beijerinckii NRRL B593, since variations in the sequence of events in the later stages of sporulation in Clostridium species has been noted (131). It did not appear that such anomalies occur in C. beijerinckii NRRL B593 (Fig. 7). Clostridium beijerinckii did not have any reversed stages, such as formation of the spore coat before formation of the cortex, or the production of appendaged outer spore coats (131). The possible reversal of the sequence of formation of the spore coat and the cortex could have had an influence on solvent production, but did not occur. Instead C. beijerinckii NRRL B593 followed the sequence of sporulation events reviewed in section III of the Introduction, as deduced from the observations recorded below.

Samples examined by phase contrast light microscopy and in thin section by electron microscopy are presented in Fig. 6 and 7, respectively. Fig. 6A is a photomicrograph of vegetative cells, stage 0 of sporulation, just prior to the air pulse. Fig. 7A shows a dividing vegetative cell from the
FIG. 6. Phase contrast photomicrographs (2400x) of *Clostridium beijerinckii* before and after exposure of a domain 2 culture to an air pulse. (A) Cells before air pulse. (B) Cells 13 h after air pulse. Arrow indicates phase-dark clostridial form. (C) Cells 24 h after air pulse. Solid arrow indicates free spore, plain arrow indicates phase-bright forespore. Numerous phase-dark forespores are visible. (D) 94 h after the air pulse with numerous free spores.
FIG. 7. Electron micrographs of thin sections of *Clostridium beijerinckii* before and after exposure of a domain 2 culture to an air pulse. (A) Dividing vegetative cell just prior to air pulse. (B) Longitudinal section of a stage II sporulating cell 19 h after the pulse, displaying the asymmetrical division of prespore formation. The separating membrane near the cell pole can be seen traversing the cytoplasm from one thickening wall to a similar thickening wall on the opposite side of the cell (arrows). (C) Two stages of sporulation: a phase-dark clostridial form, stage III, showing the faint outline of the double membrane bound forespore; a phase-bright forespore still within the sporangium in a late stage of sporulation before release. Note the electron dense spore coat surrounding the electron lucent cortex. (D) A swollen phase-dark clostridial form. The double forespore membrane can be seen as distinct from the plasma membrane of the sporangium (arrow), indicating engulfment was complete. (E) A finished spore with remnants of the sporangium still associated 43 h after the air pulse. The lamellar layer between the spore coat and the cortex (arrows) displaying many channels. The membrane on the inner side of the electron lucent cortex is clearly visible. Bars represent 0.5 μm.
same sample. Fig. 7B is a cell 23 h post pulse exhibiting forespore division, stage II of sporulation. Arrows indicate the division site where a faint unit membrane traverses the cytoplasm, forming two membrane-bound cells within a continuous cell wall. Note the inward thickening of the cell wall at the site of constriction.

Fig. 7C shows two cells, in different stages of sporulation. One (upper left) is a phase-bright sporangium displaying a forespore surrounded by the inner spore membrane, an electron lucent cortex, a darker layer, the spore coat, and the remainder of the sporangium. Note the uninterrupted mother cell wall. The other cell (lower right) is a phase-dark forespore displaying the "clostridial form" morphology, unique to these anaerobic spore-forming bacilli. Note the faint outline of the engulfed forespore at the swollen end of the cell.

Fig. 7D shows a more detailed electron micrograph of a phase-dark forespore. The arrow indicates the visible double membrane indicative of a cell that has completed stage III of sporulation. Note the absence of an electron lucent cortex, which would graduate the sporulating cell to the next stage (IV) in the scheme (Fig. 8). The swollen "clostridial form" morphology is not as striking as in Fig. 7C.

Fig. 6B is a photomicrograph of cells 13 h after the air pulse (Fig. 4B) displaying the presence of phase-dark forespores (arrow). Eleven h later, phase-bright forespores, stage IV of sporulation, are detected in the fermentor (Fig. 4B and Fig. 6C). The open arrow indicates a phase-bright forespore and the closed arrow indicates a free spore.
FIG. 8. Schematic illustrating the morphological stages in *Clostridium beijerinckii* NRRL B593 sporulation inferred from electron micrographs similar to those of Fig. 7. The opposed, unit membranes believed to constitute the thickened membrane structure surrounding the forespore in the phase dark forespore stage and the plasma membrane of the sporangium are indicated by arrows.
Free spores do not appear until 15 h after phase-dark forespores were first noted (Fig. 4B). Free spores are shown in Fig. 6D and a finished spore is shown in Fig. 7E with remnants of the sporangium still associated. The inner spore membrane surrounding the core or spore protoplasm is partially visible, surrounded by the cortex, the outer spore membrane (arrows), the electron dense inner spore coat, and finally the outer spore coat.

When sporulation was induced in a domain when solvents are not usually produced (refer to Fig. 3), only butanol was detected in association with the phase-dark forespore stage of sporulation.
DISCUSSION

Section I. Morphologic Differentiation in Escherichia coli B/r H266

I investigated the growth-rate dependent minicell formation in *E. coli* B/r H266 to determine if this unique division phenotype was in the *minB* operon and whether ppGpp might be involved in the topological regulation of MinE activity.

Various carbon/energy sources were tested; including glucose, L-alanine, succinate, lactose, and erythritol. In all cases minicell formation was not detected until the growth rate had slowed to approximately 20 h, indicating B/r H266's unique minicellulation phenotype is not an artifact of the carbon/energy source. Formation of true minicells was confirmed using the nucleoid condensation method. Small chromosome-less cells were produced; an unequal division producing two chromosome containing cells did not occur. It had been previously noted that *E. coli* B/r H266 forms minicells only in postexponential cultures (130). In this strain, minicell formation began when the culture entered domain 2 and the rate of formation decreased as the growth rate fell throughout the domain (16).

Through transduction studies, *zcf-236::Tn10* was located within an excisable cryptic chromosomal region, e14, at 25.7 min on the *E. coli* chromosome (7) (Appendix II). If the e14 region in *E. coli* K12 in which the transposon was mapped is not present in B/r strains, there would be
nonhomologous DNA sequences between the transposon and the \textit{minB} operon. When homologous recombination occurs between the \textit{K12 minD1} and the \textit{B/r H266 minD*} the transposon would be lost, thus making it impossible to recover \textit{To r B/r H266} transductants with the \textit{minD1} genotype.

The \textit{min*} mutation in \textit{B/r H266} is close to \textit{zcf-236::Tn10}, but it is not possible to determine the location of the mutation because the exact location of the \textit{zcf-236::Tn10} insertion site in \textit{B/r H266} is unknown. The accuracy of mapping two closely linked markers by this method is poor (133).

de Boer \textit{et al.} determined that the products of the \textit{minB} operon, that is MinC, MinD, and MinE, work together to block division at the cell poles and prevent division blockage at internal sites (25). MinC/MinD blocks division at the poles while MinE inactivates MinC/MinD in the midcell region (27, 26), but the topological regulator influencing MinE to function only at internal division sites of the cell is unknown (24). It appears that the \textit{min*} mutation in \textit{B/r H266} is a minicell producing mutation not in the \textit{minB} operon as the operon is described by de Boer \textit{et al.} (24, 101). The mutation in \textit{B/r H266} could be in the topologic regulator of MinE activity that has not yet been identified (24).

In \textit{B/r H266}, minicells are produced only in domain 2, after the level of ppGpp begins to increase in the cell (3, 16, 121, 122). How increasing levels of ppGpp and the \textit{min*} mutation allow division to occur at the cell poles is difficult to determine at this time. It is possible that in this strain \textit{Min*} is allowing MinE to inhibit MinC/MinD at the cell poles only after the ppGpp level begins to increase in the cell. A more extensive study of the
*gene is required to determine the exact role of ppGpp in the process of normal cell division.

Section II. Metabolic and Morphologic Differentiation in

*Clostridium beijerinckii* NRRL B593

Growth Rate Domains and Metabolic Differentiation

Recycling fermentor experiments, with their expanded time scales, present the series of metabolic and morphological differentiations observed in batch cultures in a more easily dissected form. Shifts in metabolism did not occur at a single point in the fermentation or at a single *t*_d. The sequence of production of each acid and solvent could not be organized such that either acidogenic or a solely solventogenic stage could be recognized in the way these stages are perceived in batch culture (37, 38). As *C. beijerinckii* traversed the three domains, changes in one or more of the product formation paths occurred in separate series (2, 100).

Most of the information concerning the differentiation from acidogenesis to solventogenesis in solventogenic *Clostridium* species has been obtained through batch culture studies. Batch culture is a substrate-closed, biomass-closed system (16, 17), therefore products leaving the cell accumulate in the surrounding environment. It has been shown that the accumulation of volatile fatty acids produced during acidogenesis affects the differentiation to solventogenesis (56, 115). When grown in a cell recycle fermentor, acidogenic products do not accumulate, allowing study of
metabolic differentiation without the influence of decreasing pH (8) or the effect of undissociated butyric and acetic acids in equilibrium across the cell’s membrane (43, 52, 115).

The differentiation from acidogenesis to solventogenesis is not a clear-cut shift. It began in domain 2 with a decrease in the rate of acetic acid production and ended 54 h later in domain 3 with the increases in the rates of production of both ethanol and propanol, coinciding with the appearance of butanol. Elevated levels of ppGpp were not required for regulation of metabolic differentiation since solventogenesis occurred approximately 29 h after the culture entered domain 3.

Unlike other bacterial genera tested (2, 18, 100, 121, 122), when C. beijerinckii was switched to recycle mode, its yield coefficient fell, and the culture dropped into domain 2, indicating that the trigger for the switch from domain 1 to domain 2 occurred immediately after the fermentor was switched to recycle mode. The switch to recycle mode converts the fermentor from a substrate-open, biomass-open growth system to a substrate-open, biomass-closed growth system (16). The biomass begins to increase while the substrate supply rate remains constant, resulting in an immediate decrease in the amount of substrate per cell and possibly the undersaturation of the carbon uptake system of the cell which, in turn, would actuate the 1 to 2 domain shift in C. beijerinckii (100). Therefore, it may be that the undersaturation of the carbon uptake system indicates a change in the cell’s surrounding environment from one that can be exploited to one requiring and thus necessitating a transition from domain
Another unexpected event occurred upon switching from chemostat to recycle mode, the decrease in the rate of acetic acid production without an accompanying decrease in the rate of butyric acid production. Why this occurs is not clear. As mentioned previously, the switch to recycle mode could be affecting the degree of saturation of the cell's carbon uptake system which could trigger the decrease in the rate of acetic acid production while not affecting the rate of butyric acid production. It is not likely to be in response to regulation by domain 2 levels of ppGpp and related nucleotides because the rate of acetic acid production changes too quickly. But, the unique observation of uncoordinated production of acetic and butyric acids does indicate that the regulation of these catabolic pathways are not linked.

In the recycling fermentor, C. beijerinckii NRRL B593 produced propionic acid and n-propanol (2). At no time was 1,2-propanediol detected as was seen by Forsberg et al. while studying C. beijerinckii NRRL B593 grown with rhamnose as the carbon source (33). It would seem the pathways suggested by Forsberg et al. (33) do not account for the results seen in the cell recycle studies using the same organism. More likely, it is one of the two following paths. The first pathway has been characterized in other Clostridium species; propionyl-CoA is produced from pyruvate, with succinate as an intermediate (108). The second path for conversion of pyruvate to propanol and propionic acid follows the conversion pattern observed in butyric acid and butanol production. Lactic and acrylic acids would be intermediates in the production of propionyl-CoA. In both cases,
propionyl-CoA would then be converted to propionic acid or propanol analogous to butyric acid and butanol formation (Fig. 1).

**Differentiation to Sporogenesis and its Association with Solventogenesis**

Sporulation has been shown to be temporally associated with the onset of solventogenesis in batch cultures of *C. acetobutylicum* (64). This association was not seen in batch cultures of *C. beijerinckii* at a neutral pH (37). In chemostat culture at acid pH the differentiation to solventogenesis occurred (2) (Table 3) with concurred production of phase-dark forespores.

To study the link between sporogenesis and solventogenesis we determined which stage of sporulation is associated with a single solvent or all solvents. Even though the air pulse induced and spontaneous sporulation occurred in different domains, only butanol production began during the phase-dark stage (III) of sporulation and ended with the conversion of the cell to stage IV, when they became phase-bright.

In an attempt to better represent the information obtained from the light and electron microscopy plus the butanol production data, a schematic of the morphological stages observed and their relation to butanol formation is presented (Fig. 8). The first stage, the filamentous arrangement of the nucleoid, was not seen in our samples. Stage II, the asymmetric prespore division, was visualized in thin sections using the electron microscope (Fig. 7B). The transition from stage II to III requires the "engulfment" of the prespore to become a double membrane bound forespore producing the "clostridial form" which is referred to as the phase-dark forespore (Fig 7C and D). It is this stage that is linked to solvent
production (64), but in my experiment only butanol was detected.

As a sporulating cell moves from stage III to stage IV, the forespore begins to refract light as the cortex is formed, producing forespore intermediate between phase-dark and phase-bright, and the butanol production rate begins to decrease (Fig. 4A and B). These cells were included in the phase-dark forespore counts. A stage IV sporulating cell is a phase-bright "clostridial form" having completed the formation of the cortex layer of the spore. The phase-bright cells go on to form a spore coat and are released from their sporangium upon completion as seen in Fig. 7E.

In C. acetobutylicum, the association between metabolic and morphologic differentiation is at a stage in sporulation before free spore formation (80, 132) and after prespore division (64). In C. beijerinckii, the association of sporulation and solvent production is more accurately in the formation of phase-dark forespores and the production of butanol (Fig. 4A and B and Table 3). The specific stage of sporulation linked with solvent production in C. acetobutylicum has not been determined, but appears to also be the formation of phase-dark forespores (49, 64, 80, 132) and not an earlier stage of sporulation (64).

It has been proposed in C. acetobutylicum that the association between the differentiation to solventogenesis and sporulation is due to regulation by a global regulatory system (37, 132). If this is the case in C. beijerinckii, it is not due to the attainment of full stringent levels of ppGpp (Fig. 3). From the data presented here it would appear that the association
is between formation of phase-dark forespores and butanol production and not the production of all solvents. This is most evident in the sporulation induction experiment (Fig. 4B). When sporulation was induced and phase-dark forespores were formed, only butanol was produced.

The link could be the formation of the double membrane bound forespore. Jones et al. showed that C. acetobutylicum mutants unable to complete forespore division (stage II) produced low levels of total solvents as compared to the parent (64). It may be the formation of the engulfed prespore (Fig. 7D) that creates an environment in the cell for butanol formation. The phase-dark forespore, before the cortex is formed, could still be producing butyric acid which diffuses into the mother cell creating an acid environment. The increase in butyric acid in the mother cell then stimulates the conversion of butyric acid to butanol. This would mean that it is necessary to have phase-dark forespores for solventogenesis to occur.
**Appendix I**

**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>$t_d$</td>
<td>mass doubling time</td>
</tr>
<tr>
<td>ppGpp</td>
<td>guanosine 5'-diphosphate 3'-diphosphate</td>
</tr>
<tr>
<td>TS</td>
<td>tryptic soy</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani broth</td>
</tr>
<tr>
<td>Tc</td>
<td>tetracycline</td>
</tr>
<tr>
<td>DM</td>
<td>Davis-Mingioli minimal medium</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>MGC</td>
<td>modified George and Chen medium</td>
</tr>
<tr>
<td>CB</td>
<td>cacodylate buffer</td>
</tr>
<tr>
<td>B/r H266</td>
<td><em>E. coli</em> B/r H266</td>
</tr>
<tr>
<td>K12</td>
<td><em>E. coli</em> K12</td>
</tr>
<tr>
<td>Tcr</td>
<td>tetracycline resistance</td>
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<td>D</td>
<td>dilution rate</td>
</tr>
<tr>
<td>SPR</td>
<td>substrate provision rate</td>
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Above is an enlarged section of a linear scale drawing representing the circular *E. coli* K-12 linkage map (7) from approximately 25.5 to 26.5 min on the K-12 chromosome (min being the unit of the map). This section includes the *minB* operon at 26.2 min (24) and e14, an excisable region of the K-12 chromosome from 25.6 to 25.9 min (7). It is within e14 region that *zcf-236::Tn10* maps.
LITERATURE CITED


