A STUDY OF THE COCCOID BODIES OF *PROLINOBORUS FASCICULUS* (*AQUASPIRILLUM FASCICULUS*)

by

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APPROVED:

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Abstract: Following active growth, the aquatic gram-negative rod *Prolinoborus fasciculus* (*Aquaspirillum fasciculus*) exhibited a mass conversion from its culturable rod form to a nonculturable coccoid form. Chloramphenicol did not prevent the conversion. Attempts to obtain variants that would not convert to the coccoid form were unsuccessful. Although the coccoid form fluoresced with acridine orange, agarose gel electrophoresis revealed extensive ribosomal RNA degradation. Poly-ß-hydroxybutyrate, abundant in the vegetative rods, was not detectable in the coccoid cells. The results suggest that the coccoid form of *A. fasciculus* is a degenerative form rather than part of a life cycle.

ACKNOWLEDGEMENTS

> "You may have seen many a quaint craft in your day, for aught I know;- square-toed luggers; mountainous Japanese junks; butter-box galliots, and what not; but take my word for it, you never saw such a rare old craft as this same rare old Pequod."
>
> Moby Dick, Herman Melville

Dr. Krieg has been a great influence in my life. He has given me a chance when no one else would and for that reason, I dedicate this thesis to him. He is a great scholar and an incredible friend. I am very fortunate and extremely proud to have worked under his supervision. He has always been so fiercely passionate about the discipline of Bacteriology that one can not help but compare him to Captain Ahab. And I was his Ishmael.

I would also like to thank my brother, Michael P. Koechlein, for all the words of encouragement when I needed them the most. Michael has made me laugh when I was feeling down and pushed me when I really deserved to be shoved.

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Introduction

Many microorganisms in aquatic environments have been shown to exhibit several survival strategies in response to low nutrient concentrations, temperature, and osmolarity. The cells may undergo physiological and morphological changes in order to remain viable through periods of starvation. Some of these changes may include cell elongation, increase in the number of high affinity nutrient transporters, and, in some instances, the conversion of rod-shaped vegetative cells to a coccoid (spherical) shape. Some of these altered forms, such as coccoid bodies, have been termed "viable but nonculturable" (VBNC) by Rollins and Colwell (1987). The "nonculturable" aspect of VBNC was based upon the discovery that the cells could not be recultured on the medium from which they were initially isolated or any other laboratory medium. The "viable" aspect was based on the ability of the altered cells to fluoresce when treated with acridine orange and to exhibit cell elongation when treated with nalidixic acid.

The suggestion has been made that VBNC forms of pathogenic bacteria represent a dormant, infectious form. Such forms could be implicated in reinfection of susceptible hosts after treatment, reoccurrence of infection after treatment, or survival of potentially infectious organisms in water or sewage. The existence of VBNC pathogens in drinking-water supplies has serious epidemiological importance. Examples of potential VBNC pathogens are *Legionella pneumophila*, *Campylobacter jejuni*, *Escherichia coli*, *Vibrio* spp., *Salmonella enteritidis*, and *Helicobacter pylori* .

Prolinoborus fasciculus (previously *Aquaspirillum fasciculus*) is a freshwater, gram-negative, nitrogen-fixing straight rod first described in 1971 by Strength and Krieg.

Its ability to undergo mass conversion to the coccoid form was described by Strength *et al.* The purpose of the present study was to quantify the phenomenon and examine factors such as the condition of the ribosomal RNA (rRNA) that bear on the question of whether the coccoid form is part of a life cycle or merely a degenerative form.

Literature Review

Microbial Growth

Bacterial growth is represented by a sequence of a lag phase, exponential or log phase, stationary phase, and a decline or death phase (Fig. 1) (Ingraham *et al.* 1983). When a microbial population is inoculated into a fresh medium, growth does not occur immediately, but only after a period of time called the lag phase. The lag phase happens because cells must have time to synthesize new enzymes in order for exponential growth. In the log or exponential phase, the number of cells increases by a constant factor during each unit time period. During exponential growth, all biochemical constituents are being synthesized at the same relative rates, a process known as balanced growth. When an essential nutrient of the culture medium has been exhausted or some waste product of the organism builds up in the medium in an inhibitory level, exponential growth ceases. The cells in the medium have reached the stationary phase. In the stationary phase there is no net increase or decrease in cell number. Cells in the stationary phase may continue metabolism and some biosynthetic processes. After the stationary phase, the population is said to be in the death or decline phase. During this phase, the total count (as measured by a direct microscopic count) may remain constant, but the viable count decreases (Ingraham *et al.* 1983). From studies of batch cultures limited by various elements, e.g., phosphate, magnesium, carbon, or nitrogen, populations of bacteria in the death (or

decline phase) reach an extended survival state with 2 to 4 % of the cells remaining viable as measured by the colony-forming ability (Ribbons and Dawes, 1963). This stage is termed cryptic growth, because a portion of a starved microbial population dies, and releases products of lysis and leakage that support the growth of the survivors (Postgate, 1976). Studies of bacterial growth have focused mainly on the early phases of growth while the decline phase has often been ignored because the predominance of dead cells was of no interest in studies of growth (Postgate, 1976).

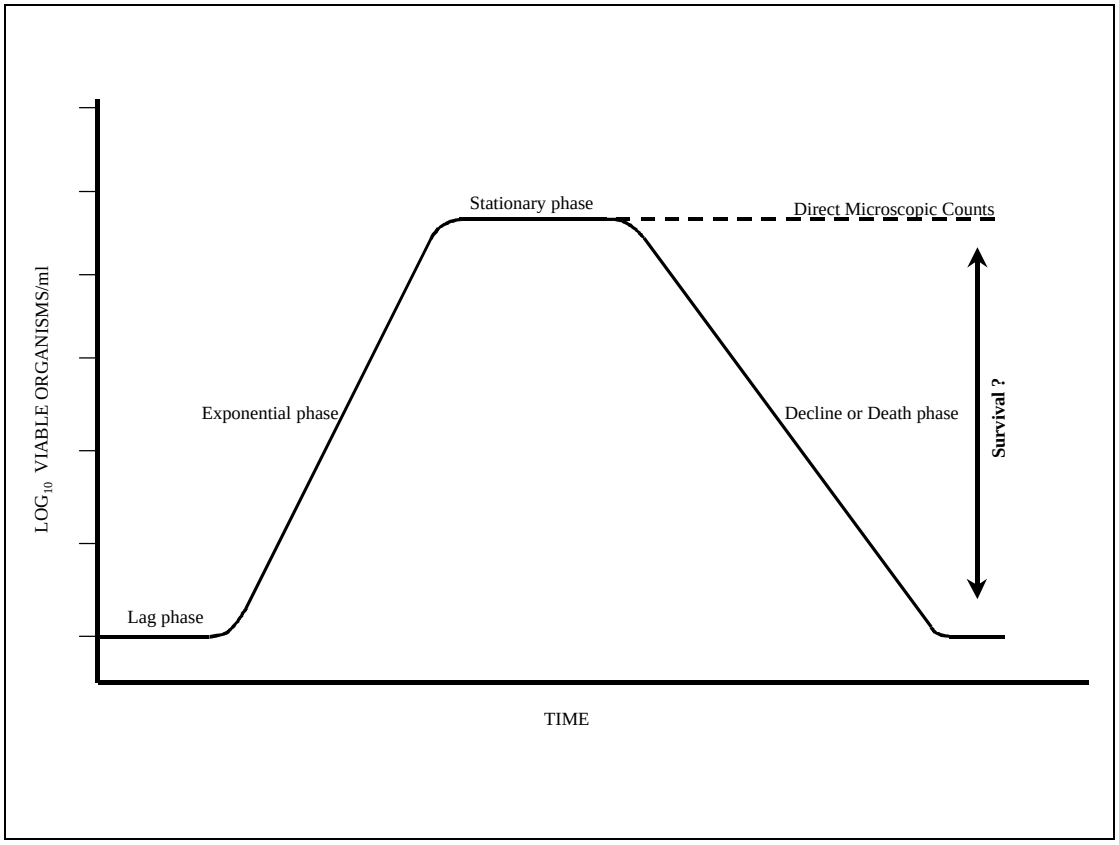

Figure 1. Typical growth curve showing the results of plate counting methods versus direct microscopic counts. “Survival?” refers to cells that could be “reactivated” or “resuscitated” from the viable but nonculturable state (VBNC). (Adapted from Ingraham *et al.* 1983)

Viable but Nonculturable Forms

The decline phase is of particular importance in microbial ecology. The environments of bacteria in nature are generally more oligotrophic than those found in laboratory conditions (Kaprelyants *et al.* 1993). Bacteria in aquatic and marine environments must adapt their metabolism to changing conditions and shift between states of growth and nongrowth. Many bacteria have evolved highly specificed mechanisms to allow them to remain viable during starvation and resume growth rapidly when nutrients become available. Some species form dormant spores, some form multicellular aggregates and fruiting bodies in response to starvation conditions (Siegele and Kolter 1992). Many bacteria, such as *Esherichia*, *Salmonella*, and *Vibrio* spp. enter a starvation induced program that results in a metabolically less active and resistant state (Baker *et al*. 1983; Cashel *et al*. 1987). Some scientists suggest that this metabolically less active state is a period of dormancy in non-sporulating bacteria until conditions become favorable (Kaprelyants *et al.* 1993). Heinmets *et al.* (1953) claimed that *E. coli* that failed to grow on solid media after a period of starvation could be "revived" or "resuscitated" after a short incubation time in the presence of suitable metabolites in broth cultures. Experiments performed by Jenkins *et al*.(1991) on *E. coli* have shown that cells synthesize proteins involved in maintaining viability during prolonged starvation. They also discovered that *E. coli* cells become much smaller and almost spherical when they enter the stationary phase.

This phenomenon is is also observed in many marine bacteria which greatly decrease in size and form ultramicrocells, as small as 0.03 μm^3 (Kjelleberg *et al*. 1987). Ultramicrocells result from cells that undergo several cell divisions without an increase in biomass and then a further decrease in their size as a result of endogenous metabolism. One possible selective advantage of these successive divisions is to improve the survival of the clonal population by increasing the probability that some cells will encounter nutrients (Morita 1986). These discoveries have led some researchers to believe in a viable but nonculturable (VBNC) phase in which cells are intact and alive but are unable to form colonies on solid media until conditions become favorable (Rozak and Colwell 1987). The phenomenon appears to reflect a mechanism of bacterial survival, with strain variation as well as variation in characteristics being related to environmental conditions and length of exposure to those conditions (Rozak and Colwell 1987). The VBNC state can be induced by a variety of factors including temperature, nutrient concentration, salinity, osmotic pressure, and pH (Huq and Colwell 1995).

The occurrence of VBNC forms led to evaluations of the usefulness of plate counting procedures (Buck 1979). Comparisons between results of plate counts, direct microscopic counts, and indirect activity show that the number of bacteria forming colonies on a solid medium is significantly less than the number actually present and metabolically active, often by several orders in magnitude (Buck 1979). The use of selective media used in traditional plating procedures are not optimal for growth even for nonstressed populations because of inhibitory dyes, mechanical stress in transfers, and

oxygen damage (Rosak and Colwell 1987). This has led to a debate on what microbiologists define as “viable” cells. Kurath and Morita (1983) refer to cells as being viable only so long as they demonstrated the capacity to reproduce on a agar medium suitable for growth of the organism. Cells were considered nonviable once they lost the ability to form a colony. However, Kurath and Morita concluded that there was a distinct subpopulation of cells that were actively respiring at a concentration 10-fold greater than the number of “viable” cells growing on agar plates. Hoppe (1976, 1978) employed autoradiographic methods on marine bacteria and observed that there was an actively metabolizing population of cells which were the predominate inhabitants that did not form colonies on agar plates. Postgate and Hunter (1962) used slide culture as detection method, and considered cells to be alive if they were capable of cell division. Cells were considered dead when they had lost the ability to divide. Postgate and Hunter conceded that some nondividing bacteria might be in some sense “alive” because they retained their osmotic barriers after “death”.

The conventional view held by many microbiologists is that a single, viable organism is represented by a cell capable of dividing and forming at least one live daughter cell when it is placed in a suitable environment (Rozak and Colwell 1987). Dormancy is defined as a reversible state of low metabolic activity, in which cells can persist for extended periods without division. Dormancy has mainly been connected with bacterial forms which are obviously morphologically specialized such as spores and cysts, structures which can be formed by only a limited number of bacterial species (Sussman and

Halvorson 1966). The argument that is often used in favor of the widespread existence of dormant foms of vegetative non-sporulating bacteria is that “in most natural microbial environments only a very small fraction of the microbes present can be enumerated using conventional agar plate techniques” (Mason *et al*. 1986). During the past decade, clinical and aquatic environmental pathogens representing more than 13 genera including members of the families *Enterobacteriaceae, Vibrionaceae,* and *Aeromonadacea* have been reported to occur in the VBNC state (Huq and Colwell 1995). Pathogens that exist in the VBNC state may escape detection if routine bacteriological culture methods are solely employed. In such situations, inaccurate and misleading reports may be generated, preventing public health officials from taking the appropriate actions. Many of these pathogenic bacteria form coccoid bodies which many researchers consider VBNC (Rozak and Colwell 1987; Mauch and Malfertheiner 1993; Jones *et al.*1991; Sörberg *et al*. 1996).

Coccoid forms

The conversion of rod-shaped, vibrioid, or helical vegetative cells to a coccoid form occurs in aging cultures of a variety of bacteria. The morphology, physiology, and biochemistry of these forms have been studied but the significance of coccoid bodies still remains a matter of speculation (Tritz and Ogg 1967; Boucher *et al*. 1994). Studies of bacteria *in vitro* have led to conflicting opinions as to whether the coccoid bodies represent a degenerative state or a viable resting stage. There are many species of bacteria

that form coccoid bodies such as *Campylobacter* spp., *Vibrio* spp., *Oceanospirilla* spp., *Aquaspirilla* spp., and *Helicobacter pylori* (McKay 1993; Sörberg *et al*. 1996; Bergey's Manual of Sytematic Bacteriology, 1989).

Bacteria that form coccoid bodies have been compared to endospore-forming organisms (Hood *et al.* 1986). A typical endospore-forming organism responds to adverse environmental conditions by producing the dormant endospore. This model of dormancy is probably the best understood of the microbial dormant forms. The spore-forming organism produces a structure with many tough protective layers such as the exosporium, spore coats, and a cortex which can resist desiccation, UV light, and high temperatures (Setlow 1983). However an aquatic organism would certainly not experience the lack of water nor probably excessive UV light, but it would experience stress due to low nutrient levels and temperature fluctuations. Thus an aquatic bacterium would not necessarily benefit from forming protective layers, but it may need to carry out some of the same macromolecular changes that occur in spore formation. The conservation of structural ribosomes and a decrease in total lipids, DNA, and protein are responses observed in some species of endospore formers (Setlow 1983). These are some of the same responses observed in coccoid body formation by some researchers (Morgan and Upton 1985; Hood *et al.* 1986; Hazeleger *et al.* 1995). Observed changes in coccoid bodies are changes in cell morphology such as retraction of the protoplast away from the cell wall to one side of the cell, an increase in surface hydrophobicity, a decrease in lipid and RNA content, changes in fatty acid composition, and utilization of reserves such as poly-β-hydroxybutyrate (for review see Kjelleberg *et al.* 1987; Kaprelyants *et al.* 1993).

However, if coccoid bodies are to be considered to be in a dormant state, one has to show that the cell of interest cannot grow under the present conditions of incubation but is able to produce daughter cells under appropriate conditions.

Evidence that coccoid bodies are nonviable degenerate forms include loss of proteins (Nilius *et al*. 1993) and nucleic acids (Morgan *et al*. 1986), decreased density in rate-zonal centrifugation (Morgan *et al*. 1986), and loss of cell wall components (Morgan *et al*. 1986; Buck *et al*. 1983). However, other evidence indicates that these forms are still viable: the ability to synthesize small amounts of DNA (Bode *et al*. 1993), the presence of polyphosphates (Bode *et al*. 1993; Bode *et al*. 1993), the putative ability of a large inoculum of coccoid forms to cause disease in laboratory animal models (e.g., see Cellini *et al*. 1994), and the resuscitation of *Vibrio cholerae* coccoid forms by an increase in temperature (Nilsson *et al*. 1991). One study suggests that *Helicobacter pylori* has two different coccoid forms, one that is a viable dormant stage, and one that is degenerate (Sörberg *et al*. 1996).

Studies involving VBNC forms

Many studies on VBNC state have focused on the induction of coccoid forms by low nutrient conditions. Hood *et al*. (1986) measured the response of *Vibrio cholerae* to low nutrient levels. They observed significant decreases in the amounts of lipids, carbohydrates, DNA, RNA, protein levels and the complete loss of poly-β-hydroxybutyrate (PHB) before the cells changed into the coccoid form. Poly-β-

hydroxybutyrate is normally used as endogenous energy source, and its structure and function will be discussed later in this review. The authors suggested that the rapid disappearance of phospholipids, PHB, and carbohydrates are to prepare the cells for dormancy. Examination of the ribosomes by electron microscopy showed that there was a decrease in the number of ribosomes, but they appeared to exhibit no structural change. However, it was argued by Kjelleberg *et al*. (1993) that these responses observed by Hood *et al*. (1986) could be due to the fact that the cells are in a degenerative state and not in preparation for dormancy. No attempts were made by Hood *et al.* (1986) to resuscitate *V. chlorae* from the VBNC state.

Steinert *et al.* (1997) published a study on the resuscitation of VBNC cells of *Legionella pneumophilia*, an aquatic bacterium responsible for Legionnaires' disease in humans. They observed that VBNC cells of *L. pneumophilia* could be "resuscitated" in the ameoba *Acanthamoeba castellanii*, a host which legionellas multiply intracellularly. VBNC forms were deemed to be viable after 125 days by 16S rRNA-target oligonucleotide probes and acridine orange direct counts (detection methods are discussed later in this review). However, reactivation of VBNC legionella was not observed when fed to mice. Yamamoto *et al*. (1995) used quanitative PCR detection of *L. pneumophilia* DNA to show that nonculturable cells retained DNA even after starvation for 300 days. However, their resusicitation trials using the protozoa, *Tetrahymena pyriformis*, were unable to recover any colony forming units after 100 days. Examination of the nucleic acids of *L. pneumophilia* showed extensive degradation of the rRNA subunits. They suggested that although the *L. pneumophilia* retained nucleic acids, these were degraded

and were probably nonfunctional. They further suggested that rRNA degradation observed in nonculturable legionella cells suggest that the ribosomes were disassembled and that their protein synthesizing machinery was inoperable (Yamamoto *et al.* 1995). The structure and function of rRNA will be discussed later in this review. The resuscitation observed by Steinart *et al.* (1997) may have been due to a few undetected culturable cells.

Nilsson *et al.* (1991) recovered *Vibrio vulnificus*, an estuarine bacterium, from the VBNC state by using a temperature upshift from 4° C to room temperature. They described this phenomenon as "resuscitation" from the VBNC state. This was the first report of recovery of VBNC coccoid cells without the addition of nutrient, and suggested that temperature may be the single most important factor in resuscictating the VBNC forms of some species of bacteria. However, Weichart *et al.* (1992) observed that although *V. vulnificus* produces a factor that causes a decline in culturability, a few cells retain the ability to grow. Thus, the "resuscitation" of the coccoid boidies reported previously by Nilsson *et al.* (1991) may have been regrowth from a few culturable cells (Weichart *et al.* 1992). The suggestion has been made that since there was no exogeneous nutrient added in Nilsson's study, the increases in culturable cell numbers might be accounted for by the regrowth of a few cells at the expense of dead cells in the population (cryptic growth) (Ravel *et al.* 1995). Whether true "resuscitation" of the VBNC population occurs or whether regrowth of one or more undetectable culturable cells results in the appearance of culturable cells has remained one of the most important questions regarding the significance of the VBNC state.

Detection of VBNC forms

Many methods have been employed to detect cells that are viable but unable to multiply. Radiolabeling techniques have been used in combination with other methods such as fluorescent labeling to estimate cellular metabolic activity (Fleirmans and Schmidt 1975; Tabor and Neihof 1982; Bode *et al*. 1993). In these studies, cells that are metabolically active will take up radiolabeled substates which can then be detected on photographic film or enumerated using a scintillation counter. Autoradiolabeling procedures have also been combined with immunofluorescence to detect specific cells within a population (Fleirmans and Schmidt 1975; Tabor and Neihof 1982). More recently, researchers have been using radiolabeled probes and the polymerase chain reaction (PCR) to detect viable but nonculturable forms (Steinert *et al.* 1997; Herman 1997).

Some methods have been developed with the assumption that differences can be observed microscopically with the use of antibiotics and by staining, including the use of fluorescent dyes. A direct viable count by microscope can be made of the elongated cells formed when viable bacteria are incubated with yeast extract in the presence of nalidixic acid. Nalidixic acid is a specific inhibitor of bacterial DNA gyrase and thus prevents cell division (Betts *et al.* 1989). One of the most widely used fluorescent dyes is acridine orange (3,6-tetramethyldiaminoacridine). From results obtained with the acridine orange direct counting method, it has been suggested that one can distinguish between living and

inactive cells (Daley 1979; Rozak and Colwell 1987; Betts *et al.* 1989; Steinert *et al.* 1997).

The use of acridine orange (AO) to determine the metabolic state of bacteria in environmental samples is widespread (Hood *et al.* 1986; Rollins and Colwell, 1986; Steinert 1997). The procedure is a simple, fast, and inexpensive method to estimate the total number of living, viable or growing, bacteria in nature (Daley 1979). The principle of AO staining is simple: the dye fluoresces orange-red when attached as a dimer to single stranded nucleic acids, and green when attached monomerically to double stranded nucleic acids (Daley 1979). Therefore, if a cell is metabolically active and growing, it would fluoresce red due to a high RNA/DNA ratio. If the DNA/RNA ratio is high, the cell fluoresces green and is considered inactive or dead because no transcription of RNA is occuring (Daley 1979). Knowledge of the sample volume, microscope magnification, counting area, dilution factor, and appropiate control counts, the number of active or growing bacteria can be determined (Daley 1979).

However, several problems exist in the AO procedure. The use of different lamps, light filters, membrane filters, and stain concentrations can affect cell enumeration. For example, the use of cellulose membrane filters causes low contrast and rapid fading while the use of polycarbonate membrane filters such as Nucleopore filters eliminates this problem (Hobbie *et al.* 1977). Nucleopore filters, however, are autofluorescent and used to be treated with the dye irgalan black in order to use the AO method (Hobbie *et al.* 1977). However, black Nucleopore membrane filters are now commercially available for

epifluorescence techniques. Another problem with the AO method is that dead cells with denatured DNA will also fluoresce red thus giving inacurate viable counts (Daley 1979).

***Prolinoborus fasciculus* (*Aquaspirillum fasciculus*)**

Aquaspirillum fasciculus VPI strain XI (ATCC 27740) is a freshwater, gram-negative straight rod first described in 1971 by Strength and Krieg. It was assigned to the genus *Aquaspirillum* by Strength *et al*. (1976) even though this genus had previously contained only helical and vibrioid bacteria. This was because the physiological characteristics of the organism were typical of those of aquaspirilla. In the view of Strength *et al.*, *A. fasciculus* was a spirillum that had "forgotten" how to be helical. This view was reinforced when Pot *et al.* (1992) found evidence that the closest phylogenetic relative of *A. fasciculus* was *Aquaspirillum serpens*. The melting temperature, i.e., the $T_{m(e)}$ of rRNA/DNA hybrids of *A. fasciculus* with *Aquaspirillum serpens* was 6°C. Pot *et al.* believed that a $T_{m(e)}$ of 6°C or was sufficient to justify the removal of *A. fasciculus* from the genus *Aquaspirillum* and they placed the organism in a separate genus, *Prolinoborus*, which contains a single species, *Prolinoborus fasciculus*. The name of the genus was based on the unusual ability of the organism to use proline as a sole source of both carbon and nitrogen. Phenotypic characters associated with the genus *Aquaspirillum* include: presence of intracellular poly-ß-hydroxybutyrate granules, inability to catabolize carbohydrates; a strictly respiratory type of metabolism; positive catalase and oxidase

reactions; a negative indole reaction; no hydrolysis of casein, starch or hippurate; no growth in presence of 3% NaCl; an optimum temperature of 30°C; the presence of bipolar flagellar fascicles; and the formation of coccoid bodies. In *P. fasciculus*, the bipolar flagellar fascicles consist of approximately 10-12 flagella and are clearly visible by phase-contrast and dark-field microscopy . The fascicles undergo changes in shape ranging from being fully extended to coiled up like springs or being wrapped about the cell body. Motility does not occur in ordinary nonviscous media; instead, the cells exhibit a "floundering about" movement. In media of high viscosity, however, the cells swim readily. The ability of *P. fasciculus* (*A. fasciculus*) to form coccoid bodies is a feature that also occurs in pathogenic vibrioid or helical bacteria such as the genera *Campylobacter*, *Helicobacter* and *Vibrio*. However, *P. fasciculus* (*A. fasciculus*) coccoid bodies are much larger in diameter than those of these other genera.

Poly-β-hydroxybutyrate

Many organisms produce an osmotically balanced carbon storage compound that is a polymer of 3-hydroxyalkanoic acids (PHAs) (Dawes and Senior 1973). Poly-β-3-hydroxybutyrate (PHB) is the most abundantly occurring unit of PHA, discovered by Lemoigne in 1923 (Tal *et. al.* 1990). Lemoigne was also the first to chemically characterize PHB (Fig. 2), and it is now known that residues up to C_7 hydroxyacids can constitute the polymer (Tal *et. al.* 1990). PHAs have gained recent industrial attention because they are a naturally produced, biodegradable polyester thermoplastic (the melting

temperature is approximately 180° C) with properties similar to isotactic polypropylene (Doi 1990). PHB is also found as a normal metabolite in human blood which suggests a number of medical uses for PHB such as controlled drug release, surgical sutures, and bone plates (Doi 1990).

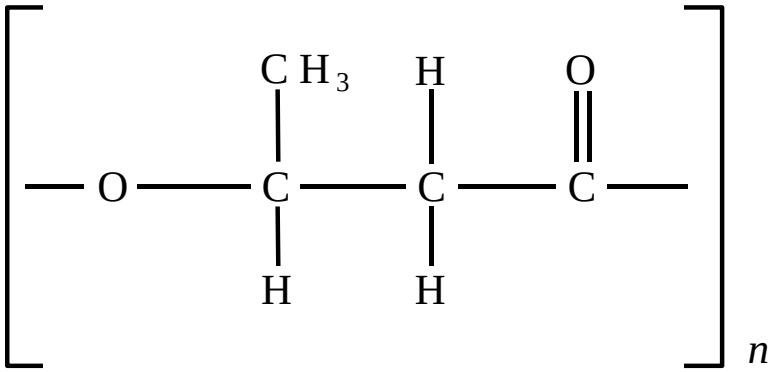

Figure 2. Structure of PHB. (Doi 1990)

A wide variety of gram negative and gram positive microorganisms accumulate PHB as a reserve material. Poly-β-hydroxybutyrate is normally accumulated in response to the limitation of an essential nutrient (reviewed by Anderson and Dawes 1990). It has been shown that levels of PHB can be drastically increased from a low percentage to over 80% of cell dry weight when growth is limited by an essential nutrient such as nitrogen, oxygen, phosphorous, magnesium, or sulfur (Doi 1990). PHB has been shown to enhance survival under starvation conditions. Studies of *Azospirillum brasilense* with a high content of PHB are better able to survive than those cells with a lower PHB content (Tal & Okon 1985). PHB can also serve as an endogenous carbon source for sporulation in *Bacillus* species and cyst formation in *Azotobacter* species (Sillman *et. al* 1986). Senior and Dawes (1971) have also proposed that PHB can be viewed as a sink of reducing power.

Synthesis of PHB begins with the formation of acetyl-CoA from an acceptable carbon source (Fig. 3). Organic acids, sugars and alcohols are examples of favorable carbon sources for PHB productions. Production of PHB is catalyzed by a series of three enzymatic reactions. 3-ketothiolase, NADH-dependent acetoacetyl-CoA reductase, and poly (hydroxybutyrate) synthase. Two molecules of acetyl-CoA are condensed by the action of 3-ketothiolase to release a free coenzyme A (CoASH) and form acetoacetyl-CoA. The intermediate is reduced to D(-)-3-hydroxybutyrl-CoA by NADPH-dependent acetoacteyl-CoA reductase. PHB is then produced by the polymerization of D(-)-3-hydroxybutyrl-CoA by the action of poly (hydroxybutyrate) synthase (polymerase) (Doi 1990). A key regulatory enzyme in PHB synthesis is 3-ketothiolase, which is inhibited by

high concentrations of free coenzyme A (Senior and Dawes 1973). Under balanced growth conditions in the presence of excess oxygen, acetyl-CoA enters the tricarboxylic acid (TCA) cycle for energy generation and the formation of amino acids. As a result, the concentration of free coenzyme A is high and the synthesis of PHB is inhibited. Under growth limiting conditions, citrate synthase is inhibited by high concentrations of NADH and acetyl-CoA levels increase, which leads to a decrease in the concentration of free coenzyme A. The inhibition of 3-ketothiolase by free coenzyme A stops and the synthesis of PHB is initiated. Isozymes of both 3-ketothiolase and acteoacetyl-CoA reductase have been discovered in several different species of microorganism. In some cases these isozymes have shown substrate specificities different from the primary enzymes (Doi 1990). Poly(3-hydroxybutyrate) synthase has proven difficult to isolate and characterize. In *Zoogloea ramigera* the synthase has been shown to exist in either a soluble form or in a form that is bound to the PHA granule (Doi 1990). In the latter case the location of the enzyme is due to the amount of PHA contained in the cell. A high amount of the polymer leads to association with the granule while low amount results in the soluble form of the synthase.

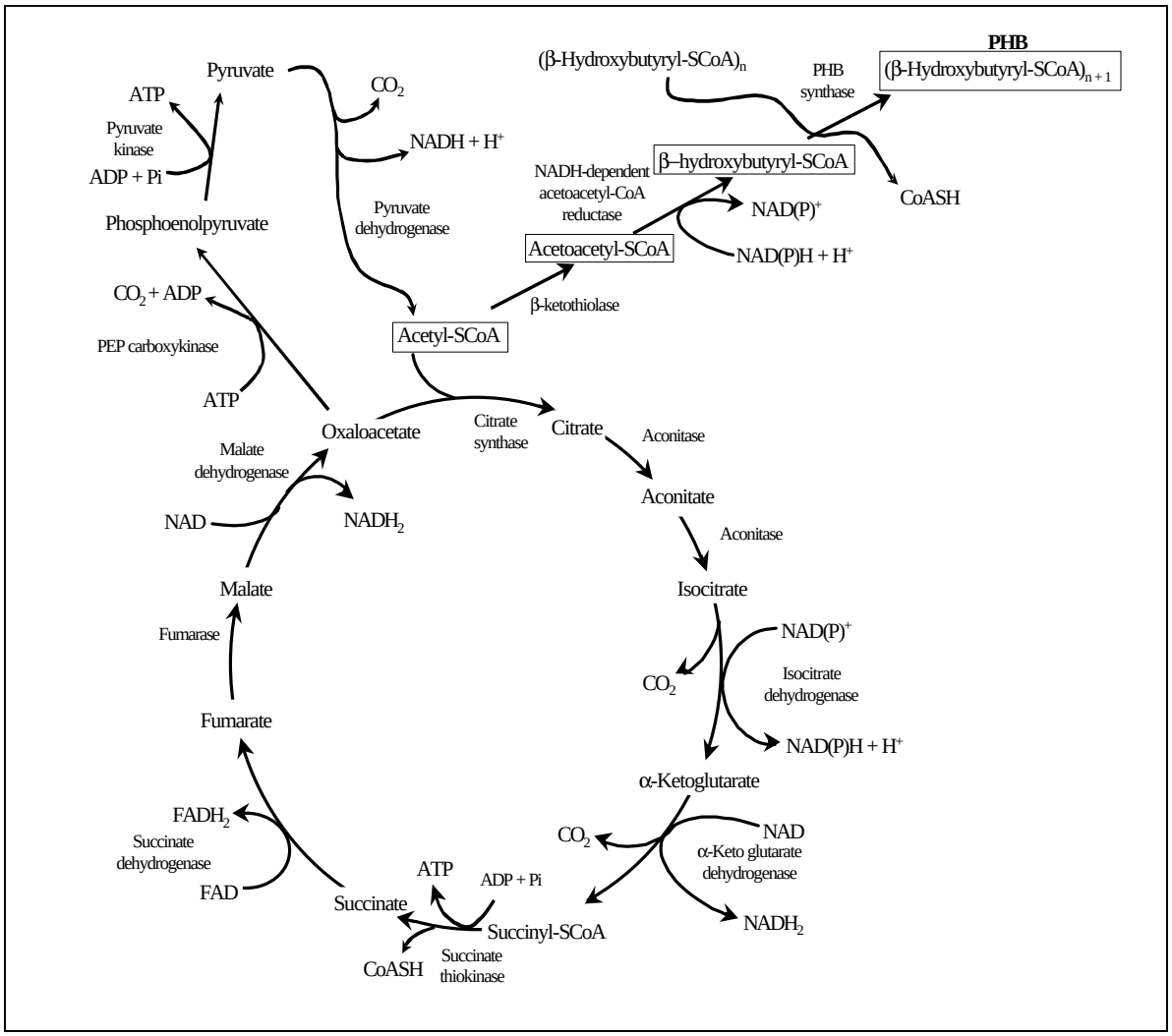

Figure 3. TCA Cycle and PHA shunt (Adapt from Doi, 1990)

Many different bacteria accumulate PHB as an endogenous energy reserve in the presence of an excess carbon source and will depolymerize PHB under nutrient-limited conditions. The pathway of PHB degradation is shown in Figure 4. Poly-β-hydroxybutyrate is first

degraded to D(-)-3-hydroxybutyric acid by the action of PHB depolymerase. 3-Hydroxybutyrate is then oxidized to acetoacetate by NAD-specific D(-)-3-hydroxybutyrate dehydrogenase (Senior and Dawes 1973). The next step is the formation of acetoacetyl-CoA from acetoacetate by acetoacetyl-CoA synthase. Acetoacetyl-CoA is common to the biosynthesis of and degradation of PHB. 3-ketothiolase, the enzyme catalyzing the first reaction of biosynthesis, catalyzes the last reaction of degradation to give acetyl-CoA. Acetyl-CoA is then used by the bacteria to generate energy via the Krebs cycle (TCA cycle).

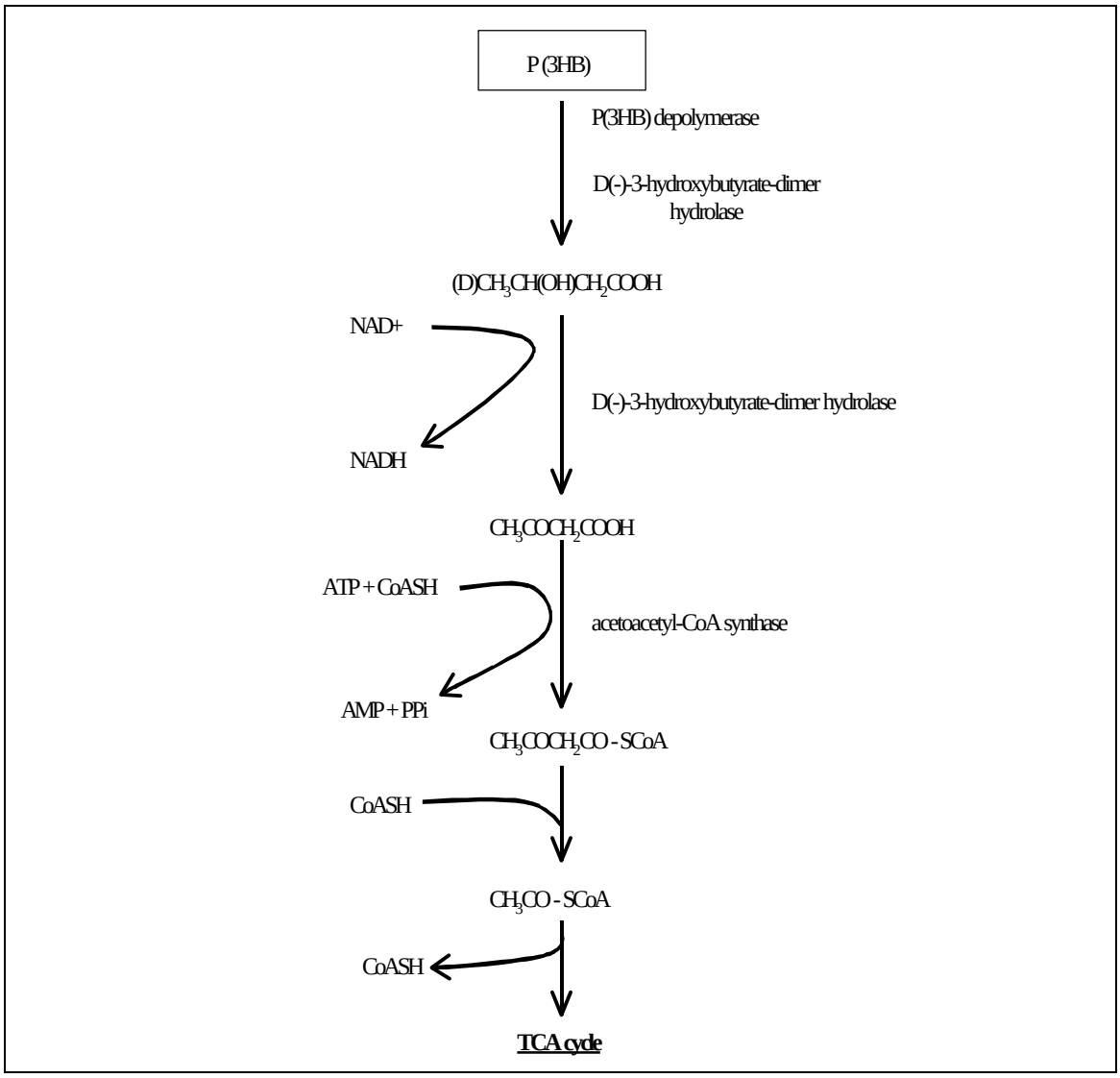

Figure 4. Degradation of PHB by *Zoogloea ramigera*.(Adapted from Doi 1990)

In the past, the presence or absence of PHB was visualized by staining with Sudan black, an extremely cumbersome procedure. A more rapid visualization procedure was design by Ostle and Holt (1982) using the fluorescent dye Nile Blue A. Poly-β-

hydroxybutyrate can be quantified by the method of Law and Slepecky (1960). Poly-β-hydroxybutyrate is depolymerized by heating in concentrated sulfuric acid to β-hydroxybutyric acid. The sulfuric acid also acts as a dehydrating agent which reduces the β-hydroxybutyric acid to crotonic acid. Crotonic acid can be quanitatively measured by the ultraviolet absorption maximum at 235 nm (Law and Slepecky 1960).

Ribosomes: Structure, Function and the Process of Translation

Ribosomes are the sites of protein synthesis in cells; a process known as translation (Watson *et al*. 1987). Palade first observed them in the electron microscope as dense particles or granules (Watson *et al*. 1987). Ribosomes are complex molecular machines that catalyze peptide bond formations between mRNA-specified amino acids (Watson *et al.* 1987). To function actively in protein synthesis, they must be bound into complete ribosomes. *E. coli* cells contain approximately 15,000 ribsomes. This accounts for approximately one-fourth the total bacterial mass, which indicates that a large fraction of cellular synthesis is dedicated to creating ribosomes (Watson *et al*. 1987). The ribosomes in bacteria have a particle mass of about 2.5 million daltons, a sedimentation constant of 70S, and are about one-third by weight protein and two-thirds by weight RNA (Fig. 5). All bacterial ribosomes are constructed from two subunits; the larger subunit (50S) is approximately twice the size of the smaller subunit (30S) (Voet 1990). The 50S and 30S refer to the sedimentation coefficient of the two subunits. This coefficient is a measure of

the speed with which the particles sediment through a cesium gradient when spun in an ultracentrifuge. Both subunits are composed of RNA (rRNA) and protein (Voet 1990). The 50S subunit is composed of two rRNA molecules whose sedimentation constants are 23S and 5S. The 50S subunit also contains 32 different proteins (Voet 1990). The smaller 30S subunit contains one rRNA molecule with a sedimentation constant of 16S plus 21 different proteins (Voet 1990). The three rRNA molecules (23S, 16S, and 5S) can be isolated and analyzed using formaldehyde gel electrophoresis (Sambrook *et al*. 1989).

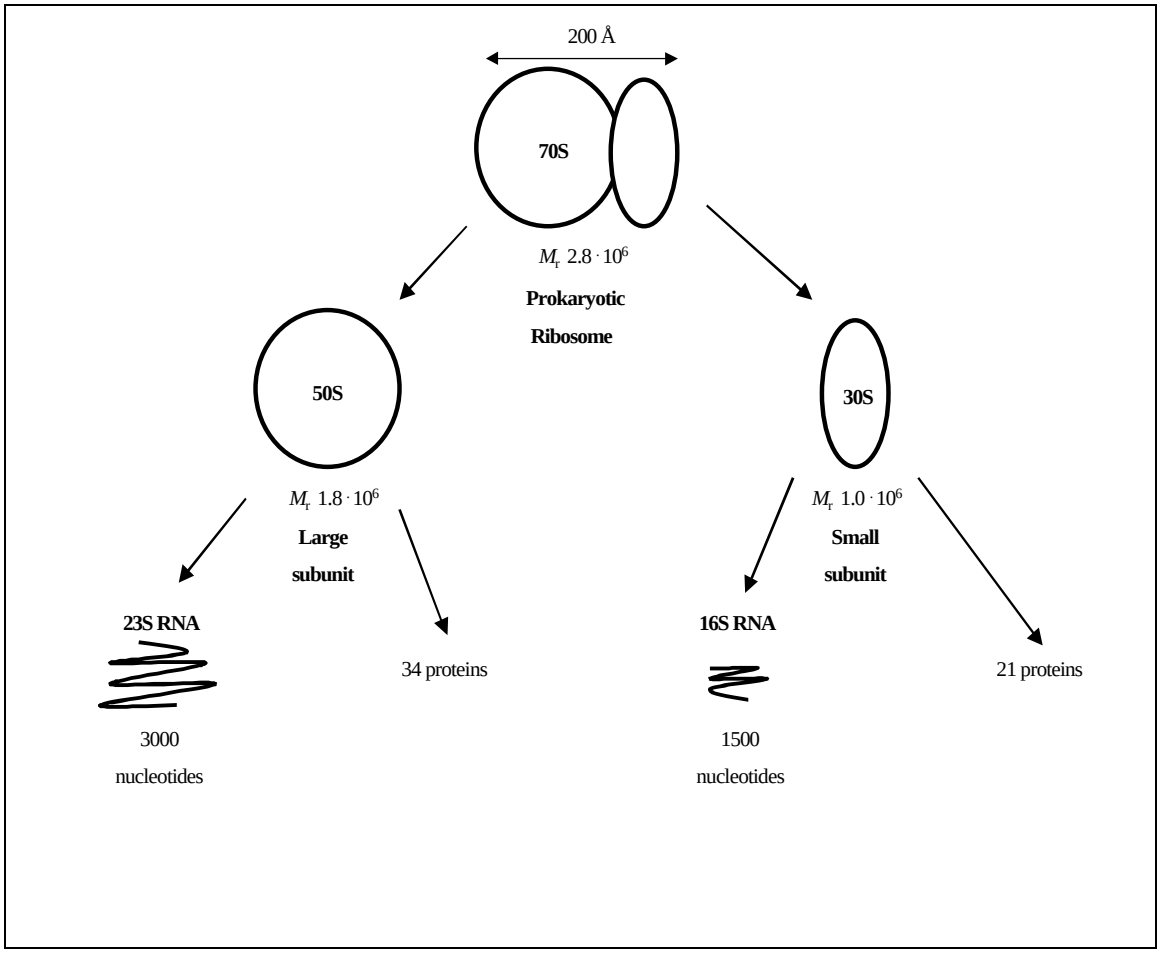

Figure 5. Simplified view of the 70S prokaryotic ribosome.(Adapted from Zubay 1988)

There are three major types of cellular RNA involved in protein synthesis: messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA). RNA polymerase transcribes messenger RNA from DNA, and its sequence contains the information for the sequence of amino acids in the polypeptide chain. Ribonucleoside triphosphates such as adenosine triphosphate (ATP), guanine triphosphate (GTP), cytosine triphosphate (CTP), and uracil triphosphate(UTP)(substituted for thymine triphosphate (TTP) in DNA) are the building blocks for this mRNA sequence. Each amino acid is represented in the mRNA by a sequence of three nucleotides known as codons. Transfer RNA (tRNA) is also transciribed from the DNA template, and it contains both a site for the attachment of an amino acid and a site, the anticodon, that recognizes the corresponding three-base codon on the mRNA. There are about 50 different kinds of tRNAs in the bacterial cell each carrying an amino acid that is enzymatically attached to the 3' end of the tRNA by a specific aminoacyl-tRNA synthase. Methionine is found at the beginning of all polypeptide chains, and it is represented by the codon AUG. For this reason, AUG is known as the start codon in the mRNA chain. Bacteria contain an enzyme that formylates the terminal amino (NH_3^+) end of the initiator methionine of the tRNA (fMet-$tRNA_f^{Met}$). When actively involved in protein synthesis, the ribosome translates the mRNA chain in the 5' to 3' direction. Most commonly, a strand of mRNA is read simultaneously by a number of ribosomes, each engaged in the synthesis of a single polypeptide chain; the resulting structure resembles a string of beads known as a polysome.

There are three steps required to initiate translation in all protein-synthesizing systems. In the first two steps, the small ribosomal subunit binds to the initiator tRNA carrying a formyl-methionine which will bind to the AUG start codon, and to the mRNA at the Shine-Dalgarno sequence which is complementary to the 3' end of the 16S rRNA. The Shine- Dalgarno sequence allows for the proper alignment of the mRNA so that the genetic message will be read starting at the 5' end. In the third step of initiation, the large ribosomal subunit joins the complex, and protein initiation factors (IF) dissociate from it (Fig. 6). In *E. coli* there are three protein initiator factors. These factors are bound to the small pool of 30S ribosomal subunits that are waiting to initiate the events of protein synthesis. One of these factors, IF-3, serves to hold the 30S and 50S subunits apart after the termination of a previous round of protein synthesis. The other two factors, IF-1 and IF-2, function to promote the binding of fMet-$tRNA_f^{Met}$ and mRNA to the 30S subunit. Binding between the two RNAs occurs in such a way that the anticodon on the tRNA is specifically complexed to the start AUG codon on the mRNA. At this point, IF-3 disassociates from the 30S subunit, permitting the 50S subunit to join the complex. This in turn results in the release of the other two initiation factors, IF-1 and IF-2. The overall binding action between the 30S and 50S requires the energy of GTP which is bound in complex with IF-2 (Zubay 1988).

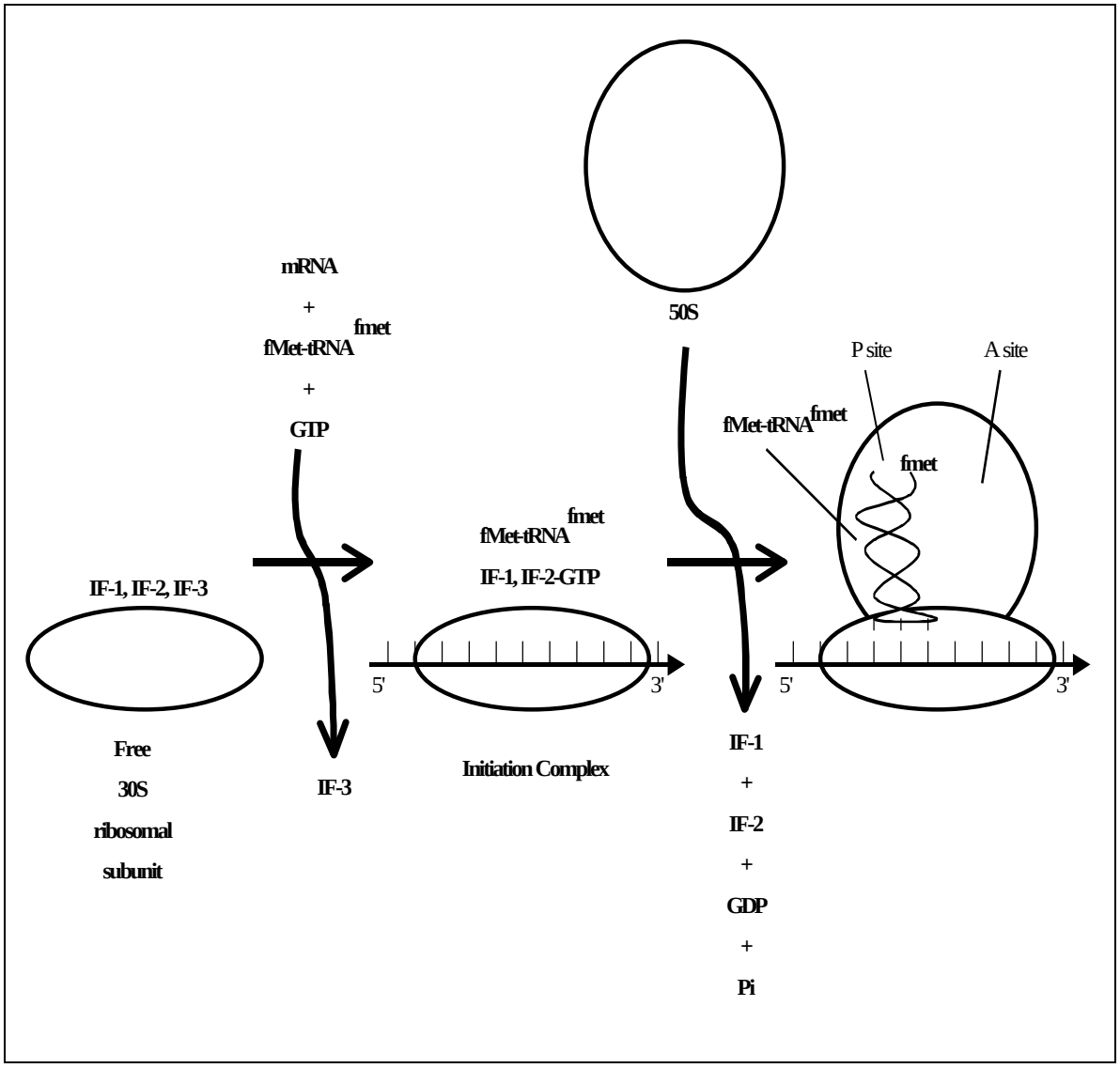

Figure 6. Initiation in Prokaryotes.(Adapt from Zubay 1988)

Once assembled, the ribosome displays a series of small grooves, tunnels, and platforms, where the action of protein synthesis occurs. The process of elongation involves all of the reactions related to protein synthesis. The catalytic center responsible for the formation of the peptide bond during protein synthesis resides entirely within the large ribosomal subunit. The specific binding of messenger RNA (mRNA) during protein

synthesis occurs on the small ribosomal unit. Elongation begins with the binding of a second aminoacyl-tRNA at a site adjacent to the methionyl-tRNA. There are two sites on the ribosome for tRNA binding known as the P site and the A site (Fig. 6). fMet-$tRNA_f^{Met}$ is the only aminoacyl-tRNA that binds directly to the P site. All of the remaining aminoacyl-tRNAs bind first to the A site where they are later translocated to the P site. Binding of an aminoacyl-tRNA to the A site is mediated by the protein complex EF-Tu-GTP. This complex forms if, and only if, the anticodon on the tRNA interacts specifically with the next codon on the mRNA chain. Once this happens, GTP is hydrolyzed to GDP and Pi and the resulting EF-Tu-GDP is released from the ribosome. A second elongation factor, EF-Ts, regenerates EF-Tu-GTP for interactions with other aminoacyl-tRNAs.

With two aminoacyl-tRNAs occupying the ribosome, a peptide bond is formed. This process is known as transpeptidation. This reaction is catalyzed by the enzyme peptidyl transferase, an enzyme that is contained entirely on the 50S subunit. The free amino group of the newly bound aminoacyl tRNA attacks the carbonyl group of the adjacently bound methionyl-tRNA in a nucleophilic displacement reaction that replaces the ester bond with a peptide bond (Zubay 1988). The net result of this reaction is a transfer of the methionine from one tRNA to the next, simultaneous with peptide bond formation.

After transpeptidation, the ribosomal P site is occupied by a deacylated tRNA and a peptidyl-tRNA occupies the A site. Through the process of translocation, the peptidyl-tRNA is moved to the P site, the deacylated tRNA expelled from the A site, and the ribosome moves to the next codon on the mRNA chain. These collective reactions require the hyrolysis of GTP and the participation of EF-G (Fig. 7). The three reactions,

absorption of an aminoacyl-tRNA to the ribosome A site, transpeptidation, and translocation, are successfully repeated until each codon has been translated to produce a fully formed polypeptide and the ribosome reaches a stop codon on the mRNA.

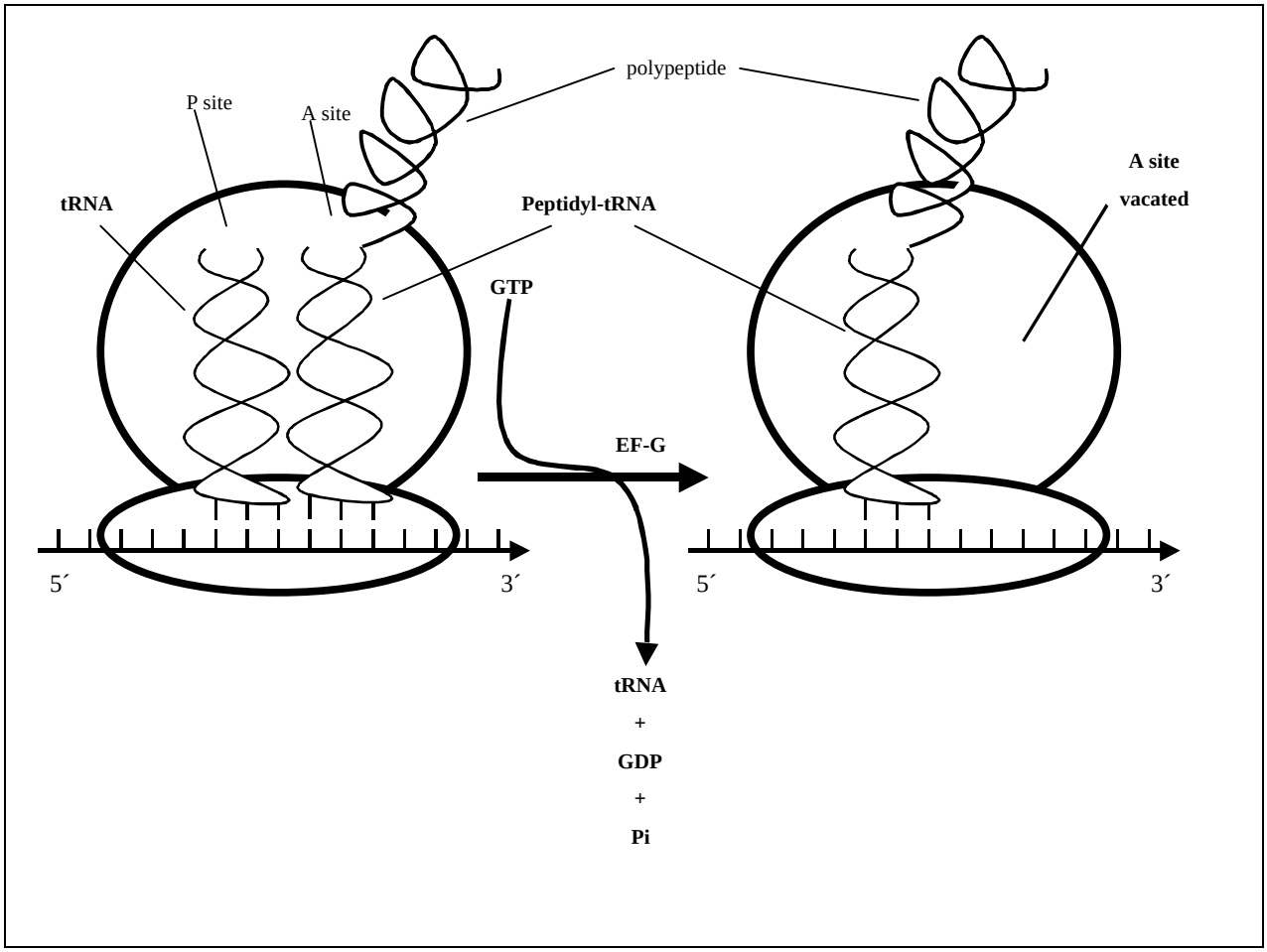

Figure 7. The translocation reaction in *E. coli*.(Adapted from Zubay 1988)

Release of the ribosomal subunits occurs with the help of release factors after the ribosome encounters a stop codon on the mRNA. The stop codons, UAA, UAG, and

UGA (also called opal, amber, and ochre codons respectively), are the only 3 of 64 codons that do not specify amino acids. In *E.coli*, there are three GTP dependent release factors designated RF-1, RF-2, and RF-3. RF-1 responds specifically to UAA and UAG, while RF-2 responds specifically to UAA and UGA. RF-3 does not have release activity by itself, but stimulates the reaction catalyzed by either RF-1 or RF-2. When the termination codons are bound to the decoding site on the ribosomes, the release factors bind to the A site (Fig. 8). This binding causes a change in the peptidyl transferase so that it transfers water to the completed peptide chain rather than an amino group of an aminoacyl-tRNA. Subsequently, the polypeptide is released, the final tRNA is released, the mRNA disassociates from the ribosome, and the two ribosomal subunits separate.

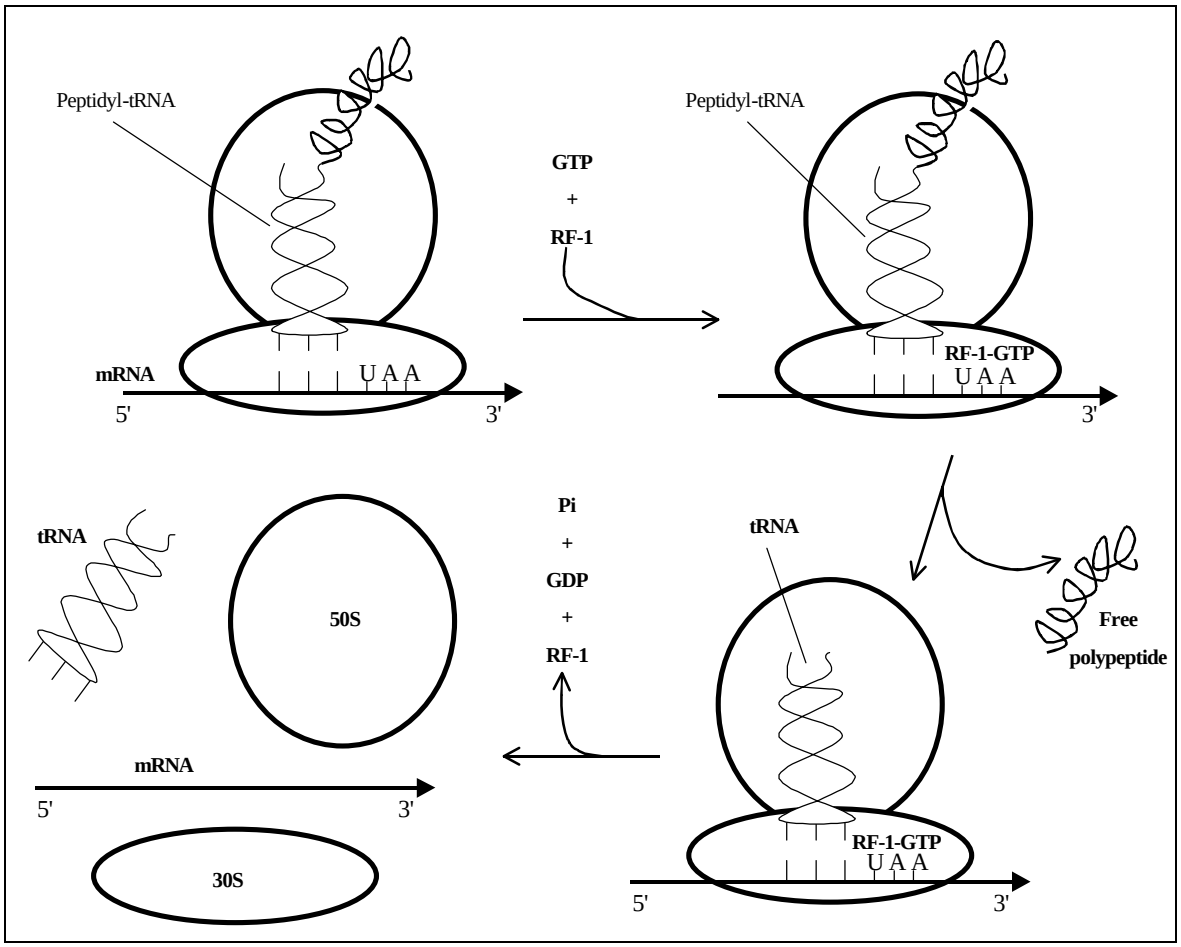

Figure 8. The release reaction in *E. coli*.(Adapted from Zubay 1988)

The antibiotic chloroamphenicol inhibits protein synthesis in a wide range of both Gram positive and Gram negative bacteria, and it was the first broad-spectrum antibiotic to be used clinically. Eukaryotic cells are generally resistant to the effects of chloroamphenicol, but clinical use has been severely curtailed due to toxic side effects caused by the antibiotic on mitochondrial ribosomes. At minimum inhibitory levels, one molecule of the antibiotic binds to the 50S ribosomal subunit and inhibits the peptidyl

transferase reaction. As a result of this binding, peptide bonds are not formed and all protein synthesizing reactions stop. Many studies show that the addition of choroamphenicol does not inhibit the transformation from the spiral or vibroid form to the coccoid form (for review see Koenraad *et al.* 1997). These findings suggest that transformation to the coccoid state does not actively involve protein translation and that the coccoid form is a degenerative state. The addition of chloroamphenicol to sporulating species of bacteria prevents the formation of endospores (Setlow 1983).

Manuscript in press for publication in the Canadian Journal of Microbiology :

Viable but nonculturable coccoid forms of *Prolinoborus fasciculus* (*Aquaspirillum fasciculus*)

Aging cultures of certain rod-shaped, vibrioid, or helical bacteria may exhibit mass conversion of the vegetative cells to a coccoid form (Bode et al. 1993a, 1993b, Krieg 1976, Morgan and Upton 1986). The morphology, physiology, and biochemistry of these coccoid forms have been studied but their potential role as part of a life cycle in these bacteria remains a matter of speculation (Tritz and Ogg, 1967; Boucher et al. 1994). *In vitro s*tudies have led to conflicting opinions as to whether the coccoid bodies represent a degenerative state or a viable resting stage. Evidence that coccoid bodies are nonviable, degenerate forms include their loss of proteins and nucleic acids, decreased density in rate-zonal centrifugation, and loss of cell wall components (Morgan and Upton 1986; Nilius et al. 1993). However, other evidence indicates that these forms may still be viable, such as their ability to fluoresce when stained with acridine orange (AO) or other "vital" stains, elongation in the presence of nalidixic acid, uptake of amino acids, an ability to synthesize small amounts of DNA, and the presence of polyphosphates (Bode et al. 1993a, 1993b; Rollins and Colwell 1987). The putative ability of a large inoculum of the coccoid forms of pathogenic bacteria to cause disease in laboratory animal models (e.g., see Cellini et al. 1994) and the resuscitation of *Vibrio cholerae* coccoid forms by an increase in temperature (Ravel et al. 1995) also supports the idea that the coccoid forms are viable. One study suggests that *Helicobacter pylori* has two different coccoid forms, one that is a viable dormant stage and one that is degenerate (Sörberg et al. 1996).

Prolinoborus fasciculus (previously *Aquaspirillum fasciculus*) is a freshwater, gram-negative, nitrogen-fixing straight rod first described in 1971 by Strength and Krieg. Its ability to undergo mass conversion to the coccoid form was described by Strength et al. (1976). The purpose of the present study was to quantify the phenomenon and examine factors such as the condition of the ribosomal RNA (rRNA) that bear on the question of whether the coccoid form is part of a life cycle or merely a degenerative form.

Prolinoborus fasciculus VPI strain XI (ATCC 27740) (formerly *Aquaspirillum fasciculus* and reclassified by Pot et al. 1992) was maintained in the peptone-fumarate salts (PFS) medium described by Strength et al. (1976) at 30°C with weekly serial transfer. For characterizing the conversion from rods to cocci, 1-L portions of PFS broth were inoculated with 10 mL of a 24-h-old culture in PFS broth and incubated at 30°C on a reciprocating water bath shaker at 84 oscillations per min. Samples (1 mL) were removed periodically for enumeration of cells. Colony forming units (CFU) per ml were estimated by spreading 0.1-mL samples of dilutions of broth cultures onto PFS agar plates. Total direct counts (TDC) were estimated with a Petroff-Hauser counting chamber. Preparations stained with acridine orange by the method of Hobbie et al. (1977) were observed with a Zeiss epifluorescence microscope. The method of Ostle and Holt (1982) using Nile blue A was employed for visualization of poly-ß-hydroxybutyrate (PHB) inclusions by epifluorescence microscopy. For quantification of PHB, cells were harvested by centrifugation, dried at 90°C, and extracted with hot chloroform. Two volumes of ice-cold acetone were added to precipitate the PHB. The PHB was redissolved in hot chloroform and the precipitation repeated several times. Quantification was done by the method of Law and Slepecky (1960) by converting the PHB to crotonic acid with hot concentrated sulfuric acid.

For rRNA characterization, 100 ml of bacterial cells were harvested by centrifugation at 4°C and washed twice with ice cold 50 mM potassium phosphate buffer (pH 7.0). Isolation of RNA was done by the procedure of Chomczynski and Sacchi (1987) except that the nucleic acids were pelleted by centrifugation, washed with 75% (v/v) ethanol, dried, and suspended in formamide to a final concentration of 5 µg/µl (Appendix I). Ten µg of RNA sample was denatured in gel loading buffer containing formamide and 50 mM EDTA in a ratio of 5:1 (vol/vol); Orange G (Sigma) was added to a concentration of 0.15%. After incubation for 10 min at 70°C the mixture was chilled on ice for 5 min and electrophoresis of RNA was performed using the Reliant RNA gel

system (FMC Bioproducts, Rockland, Maine) at 5 V cm^{-1}(Appendix II). The electrophoresis buffer was the formaldehyde gel-running buffer described by Sambrook et al. (1989).

The direct microscopic counts and colony counts at various incubation times are shown in Fig. 1. As the coccoid forms increased and the rod forms decreased the CFU per mL decreased. The major period in which conversion occurred was between 50 and 80 h. At 120 h there were still 8×10^7 rods per mL but a 10-fold greater number of coccoid forms; thus not every rod converted to the coccoid form. The addition of 8 times the minimum inhibitory concentration (MIC) of chloroamphenicol (MIC = 0.025 μg/ml) did not prevent conversion. AO staining indicated that at 20 h all of the cells (entirely rod forms) fluoresced orange, indicating a high ratio of RNA to DNA. In contrast, at 120 h the rod forms that were observed microscopically were green whereas the coccoid forms fluoresced orange. Despite this indication of viability of the coccoid forms, the CFU per mL (1.2×10^4) was approximately 68,000 times less than the number of coccoid forms per mL (8.25×10^8). It is difficult to say whether the few colonies that did form at 120 h were derived from a few coccoid forms that were able to be resuscitated or from a few rods that had not yet become nonviable. It is clear, however, that most of the coccoid forms were nonculturable, at least under the growth conditions used, despite their orange fluorescence.

Inclusions of PHB were observed by phase-contrast microscopy and by Nile blue A staining (Ostle and Holt 1982) in the actively growing rod-shaped cells but not in the coccoid forms. Inclusions of PHB were also reported by Strength and Krieg (1971) in ultrathin sections observed by electron microscopy. Extraction of PHB and conversion to crotonic acid indicated that 24-h-old cultures (consisting of <3% coccoid forms) contained 36 μg crotonic acid per mg dry weight of cells whereas in 30-h-old cultures no detectable PHB could be found (Fig. 2). At 30 h the culture contained less than 10% coccoid cells;

thus conversion to the coccoid form begins after most of the rods have depleted their endogenous reserves of this stored oxidizable energy source.

The fact that the coccoid cells fluoresced when stained with AO suggested that these cells, although nonculturable on peptone-fumarate agar, may still possess the ability to synthesize proteins and thus might be able to be resuscitated under suitable conditions. Most of the RNA in bacterial cells is rRNA, and examination of the condition of the RNA from 9-to 12-h-old cultures (mid-log phase) indicated distinct, undegraded bands of 16*S* and 23*S* rRNA (Fig 3). In contrast, when the culturable cell count had decreased to 1.2×10^5 the RNA had undergone extensive degradation as indicated by a smearing on agarose gel electrophoresis (Fig. 3). The RNA extractions and analyses were repeated 12 times with similar results. No decrease in the amount of RNA per ml of culture occurred after the log phase; indeed, there was an apparent increase during the stationary phase. This was probably due to the extensive degradation of the nucleic acids by endogenous nucleases which resulted in higher optical densities when measuring the RNA.

The RNA degradation was not incompatible with AO staining because degenerative forms of rRNA also can bind to fluorochrome vital stains (Yamamoto et al. 1996). A similar degradation of rRNA has been reported for the nonculturable forms of *Legionella pneumophila* (Yamamoto et al. 1996). Tolker-Nielsen and Molin (1996) have suggested that the degradation of rRNA is a direct cause of bacterial cell death. These results suggest that the inability to cultivate the coccoid forms may be due to lack of integrity of the rRNA..

In summary, the conversion of the rod form of *Prolinoborus fasciculus* to a nonculturable, coccoid form has been described. Lack of culturability, loss of intracellular PHB, and the degraded condition of the rRNA suggest that the coccoid form of *P. fasciculus* may be a degenerative form rather than part of a life cycle.

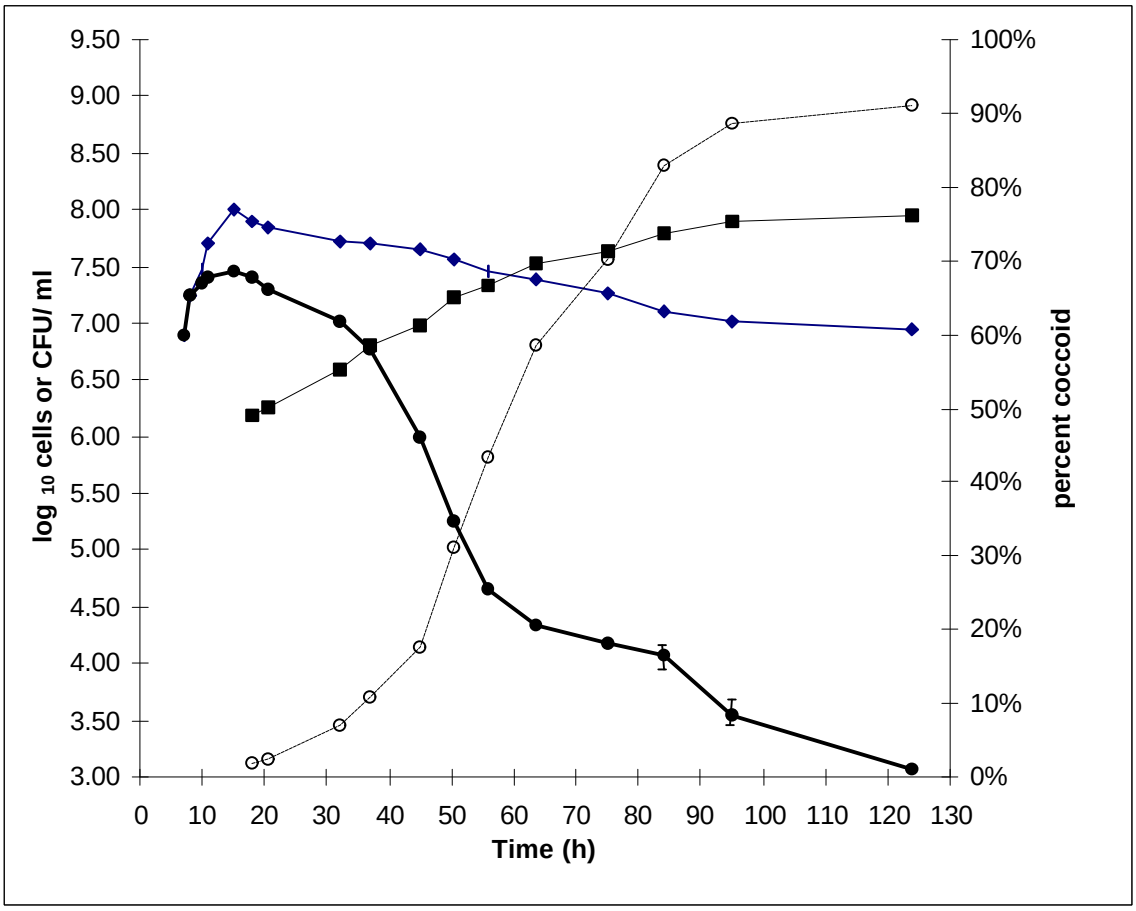

FIG. 1. Direct microscopic counts per mL and CFU per mL during growth of *Prolinoborus fasciculus*. Each data point represents the mean from four different experiments, with triplicate plates being used in each experiment for the colony counts. Standard deviations are shown when they exceed the size of the data symbol. ◆ = rods by direct microscopic count; ■ = coccoid forms by direct microscopic count; ● = CFU per mL; ○ = percent coccoid forms.

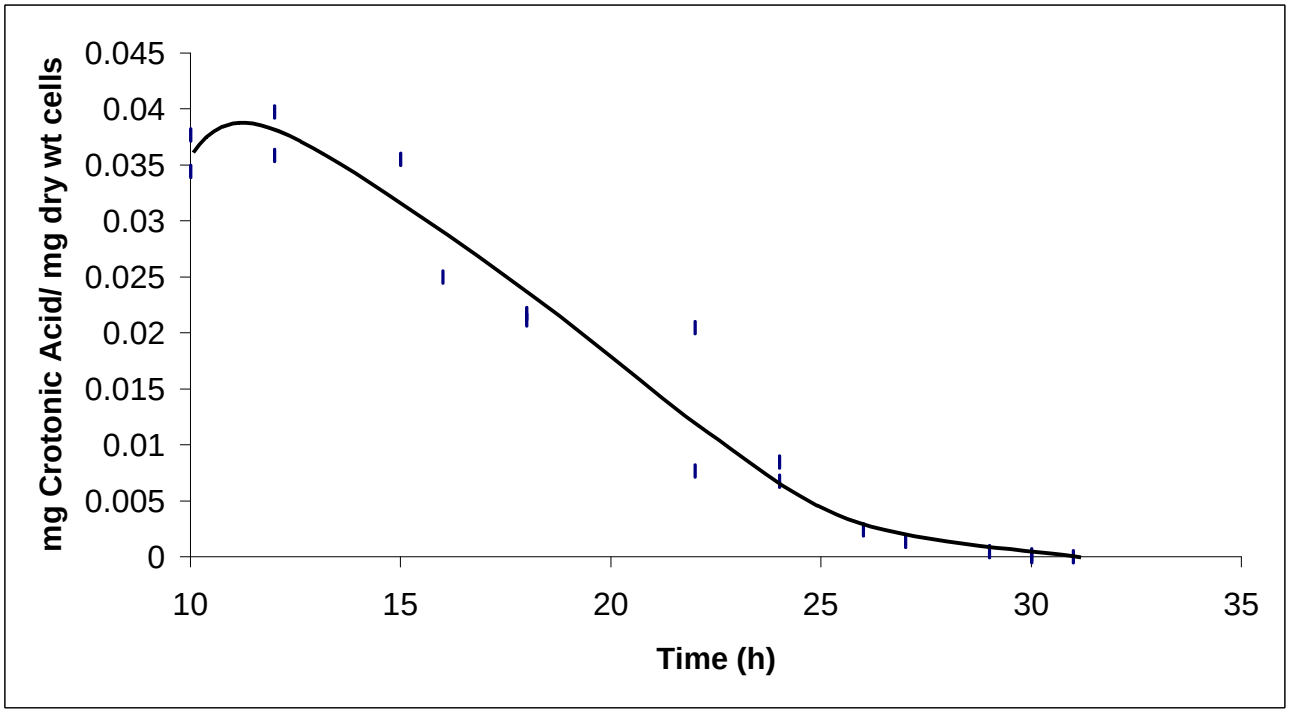

FIG. 2. Change of poly-β-hydroxybutyrate (expressed as crotonic acid) content of *Prolinoborus fasciculus* with time.

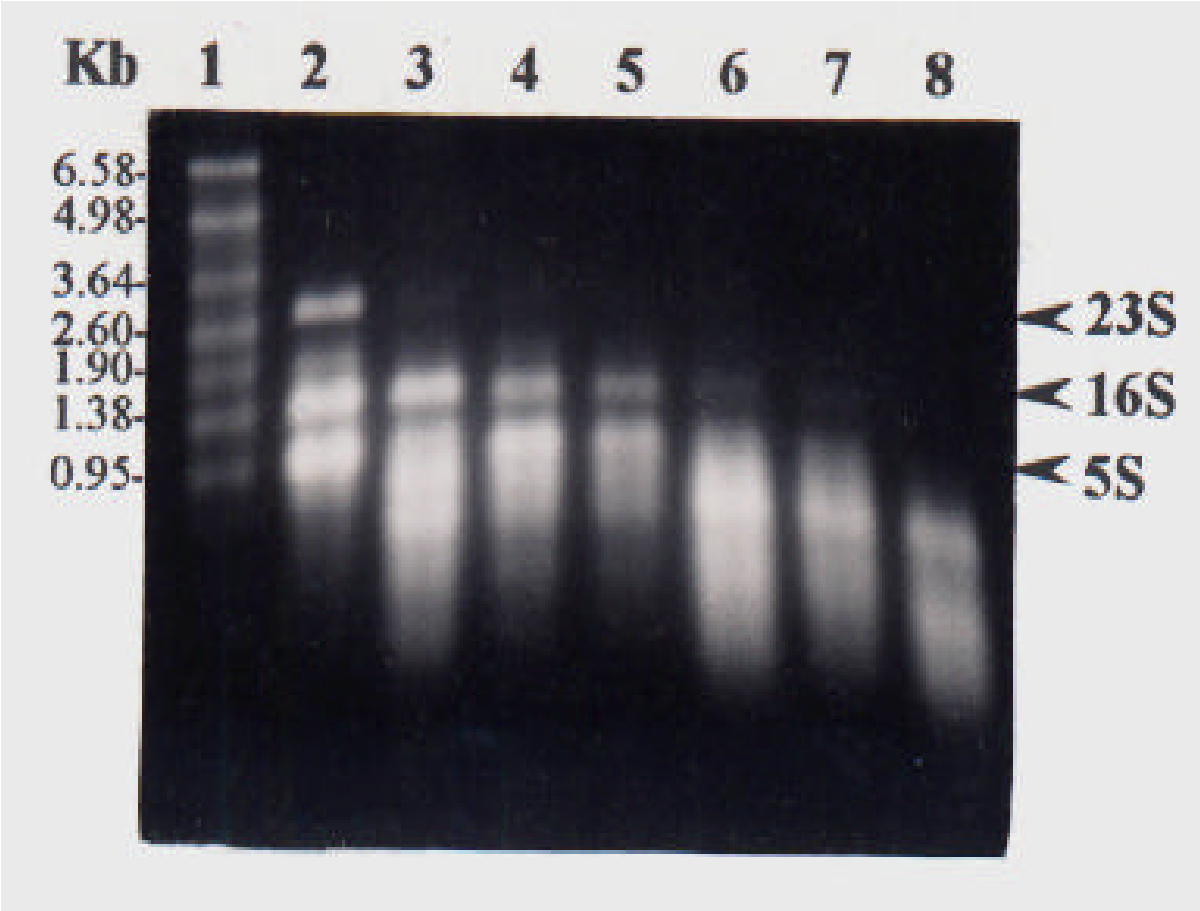

FIG. 3. Agarose gel electrophoresis of rRNA from cultures of *Prolinoborus fasciculus.* Lane 1, molecular weight markers; lane 2, from 9-h-old culture (rod-forms only); lane 3, from 23-h-old cultures (mainly rod-forms plus a few coccoid forms); lane 4, from 48 h-old cultures (40% coccoid forms); lane 5, from 72-h-old cultures (>50% coccoid forms); lane 6, from 65 h-old cultures (>60% coccoid forms); Lane 7, from 90 h-old cultures (>80% coccoid forms); and lane 8, from 120-h-old cultures (>90% coccoid forms).

Future Research

Prolinoborus fasciculus would be desireable for comparing the coccoid form with the spiral form. Isolation of the coccoid forms of *Campylobacter*, *Helicobacter*, and *Vibrio* spp. into a homogenous population has been difficult. Morgan *et al* (1986) observed that the coccoid forms of *Campylobacter jejuni* were less dense than the spiral form in rate-zonal centrifugation. This suggests that it may be possible to separate the rod form of *P. fasciculus* from the coccoid form using sucrose gradients. Once the coccoid forms have been separated into a homogenous population, comparisons between the rods and coccoid forms could be established. Examination of ATP levels, O_2 uptake, peptidoglycan components such as *N*-acetylglucosamine and *N*-acetylmuramic acid, and cell cycle intermediates in central metabolic pathways between the rod and coccoid forms could be done. These studies would indicate if the coccoid forms of *P. fasciculus* are able to maintain signs of viability.

It may be possible to resuscitate the coccoid forms of *P. fasciculus* by a change in the growth temperature. Nilsson *et al*. (1991) reported resuscitation of starved *Vibrio vulnificus* cells by an upshift in temperature. These bacteria were starved at 5° C and exhibited a time dependent loss in viability on agar plates. Direct microscopic counts showed that the cell numbers remained constant during this decline in viability. The viability could be completely restored by incubation of the culture at room temperature in salt media without any added nutrition. Transition from rods to small cocci (the end of starvation) and vice versa were observed. No increase in total cell numbers was detected. Whitesides and Oliver (1997) obtained similar results. Temperature and low nutrient levels have been implicated by many researchers as being the most important parameters in the

resuscitation of VBNC forms (Huq and Colwell 1995; Nilsson *et al.* 1991; Whitesides and Oliver 1997; Ravel 1995). Whitesides and Oliver (1997) suggested that harvesting early logarithmic phase cells of *Vibrio vulnificus* before incubation at a lower temperature was crucial in the resuscitation of this organism from a viable but nonculturable state. In the case of *P.fasciculus*; cells could be isolated when the population reaches 10^5 and then transferred into sterile distilled water. This culture would be immediately placed at 5° C. Aliquots would be taken periodically plated onto PFS agar at 30° C to determine culturability. Careful monitoring of total cell numbers would be done periodically by direct microscopic counts to ensure that the total number of cells remains constant during the decline in culturability. When culturability has been lost and the cells have reached the coccoid state, 1ml samples of the culture would be transferred to 30° and allowed to incubate overnight. Culturability would be determined following plating on PFS agar. Whitesides and Oliver (1997) reported no increase in cell number after the resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Similar results should be observed if the coccoid forms of *P. fasciculus* revert to the rod forms.

If these studies fail to result in satisfactory results, artificial coccoid forms could be generated using a combination of polymyxin B and lysozyme and compared with "natural" coccoid forms in regard to their physiological and biochemical properties. The coccoid forms of *P. fasciculus* do not lyse under hypotonic conditions. The standard dogma of bacteriology states that peptidogylcan is responsible for preventing the cell from bursting due to tugor pressure caused by water uptake in a hypotonic environment. This is certainly true in regard to gram positive bacteria. For gram negative bacteria, this statement is based in experiments in which destruction of the peptidoglycan of the cell wall results in

osmotically labile spheroplasts. However, these spheroplasts may have been damaged severely in other ways besides the loss of peptidoglycan, and the view that the peptidoglycan is solely responsible for keeping the cell from bursting is open to question. Studies have shown that coccoid bodies of *Campylobacter* spp. have lower amounts of peptidoglycan compared to the spiral, vegetative cells. Buck *et al.* (1983) used electron microscopy techniques in this regard, whereas Amano *et al.* (1992) concentrated on the amount of soluble peptidoglycan that could be retrieved from the coccoid forms compared to *Campylobacter* spp. spiral counterparts. Baker and Park (1975) reported that the formation of spheres in *Vibrio cholerae* is correlated with changes in the peptidoglycan of the cell wall. They suggested that during the conversion of rods to spheres, peptidoglycan that is not linked to other cell wall components is attacked by murein hydrolases or autolysins. As the peptidoglycan is no longer intact, the cells lose rigidity and become spherical.

In 1991, Cover *et al.* prepared osmotically stable spheroplasts of *Escherichia coli* using polymyxin B and lysozyme (a muramidase found in egg white, lachrymal fluid, and white blood cells). Previous researchers had used a Tris-EDTA-lysozyme technique introduced by Repaske (1958) to create spheroplasts, and this technique had become the "standard" method for preparing spheroplasts. However, as a result of the Tris-EDTA treatment, which causes extensive damage to the outer membrane of the *E. coli* cell, such spheroplasts become osmotically sensitive. EDTA causes the release of lipopolysaccharide (LPS) and causes a rearrangement of the components in the lipid bilayer which results in the formation of a more hydrophobic surface (Nikaido 1979; Nikaido and Nakae 1979). In contrast, a brief treatment with polymyxin B allows the outer membrane to become

permeable enough to allow the lysozyme to solubilize the peptidoglycan (Cerney and Teuber 1971). In the study by Cover *et al.*, all of the peptidoglycan was destroyed, yet the resulting spheroplasts failed to lyse when suspended in hypotonic solutions. Thus the peptidoglycan could not be the cell's sole protection from turgor pressure. The study indicates that the outer membrane of gram-negative bacteria not only functions as a permeability and structural barrier, but also contributes to resistance to cell lysis from exposure to conditions of low osmolarity.

Why some bacteria make coccoid bodies and others do not is not known (as examples, *Campylobacter jejuni* cells convert into coccoid bodies in 48-h-old cultures whereas *Campylobacter fetus* cells do not; *Oceanospirillum maris* forms coccoid bodies whereas *Oceanosprillum japonicum* does not). It seems reasonable to think that in those species that do form coccoid bodies, autolysins that are normally held in check in the cell wall become unleashed and proceed to destroy the peptidoglycan, thereby conferring a spherical shape to the cell without damaging the integrity of the outer membrane. Autolysins have not been studied in any of the helical bacterial species that develop the coccoid form. It is possible that autolysins might become activated at the end of the active growth phase. That would then convert the rod form to the coccoid form. Autolytic activity can be detected and characterized by electrophoresis in acrylamide gels (SDS PAGE) containing a turbid peptidoglycan substrate; clear bands would result from the destruction of the peptidoglycan.

The occurrence of the autolysins would be determined by disrupting the cells with sonic oscillation. High-speed centrifugation would pellet the cell wall fraction, and the cytoplasmic fraction would be in the supernatant. The pellet and the supernatant would be

subjected to SDS PAGE using *Micrococcus luteus* peptidoglycan and, if necessary, homologous peptidoglycan as substrates for the autolysins (Leclerc and Asselin, 1989). Since autolysins are normally bound tightly to their substrate (peptidoglycan), one would expect to see activity mainly in the pellet (Rogers *et al*., 1980). Comparisons of autolytic activity will be performed using a 24-h-old culture of *A. fasciculus* vs. an older culture that has nearly 100% coccoid body culture of *A. fasciculus*.

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Appendices

Appendix I:

RNA isolation from *Prolinoborus fasciculus* (*Aquaspirillum fasciculus*)

1. Materials and Solutions

1 1.7 ml microcentrifuge tubes (RNase free)

2 0.2% Diethylpyrocarbonate (DEPC) treated deionized water (ddH_2O), autoclaved

3 Phenol, pH 4.0 (water-saturated, RNase free)

4 Chloroform (49:1 isoamyl alcohol (IAA), RNase free)

5 2 M Sodium acetate, pH 2.0

6 Sarkosyl

7 Guanidinium-thiocyanate

8 0.75 M Sodium citrate, pH 7.0

9 Ethanol 100% and 75% (EtOH)

10 Centrifuge

11 Formamide

12 RNase free pipette tips (1000 µl, 200 µl, and 10 µl)

13 Vortexer

14 100 ml and 1 L Wheaton bottles, oven sterilized (cover with aluminum foil, shiny side down, before sterilization)

15 50 mM Potassium phosphate buffer, pH 7.0 (PPB) (Does not have to be RNase free)

16 GT-Stock (4 M guanidinium-thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl)

To 100 ml Wheaton bottle add:

48.3 ml DEPC ddH_2O

1.7 ml 0.75 M Sodium citrate, pH 7.0

23.63 g Guanidium-thiocyanate

0.25 g Sarkosyl

Use RNase free chemicals and containers. Mix thoroughly. Cover bottle with aluminum foil to protect from light and store in a dark place at room temperature.

17 Solution D (4 M guanidinium-thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% sarkosyl, 0.1 M β-mercaptoethanol)

To 50 ml GT-Stock add:

7.8 µl β-mercaptoethanol

Store Solution D in a dark place at room temperature. Lysis solution is good for 1 month.

NOTE: In order to maintain RNase free conditions, gloves must be worn through out the entire procedure. Change gloves frequently to maintain RNase free conditions. All solutions must be made with DEPC treated deionized water. Add 0.2 ml DEPC for every 100 ml ddH_2O. Shake vigorously to get DEPC into solution. Autoclave the solution in order to inactivate DEPC.

2. Procedure

1 Harvest 100 ml cells by centrifugation. Spin for 10 min at 6,000 r.p.m. Wash cells twice with ice-cold PPB. The last wash of the cells should be performed in a 1.7 ml microcentrifuge tube.

2 Lyse pelleted cells in 500 µl of Solution D. Vortex cells on the lowest setting until the cell pellet is completely suspended in solution. Can use a wide bore 1000 µl RNase free pipette tip to aid in suspending pellet. Let solution sit for 30 seconds or until the solution has cleared.

3 Add 1/10 volume (50 µl) of 2 M sodium acetate (pH 2.0) and vortex vigorously for 3 sec.

4 Add 500 µl of water saturated phenol (pH 4.0) and vortex vigorously for 3 sec.

5 Add 120 µl chloroform (IAA) and vortex vigorously for 10 sec and immediately place the final suspension on ice for 15 min.

6 Spin at 10,000 rpm for 20 min. at 4° C

7 Collect upper aqueous phase and extract once with 120 µl of chloroform (IAA). Invert tube several times and give a quick spin to separate the phases at room temperature.

8 Collect the upper aqueous phase and add 1 ml of 100% EtOH. Invert the tube several times and store tube at -20° C for 20 min.

9 Spin tube at 10,000 rpm for 10 min at 4° C.

10 Wash pellet in 75% EtOH. Remove all residue EtOH with pipette.

11 Resuspend RNA pellet in formamide at a final concentration of 5 µg/µl. If pellet does not resuspend immediately, allow pellet to sit in formamide at 4° C for a few hours (or overnight) until solubilized.

Appendix II:

RNA Denaturation and Electrophoresis

1. **Materials and Solutions**

1 Mini submarine gel electrophoresis tank

2 Power supply

3 Orange G dye (Sigma Chemical Co., St. Louis, Missouri)

4 20 µl RNase free pipette tips

5 70° C Thermal block (or 70° C water bath)

6 RNA ladder (Ambion, Austin, TX.)

7 Formamide

8 1mg/ml solution of ethidium bromide (EtBr)

9 Reliant RNA gels (FMC Bioproducts, Rockland, Maine)

1.5% gels can also be made using RNase free agarose and RNase free 1X MOPS running buffer

To 250 ml flask:

100 ml 1X MOPS running buffer

1.5 g RNase free agarose

Use RNase free chemicals and containers. Add reagents and melt agarose completely using a microwave. Allow the solution to cool to 60 ° C before pouring into RNase free gel caster.

10 0.5 M EDTA, pH 8.0

To a 100 ml Wheaton bottle add:

1.86 g Na_2EDTA

Use RNase free chemicals and containers. Add 80 ml ddH_2O and mix thoroughly. Adjust pH to 8.0 with 10 N NaOH. Adjust final volume to 100ml. Add 0.2ml DEPC and shake vigorously to get DEPC into solution. Autoclave.

11 10X MOPS running buffer stock (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA)

To a 1 L Wheaton bottle add:

41.86 g MOPS (free acid)

6.80 g Sodium acetate

3.72 g EDTA

Use RNase free chemicals and containers. Mix chemicals with 850 ml ddH_2O. Adjust pH to 7.0 with 10 M NaOH. Adjust the final volume to 1 L. Add 2 ml of DEPC and shake vigorously to get DEPC into solution. Filter solution through a 2 μm nitrocellulose filter and autoclave. After autoclaving, solution will have a yellow straw coloration. Cover bottle with

aluminum foil and store in a dark place at room temperature. 10X buffer is good for 2 months.

12 Gel loading buffer (5:1 (v/v) formamide:50 mM EDTA , 0.125 % Orange G)

To a 5 ml Kimax bottle add:

4 ml Formamide

0.5 ml 0.5 M EDTA, pH 8.0

0.5 ml DEPC treated H_2O

6.25 mg Orange G

Use RNase free chemicals and containers. Mix chemicals until in solution. Store at room temperature.

2. Procedure

1 Prepare samples by mixing in RNase free 1.7 ml microcentrifuge tubes:

RNA (10 µg)	2.0 µl
10X MOPS running buffer	2.0 µl
1mg/ml EtBr	1.0 µl
Gel loading buffer	15 µl

2 Denature RNA samples by placing in a 70° C thermal block for 10 min.

3 Remove tubes and immediately place on ice for 5 min.

4 While samples are on ice, dilute 10X running buffer to 1X with RNase free ddH_2O.

5 Remove the cover from the Reliant gel tray. Remove the protective tape strips on the bottom of the tray, and press the tray onto the electrophoresis chamber platform.

6 Pour the 1X MOPS buffer into the electrophoresis chamber, covering the gel tray flange to a depth of 5 mm

7 Load 20 µl of each RNA sample to the wells of the gel.

8 Electrophorese for 2 hr at a field strength of 3.5 V/cm.