CHARACTERIZATION OF SWIMMING MOTILITY IN A MARINE UNICELLULAR CYANOBACTERIUM

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Dedicated with love and respect
to my grandmother,

Edna M. Conover

Characterization of swimming motility in a marine cyanobacterium

by

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Abstract

The structural mechanism, behavior, energetics and functional significance of the unique swimming motility displayed by some oceanic isolates of the cyanobacterium Synechococcus was investigated. A variety of analytical techniques confirmed that these strains swam through liquids without flagella or flagellar-like appendages. No extracellular structures were observed in a broad range of cell preparations examined by transmission electron microscopy (TEM), or by high-intensity dark field microscopy. The possibility that a structure might be present that eluded visualization was eliminated by the lack of motility-dependent amplitude spectra, the absence of discrete circulation of microspheres around the cell body and the inability of shearing forces to arrest motility. TEM and gel electrophoretic analysis of spheroplasts, cell wall-enriched fractions from motile and nonmotile strains, and cell material collected following the application of a flagellar hook-basal body complex isolation technique to a motile Synechococcus strain provided no further evidence of a structure or protein unique to motile strains.

The motile Synechococcus strains represent the only cyanobacterium reported to date capable of swimming rather than gliding motility. Swimming behavior was characterized by several features: between 50 - 80% of cells were actively motile during logarithmic phase of growth, with speeds that ranged from 5 - 40 um s^{-1}; the average speed was 13 um s^{-1}. Swimming patterns were entirely random, consistent with the absence of bacterial flagella. Synechococcus motility resembled flagellar-mediated motility in that thrust (forward motion) was accompanied by torque (cell rotation) as demonstrated by i) dividing cells which swam with the daughter cells at an angle, ii) individual cells that were sometimes seen to rotate end over end at a rate of 3 to 5 rev s^{-1}, iii) polystyrene beads attached to the cell body served as a point of reference as the cell rotated concomitant with translocation and iv) cells attached to the coverslip or slide spun about one pole at an average rate of 1 rev s^{-1}. When observed in the same plane of focus, 50% of the cells spun clockwise and 50% spun counterclockwise, but unlike flagellated cells, Synechococcus was never seen to change direction of rotation, as would be predicted if the cell body were rotating as a single unit and the motility apparatus were incapable of reversing direction of rotation. This motility apparatus appeared to operate at a constant torque, as indicated by the relationship between swimming speeds and the fluidity of the surrounding medium.

Investigation of the energetics of motility in Synechococcus WH8113 demonstrated that swimming was sodium coupled. There was a specific sodium requirement such that cells were immotile at external sodium concentrations below 10 mM, with speeds increasing with increasing sodium to a maximum speed at 150 to 250 mM sodium, pH 8.0 to 8.5. The sodium motive force increased similarly, but other energetic parameters including proton motive force, electrical potential, and the proton and sodium diffusion gradients lacked correlation to levels of motility. When components of the sodium motive force were diminished by monensin or carbonyl cyanide m-chlorophenylhydrazone, motility was arrested. Motility was independent of the magnitude of internal ATP pools, which were depleted to 2% of control values without affecting cell motility. These results suggest that the direct source of energy for Synechococcus motility is a sodium motive force, and that the devise driving motility is located in the cytoplasmic membrane, as is the case for flagellated bacteria.

The ecological role of Synechococcus motility was explored and several lines of evidence indicated that cells lacked behavioral photoresponses but were able to detect and respond to very low concentrations of simple nitrogenous compounds. When 23 compounds were tested in spatial gradients established in blind well chemotaxis chambers, cells displayed positive chemoresponses only when placed in gradients of NH_4Cl, $NaNO_3$, urea, glycine and alanine. Cells also failed to respond in chambers which lacked gradients due to the presence of only seawater or an equal distribution of chemoeffector, demonstrating that a gradient was required to elicit a response. The apparent threshold levels of 10^{-10} M - 10^{-9} M for Synechococcus chemoresponses are 4 to 5 orders of magnitude lower than those for most other bacteria and place them in the range of ecological significance. The presence of chemotaxis in this oceanic cyanobacterium may help support the notion that nutrient enriched microaggregates may play an important role in picoplankton nutrient dynamics.

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CHAPTER 1

INTRODUCTION TO PROCARYOTIC MOTILITY AND SCOPE OF THESIS RESEARCH

Introduction

Procaryotes typically display one of two types of motility: flagellar-mediated swimming or surface-associated gliding. Both gram-positive and gram-negative bacteria swim through liquids by the rotation of one or more extracellular semi-rigid helical filaments anchored in the cytoplasmic membrane (Berg and Anderson, 1973; Silverman and Simon, 1974; Macnab, 1978), whereas only gram-negative bacteria are capable of gliding motility which occurs exclusively over solid substrates and at air-water interfaces. In contrast to flagellar motility, for which there is a good understanding at both the morphological and molecular levels (Macnab, 1978; Simon et al., 1985), the structural and mechanistic features of gliding motility remain obscure (Buchard, 1981; Castenholz, 1982; Hader, 1987), although a number of models have been proposed (Halfen and Castenholz, 1970; Ridgway, 1977; Daryell-Hart and Buchard, 1979; Pate and Chang, 1979; Glagoleva et al., 1980, Hader and Poff, 1982; Lapidus and Berg, 1982; Keller et al., 1983; Dworkin et al., 1983).

Flagellar-mediated swimming requires both thrust and torque as evidenced by translocation coupled with counterrotation of the flagellar filament and cell body (Macnab, 1978). Any cell moving at a constant velocity has thrust and torque poised at equilibrium, thus the net force acting upon the cell is zero. In all flagellated bacteria, thrust is balanced by the viscous drag generated by movement of the cell through the medium (Berg, 1976; Berg, 1983). Torque produced by rotation of flagellar filaments is balanced by the viscous drag that results from counterrotation of the cell body (Berg, 1983).

Unlike the external flagellar filaments found in other flagellated bacteria, swimming motility in spirochetes is driven by endoflagellar rotation. The bipolar flagella of these helically-shaped microbes are located between the periplasmic cylinder and the outer sheath (Canale-Parlola, 1978). The endoflagella and the outer sheath rotate counter to the periplasmic cylinder. Torque is maintained by the viscous shear at the sheath outer surface (Berg, 1976).

Motility in both gliding and flagellated bacteria is driven by either proton motive force (Larson et al., 1974; Thipayathasana and Valentine, 1974; Manson et al., 1977, Matsuura et al., 1977; Miller and Koshland, 1977; Glagolev and Skulachev, 1978; Ridgway, 1977; Pate and Chang, 1979; Daryell-Hart and Buchard, 1979; Duxbury et al., 1980; Gusev, 1979; Glagoleva et al., 1980; Khan and Macnab, 1980), or as is the case in some alkalophilic bacteria, by a sodium motive force (Hirota et al., 1981; Hirota and Imae, 1983; Dibrov et al., 1986a,b). This is in contrast to eucaryotic motility which requires the hydrolysis of ATP (as reviewed by Satir, 1976).

Functionally, all motile microorganisms are united by the ability to detect changes in important environmental parameters. This allows a population to migrate either toward an attractant or away from a repellent (positive or negative chemotaxis, respectively), or seek out regions of optimum oxygen tension (aerotaxis) or illumination (photoresponses) (as reviewed by Berg, 1975; Macnab, 1978).

In 1982, it was discovered that several open ocean isolates of the marine cyanobacterium Synechococcus (sensu Rippka et al., 1979) were capable of swimming motility. Preliminary investigation indicated that these strains were morphologically and physiologically similiar to other marine strains, with the exception of swimming motility. As such, they were the first cyanobacterium observed to swim rather than glide. Moreover, several lines of evidence suggested they lacked flagella or any other extracellular appendage that might be responsible for their motility. Thus the goal of this research has been to elucidate the morphological, mechanistic and functional features of the motility displayed by this unusual cyanobacterium.

This research has necessarily relied on what is currently known of other forms of procaryotic motility and the experimental approaches used by investigators in this field. Therefore, a brief review of flagellar-mediated swimming and gliding motility follows. This is not intended to be a comprehensive review; such reviews are cited below. Instead, it should provide sufficient background information to facilitate understanding of the rationale used in designing and implementing research on the motile strains of Synechococcus.

Flagellar-mediated swimming motility

All flagellated bacteria possess the same basic motility and taxis system. The best studied swimming bacteria include *Escherichia coli*, *Salmonella typhimurium* and *Bacillus subtitis*, which are peritrichously flagellated, having a number of flagella randomly inserted about the cell. As observed when cells are tethered to microscope slides with anti-flagellar antibody (Silverman and Simon, 1974), single flagella spin both counterclockwise (CCW) and clockwise (CW). When cells are suspended in liquid, flagella form a polar bundle which when rotating CCW generates smooth swimming (runs). Clockwise flagellar rotation disperses the flagellar bundle, causing the cell to "tumble" (Macnab 1977, Macnab and Ornston, 1977) which almost completely eliminates net displacemnent. Cells then resume swimming in a new direction, randomly determined by Brownian motion. This random pattern of motility, runs lasting roughly a second punctuated by brief tumbles of only a fraction of a second, characterizes peritrichously flagellated swimming behavior in uniformly mixed environments (Berg, 1975, Macnab, 1978).

In contrast, the swimming pattern in cells with a single polar flagellum generally features forward and backward movements produced by CW and CCW flagellar rotation, respectively (Wegenknecht et al., 1982; Alam and Oesterhelt, 1984). The single polar flagellum of *Rhodobacter sphaeroides* is capable only of CW rotation. Instead of reversals or tumbles, cells stop frequently and coil the flagellum closely to the cell body, and resume swimming in a new direction (Armitage and Macnab, 1987).

The swimming behavior of the helically-shaped, bipolarly flagellated spirilla is similar to that of unipolarly flagellated bacteria. When swimming, flagella at both poles (either two single flagella or flagellar tufts) are directed to the rear of the cell. Reversals in swimming direction are produced when the direction of flagellar rotation at both poles is simultaneously reversed. Stopping occurs when flagella at both ends are either pointed toward or away from the cell. Counterrotation of flagellar filaments coupled with a helically-shaped body results in a quivering appearance when the spirillum is stopped (Krieg, 1976).

In spirochetes, rotation of the bipolar endoflagella in the same direction produces smooth swimming. However this is interrupted not only by reversals but also by flexing caused by flagellar rotation in opposite directions (Berg, 1976).

Average swimming speeds among all flagellated bacteria range from 20 to 30 um s^{-1}. Polarly flagellated bacteria generally swim faster than pertrichously flagellated cells and have been measured at speeds as great as 80 um s^{-1}(Macnab, 1978; Armitage and Macnab, 1987).

Despite the broad diversity of flagellated bacteria, the general architecture of the flagellar apparatus is very similar. Intact flagella are constructed of three components: a 5 to 22 um semi-rigid helical filament attached to an L-shaped hook which is anchored in the cytoplasmic membrane (CM) by the basal body which consists of a series of rings and a central rod. The M ring, thought to be the motor, is positioned in the innermost face of the CM and is stabilized by the S ring on the outer surface of the CM. Gram-negative bacteria have two additional rings, the L and P to further stabilize the basal body in the outer cell wall (as reviewed by Macnab, 1978). Negatively stained preparations of flagellated bacteria and intact flagella have permitted the visualization of all these structures by electron microscopy (Depamphilis and Adler, 1971a,b; Alam and Oesterhelt, 1984; Swan, 1985).

The flagellar filament is composed of subunits packed without covalent interactions, making it easily and reversibly depolymerized by heat or suspension in acidic medium. The monomeric form, flagellin, in E. coli, Salmonella, and Bacillus is a single protein, functionally identical but with wide interspecific differences in molecular weight (Macnab, 1978). The filament may have more than one flagellin, such as in Halobacterium halobium which has three distinct proteins (Alam and Oesterhelt, 1984).

The hook is considered important in transmitting torque from the basal body to the filament (Berg and Anderson, 1973). In addition to the hook protein itself, three other hook associated proteins have been identified in enteric bacteria. Two of these are at the filament-hook junction and the third functions as a filament cap. It is required for the addition of new flagellin subunits which are transported through a central core and added to the distal end of the filament (Homma el al., 1987).

The flagellar filament and hook of genera such as Vibrio (Follet and Gorden, 1963), Bdellovibrio (Seidler and Starr, 1968), Rhizobium lupini H 13-3 (Schmitt et al., 1974), and some Pseudomonas species (Lowy and Hanson, 1965; Fuerst and Hayward, 1969) are sheathed. Electron microscopic and chemical analyses indicate that the sheath is an extension of the outer cell wall. Helical wavelength, total length and translational velocities do not vary significantly from unsheathed flagellated bacteria (Follett and Gordon, 1963; Seidler and Starr, 1968; Schmitt et al., 1974). It is thought that the sheath may serve to protect the flagellum (Doetsch and Hageage, 1968).

The basal body in S. typhimurium and E. coli consists of at least eight proteins. Those for the L and P rings have been identified in E. coli and the cellular location of several of the S. typhimurium basal body proteins have recently been elucidated (Homma et al., 1987). Berg and Anderson (1973) first suggested that flagellated bacteria swim by the rotation of rigid flagellar filaments; Glagolev and Skulachev (1978) proposed that the M ring served as the rotary motor. Empirical evidence has since been provided by Block and Brown (1984) who observed sixteen incremental increases in the angular velocity of tethered E. coli cells. These workers regulated the level of expression of the motor gene (motB) product by constructing a lacZ-motB gene fusion. This result corresponds with the sixteen stud-like processes visualized around the M ring by freeze-etch electron microscopy of Aquaspirillum serpens (Coulton and Murray, 1978). Since these are presumed to be the site of proton translocation which generates the mechanical work of motility, these studs have been termed "force-generating units" (Block and Berg, 1984).

The frequency of CCW-CW switching is an inherent property of each flagellum (Macnab and Han, 1983) and and is transiently modified in response to environmental stimuli (Berg and Brown, 1972). Most chemoeffectors are detected by methyl-accepting chemotaxis proteins (MCPs). These transmembrane proteins bind specific ligands on their periplasmic face which results in a change in level of methylation at sites on the cytoplasmic side of the protein, thereby initiating signal transduction from the chemoreceptor to the flagellar motor (as reviewed by Boyd and Simon, 1982; Simon et al., 1985). Photoresponses, aerotaxis and responses elicited by sugars transported by the phosphotransferase system are MCP-independent; signals are initiated at the photosynthetic or respiratory electron transport chain (Melton et al., 1978; Hazelbauer and Engstrom, 1980; Taylor, 1983).

Following signal reception, a series of cytoplasmic events alters the switching frequency of the flagellar motor to predominantly CCW or CW rotation marked by smooth swimming (CCW) when an attractant has been detected, or tumbling (CW) in response to repellents. This modulation of runs and tumbles as determined by gradients of stimulants is best described as a "biased random walk" (Berg and Brown, 1972).

The ability to probe flagellated bacteria genetically has been fundamental to the advance of the field of motility and chemotaxis. Based on manipulation of the roughly fifty loci involved, there are several classes of motility and chemotaxis mutants among the enteric flagellated bacteria (as reviewed by Parkinson, 1981; Boyd and Simon, 1982; Simon et al., 1985). These are:

a) Motility mutants which have defects in either structural or regulatory genes preventing the proper synthesis or assembly of specific flagellar components. These include mutants with abnormal filaments (hag), those that lack flagella (fla), and paralysed mutants (mot) which possess flagella unable to rotate.

b) Specifically nonchemotactic mutants exhibiting wild-type motility but lack chemotactic responses to a few structurally related compounds, as determined by the chemoreceptor involved. Thus, specifically nonchemotactic mutants are classed by receptor: the tsr protein binds serine and mediates the chemotactic response to this amino acid as well as to the repellents indole and leucine. Since the tar protein binds aspartate and the charged maltose binding protein, these chemoattractants and the repellents nickel and cobalt do not elicit any response in these mutants. Tgr mutants fail to sense ribose and galactose (Parkinson, 1981). The most recently isolated mutants involve the tap receptor which mediates the behavioral changes induced by dipeptide reception (Slocum et al., 1987).

c) Generally nonchemotactic mutants (che) display either wild-type motility or a permenant CW or CCW bias. Due to mutations in genes that "centrally process" chemotactic signals, these cells do not respond to any changes in chemoeffector concentration.

By comparing past with present chemoeffector(s) concentration, a bacterial cell employs a temporal sensing mechanism to respond to changes in spatial gradients of stimulants (Macnab and Koshland, 1972). This form of bacterial "memory" integrates the chemotactic signal over time, reducing unavoidable statistical fluctuation of molecule concentration at the receptor site. In addition, temporal sensing obviates the need for extreme analytical accuracy since chemoreception over time allows changes in concentration over a distance much greater than one cell length to be discerned. For example, by comparing concentrations over a 2 mm distance rather than the 2 um cell length, temporal sensing reduces the calculated sensitivity a cell would require from 1 part in 10^4 to 1 part in 10 (Macnab and Koshland, 1972).

Based on the behavior of both wild-type and mutant phenotypes, the transient response to changes in chemoeffector concentration involves five conceptually separate mechanisms: a) chemoreception as an ongoing measure of current effector concentration b) a means to compare present and past chemoreceptor concentration c) generation of a coherent signal by integration of the level of effector at each receptor site d) transmission of the signal to the flagellar motor and e) interaction of the signal with the motor resulting in the modulation of CCW-CW switching frequency (Boyd and Simon, 1982).

Intensive research within the past fifteen years as yielded a great deal of information regarding these mechanisms, however the nature of signal transduction and the flagellar motor switching mechanism remains widely debated. The level of sophistication employed by investigators in this field is exemplified by the analysis of the chemotactic response of tethered bacterial cell envelopes with functional flagella (Ravid and Eisenbach, 1984; Szupica and Adler, 1985) and the construction of strains "gutted" of chromosomally encoded che genes so that only selected che genes can be expressed in cells transformed with plasmids carrying trp-che fusions (Wolfe et al., 1987).

Gliding motility

There are three major bacterial groups capable of gliding motility: the cyanobacteria and their nonpigmented counterparts such as Beggiotoa and Achromatium, the Chloroflexaceae, and the flexibacteria, including the myxobacteria and Cytophaga spp (Reichenbach, 1981). Among the cyanobacteria, gliding is broadly dispersed, occuring in both unicellular and filamentous forms (Castenholz, 1982). Of the many mechanisms proposed, none unify observations made in all gliding bacteria.

The pattern of gliding motility does not resemble the runs and tumbles of swimming motility. Instead movement is generally nonrandom, marked by forward and backward movement along the long axis of the cell, occassionally rolling, bending or curling about one pole (as reviewed by Weibull, 1960; Doetsch and Hageage, 1968, Buchard, 1981). Speeds of translocation are much slower than flagellar-mediated swimming, averaging from 1 to 3 um min^{-1}, and is a function of the composition of the substrate and its level of hydration, temperature and associations with other bacteria (Buchard, 1981, Castenholz, 1982). In addition, many gliding organisms move in coordinated multicellular configurations forming swarms (Buchard, 1981; Reichenbach, 1981).

Although the motile Synechococcus strains are capable only of swimming motility, taxonomically and physiologically they are related to gliding rather than swimming microorganisms. It has been suggested that mechanisms thought to be involved in gliding motility may be important in Synechococcus swimming motility as well. A brief review of those models most frequently mentioned follows.

a) Slime secretion: A relationship between gliding motility and slime production was first documented in the nineteenth century (as reviewed by Doetsch and Hageage, 1968). In 1951, Hosoi noted that Oscillatoria princeps slime continued to flow in a spiral fashion even after trichome movement had stopped. It has been suggested that pores found in the outer wall of some cyanobacteria are responsible for the forceful secretion of slime. The role of slime secretion in myxobacteria has focused on either jets of slime from polar pores or breaking and retracting slime threads.

Several lines of evidence indicate that slime production is not directly responsible for gliding motility. In both the cyanobacteria and the myxobacteria, slime secretion is highly variable lacking any correlation between quantity of slime produced and gliding speed. There is no theoretical or empirical means by which slime secretion could account for the directional changes or filament rotations observed during gliding. Finally, the quantity of slime needed to propel a gliding cell exceeds the cell volume, thus the cell would have to void its contents many times a second (Holton and Freeman, 1965). Dodd's suggestion (1960) that slime may act as a lubricant enhancing locomotion is considered more tenable.

b) Contractile waves and/or fibrils: The propagation of contractile waves has been cited as the means of gliding among filamentous cyanobacteria, Beggiotoa and some myxobacteria. Slime appendages, fibrillar protein tubes ("rhapidosomes") and protein helicies or fibrils attacted to the inner surface of the cell have been reported, but not unequivocally demonstrated (Doetsch and Hageage, 1968; Halfen and Castenholz, 1970; Halfen, 1973; Buchard, 1981).

Ultrastructural and chemical characterization of O. animalis and O. princeps reveals a band of parallel fibrils within the cell wall oriented in the same pitch as the rotating trichomes during gliding. These fibrils are thought to have contractile properties, propagating waves down the length of the filament (Halfen and Castenholz, 1970; Halfen, 1973). Like the function of slime appendages and rhapidsomes, the propulsive properties of these fibrils is obscure. They are not analogous to eucaryotic microfibrils since trichomes continue to glide in the presence of cytochalasin B (Schimz, 1981) and gliding is driven by a proton motive force rather than ATP hydrolysis (Glagoleva et al., 1980). Flexibacter polymorphus was once though to possess similar fibrils (Pate and Ordal, 1967), but these were proved to be glutaraldehyde fixation artifacts when examined by freeze-etch electron microscopy (Buchard and Brown, 1973).

c) Rotary discs: Pate and Chang (1979) report numerous rotary assemblages within the cell envelope of Flexibacter columnaris and Cytophaga johnsonae, presumed to be similar to flagellar basal bodies. The movement of latex spheres on the cell surface of these bacteria is thought to be correlated with the distribution of these discs. Other workers have been unable to find such discs in the cell envelopes of other related bacteria (Lapidus and Berg, 1982).

d) Cell surface-substrate interactions such as outer membrane adsorption sites: Since gliding is always surface-associated and gliding microorganisms are immotile in liquid, many investigators have studied the interaction between the cell surface and substrate. Benthic cyanobacteria may adhere to substrates by hydrophobic interactions (Fattom and Shilo, 1977). Lapidus and Berg (1982) observed the movement of polystyrene latex beads along discrete tracks on the cell wall of Cytophaga sp. strain U67. A model is presented whereby these binding sites attach to the substratum and move within the fluid outer membrane along tracks encircing the rigid peptidoglycan layer. However, like other theories regarding the mechanism of gliding motility, the structural apparatus has not yet been found.

The isolation of C. johnsonae mutants (Chang et al., 1984) further substantiates the importance of the cell surface-substratum interaction. All nongliding mutants were found to have defects in cell surface-associated functions such as phage sensitivity and chiton digestion. Recently, sulfonolipids have been implicated in the gliding motility of C. johnsonae since their synthesis is solid-substrate dependent (Abbanat et al., 1987).

Like swimming bacteria, the tactic behavior among gliding procaryotes include aerotaxis, chemotaxis and photoresponses. Gliding cyanobacteria, which evolve O_2 and are exclusively photoautotrophic, demonstrate only photokinesis, phototaxis and photophobic reactions (as reviewed by Nultsch and Hader, 1979; Hader, 1987). Photokinesis is simply an increase in gliding speed thought to be directly related to a light-induced increase in cellular energy levels (Hader, 1987).

Phototaxis can be either positive or negative, depending on the orientation of bacterial filimants with respect to a spatial gradient of light. The phototactic behavior of Phormidium uncinatum is analogous to chemotaxis: reversals are supressed during positive phototaxis and increased during negative phototaxis until the filaments are positioned in a specific light environment. In contrast, filament movement in Anabaena variabilis is nonrandom; cells move directly toward the region of optimum illumination. It is thought that in this organism, phototaxis may be mediated by an intracellular gradient of light or some light-incuded molecule or molecular process, as is the case in the eucaryote Dictyostelium (Nultsch et al., 1979) rather than a mechanism analogous to temporal sensing, common in unicellular flagellated bacteria (Macnab and Koshland, 1972).

Photophobic responses (PPR) are described as transient changes in gliding behavior following sudden spatial or temporal increases (step-up) or decreases (step-down) in light intensity or spectral quality. Two models have been proposed in the PPR exibited by P. unicinatum. When exposed to a spatial step-down, photoreceptors in the leading trichomes of this cyanobacterium initiate a corresponding decline in pmf (Hader, 1979) which presumably induces the opening of voltage-dependent Ca^{2+} channels. This increase in electrical potential reverses the potential difference between the leading and trailing end of the cell which ultimately reverses the gliding direction (Hader, 1987). This hypothesis is substantiated by the loss of PPR on calcium-free agar, a PPR-induced transient influx of $^{45}Ca^{2+}$ (Hader and Poff, 1982) and PPR sensitivity to Ca^{2+} blocking agents (Hader, 1982).

Alternatively, based on the response to specific inhibitors of photosystem II and artificial electron donors, Gabai and Glagolev (1985) suggest that photophobic responses are initiated by brief changes in the plastiquinone redox level which may or may not correlate with changes in pmf. Counter to the role of the electron transport chain in mediating responses to light in other bacteria (Taylor, 1983), the incorporation of $^{14}CH_3$ from [^{14}C]-methionine into a membrane-bound protein has led these workers to suggest that the PPR in P. uncinatum involves an MCP-like receptor. Like the transduction of chemically induced signals, the mechansism by which changes in light modulates the motility apparatus and ultimately alters bacterial behavior remains unknown.

Scope of thesis research

At the time this research was started, it had not been unequivocally demonstrated that these cyanobacteria lacked an extracellular swimming appendage, nor was the functional significance of swimming motility clear. The goals of this thesis research were to:

1. Conclusively establish the presence or absence of bacterial flagella or some other structure that might be responsible for the observed motility.

2. Physically describe the swimming behavior of the motile strains of *Synechococcus*.

3. Identify the source of energy for motility since this is physiologically and ecologically relevant and provides a basis for comparison with other motile organisms.

4. Examine the physiological and functional significance of swimming motility in these open ocean strains of *Synechococcus*. Specifically, what were the tactic capabilities of these cyanobacteria?

5. From morphological, behavioral and energetic parameters, further elucidate the mechanism by which these cells swim and develop a model to define the ecological role of *Synechococcus* motility in the oligotrophic ocean.

Initially, the experimental designs and techniques used in the visualization, isolation and behavioral characterization of flagellated procaryotes were essential in proving the presence or absence of bacterial flagella or some other motility apparatus in motile *Synechococcus*. Thus in some experiments, previously described methods were precisely followed. However, the physiological and behavioral differences between the commonly studied flagellated enteric bacteria and *Synechococcus* frequently demanded either modification of such techniques or the development of new methods. Flagellated bacteria and/or nonmotile *Synechococcus* strains were used for control purposes when appropriate. The research approaches thus taken included:

1. Light and electron microscopic analyses of the morphology and ultrastructure of the motile and some nonmotile *Syenchococcus* strains.

2. Manual and computer analyses of video taped preparations of swimming cells observed with light microscopy.

3. Examination of motile *Synechococcus* strains under experimental conditions known to alter flagellar-mediated swimming motility such as mechanical shearing and changes in medium viscosity. Also, the presence of specific membrane proteins was addressed using fractionation techniques developed for cyanobacteria and flagellated bacteria.

4. An assessment of the energetics of *Synechococcus* motility based on investigations in which the energetics of other motility and transport systems have been identified. Hence the role of ATP and proton and sodium motive forces were considered.

5. An evaluation of the photo- and chemoresponses in motile *Synechococcus*, which provided the basis for assessing the physiological and ecological role of swimming motility in this cyanobacterium.

CHAPTER 2

MORPHOLOGICAL AND BEHAVIORAL CHARACTERIZATION OF SWIMMING MOTILITY IN *SYNECHOCOCCUS*

Introduction

Since 1981, when swimming motility was first observed in open ocean *Synechococcus* isolates, our collection of motile strains has grown from the original four to over thirty strains out of approximately seventy marine isolates. Thus what was originally thought to be an interesting but relatively rare phenomenon now appears to be fairly widespread.

The study of motility in marine *Synechococcus* began with an effort to visualize the flagella presumed to be responsible for the observed swimming motility. When initial electron microscopic investigation failed to reveal morphological features unique to the motile strains including any sort of extracellular organelle that might propel the cell, a broader variety of analytical approaches were sought to address the structural mechanism of *Synechococcus* motility. These analyses included:

1. Additional transmission electron microscopy (TEM) studies including negative staining, thin sectioning with and without the acid polysaccharide-specific dye, ruthenium red and freeze fracture and etching.

2. Examination by high-intensity dark field microscopy capable of visualizing single bacterial flagella (Macnab, 1976; Macnab and Ornston, 1977).

3. Computer analysis of motility-associated amplitude spectra of swimming *Synechococcus* cells performed by Dr. Howard Berg at Harvard University.

4. Light microscopic examination of actively motile *Synechococcus* cells in suspension with a variety of polymer-based beads in an effort to discern specific motility-associated microcurrents around the cell body.

5. The effects of shearing forces in a Waring blender on cell motility.

6. The examination of proteins and the electron microscopic visualization of spheroplasts, enriched cell wall

fractions and cell material prepared by a flagellar hook-basal body complex (HBB) isolation technique.

7. An assessment of the response of swimming cells to changes in medium viscosity.

8. A description of the physical behavior of *Synechococcus* swimming.

The collective results of these experiments served as conclusive evidence that motile strains of open ocean *Synechococcus* translocated through liquids without bacterial flagella. These cyanobacterial strains thus represent a novel form of procaryotic motility.

Materials and Methods

Isolation and growth of motile strains: Motile strains were enriched from samples collected on several cruises and maintained in culture as described by Waterbury and Willey (in press; Appendix I). Growth experiments requiring a defined medium were performed in medium AN with modifications as indicated.

Electron microscopy: Cell suspensions were prepared from actively motile cells in mid to late logarithmic phase of growth. Cells with or without fixation in glutaraldehyde or osmium tetroxide (solution or fumes) were negatively stained for TEM with uranyl acetate or phosphotungstic acid at a variety of concentrations and pH values. Formvar coated, glow discharged copper grids with or without a film of carbon were used in all negatively stained preparations.

Fixation and embedding for thin sectioned preparations was conducted as described by Waterbury and Stanier (1978). Where indicated, preparations were stained with ruthenium red using the method of Luft (1971), with the following modifications: Samples were suspended in marine buffer (Waterbury and Stanier, 1978) and prefixed for 30 minutes in 0.5% glutaraldehyde and an equal volume of ruthenium red, final concentration 1500 ppm. Fixation in 3.5% glutaraldehyde plus an equal volume of ruthenium red for 1 hour was followed by fixation in a minimal volume of osmium tetraoxide and an equal volume of dye.

The quick-freezing technique of Heuser et al. (1979), performed in the laboratory of Dr. Thomas Reese (Marine Biological Laboratory, Woods Hole) was used to prepare actively motile cells, that were concentrated by centrifugation at 600 x g for 10 minutes, for freeze fracture and etching. Because this technique freezes samples in 2 msec or less, cryoprotectants such as glycerol were unnecessary. Samples were fractured and etched with a Balzers 300 freeze ethching apparatus under the conditions described by Coulton and Murray (1978). A Philips 300 transmission electron microscope was used to visualize all sample preparations.

High-intensity dark field microscopy: Synechococcus WH 8103 was examined under high-intensity dark field microscopy in the laboratory of Dr. Robert Macnab (Yale University) with the technique described by Macnab (1976), except a filter that eliminated that portion of the high-intensity light spectrum less than 500 nm was employed. This strain of swimming Synechococcus has an unusually high concentration of phycourobilin (PUB) which has a peak absorbance of 495 nm (Ong et al., 1984), thus this filter prevented photoinhibition. Photoinhibition could not be prevented in other motile strains which have a higher phycoerythrobilin:phycourobilin ratio, because the appropriate combination of filters could not be assembled.

Glass bead-cell suspensions: Beads (0.23 - 0.25 um in diameter, unless otherwise indicated) purchased from Polyscience, Inc., Warrington, PA, included: carboxylate polybead monodisperse, polystyrene monodispersed latex, hydroxylate polybead monodisperse, butadiene polybead styrene, PMMA polybead monodisperse, amino acid polybead microsphere (0.19 um), polystyrene polybead microsperes (0.06 um) and carboxylate polybead monospheres (0.11 um). Silcate (0.38 um) and titanium dioxide (0.1 - 0.2 um and 0.5 um) beads were gifts of Dr. Howard Berg and polystyrene beads (0.38 um) were a gift of Dr. Stanley Watson (Woods Hole Oceanographic Institution). In all cases, cells in the mid-logarithmic phase of growth were harvested and resuspended in buffer AN to reduce possible ionic interactions between the seawater-based medium and the beads. Bead-cell suspensions were examined by phase contrast microscopy; the contrast levels of the suspensions were too low to permit video recording, even when examined by dark field microscopy.

Determination of amplitude spectra: Dr. Howard Berg kindly tested for the presence of a discernible motility-dependent amplitude spectra in images of actively swimming *Synechococcus* WH 8113 cells using the system described by Lowe (1987). This method is based on the observation that the torque generated by flagellar rotation is balanced by counterrotation of the cell body. An image of the cell body as it translocates thus has a specific wobble, if the long axis of the cell body and the flagella are not collinear. In addition, if there is a net imbalance on the forces perpendicular to the axis of the flagellar helix, a vibrational frequency equal to the rotational frequency of the flagellar bundle is observed. In flagellated bacteria, fast Fourier transforms of digitized video recordings of photomultiplied cell images yield spectra that contain a low frequency peak generated by the roll of the cell body and a high frequency peak due to vibrational movement (Lowe et al., 1987).

Shearing experiments: A Waring two-speed blender was used to conduct shearing experiments as described by Waterbury et al. (1985; Appendix II).

Spheroplast preparation, cell fractionation and protein analysis: Spheroplasts were prepared from the nonmotile strain *Synechococcus* WH 7803 and the motile strain WH 8113 by the method of Peschek (1983). Enriched cell wall fractions were prepared from the nonmotile strains *Synechococcus* WH 7803 and WH 8018 and the motile strains WH 8103 and WH 8113 by the technique of Resch and Gibson (1983). Cell wall fractions were analyzed for the presence of thylakoid and carotenoid pigments by scanning spectrophotometry (Shimadzo UV-visible spectrophotometer UV-260) from 300 to 700 nm. Cell material was also obtained from *Synechococcus* WH 8113 by the HBB isolation method of Aizawa et al. (1985), as modified by Brahamsha and Greenberg (submitted for publication). Spheroplasts, cell wall fractions and cell material from the flagellar isolation procedure were negatively stained for TEM as described previously. SDS-PAGE was performed by the method of Laemmli (1970). Protein concentration was determined by the Folin-phenol technique of Lowry et al. (1951).

Viscosity experiments: Synechococcus WH 8103 and WH 8113 and E. coli H102 were grown to the mid-logarithmic phase of growth in medium SN and seawater-based LB broth (Maniatis et al., 1982), respectively, and harvested by centrifugation at 3000 x g for 10 minutes at 4°C. Cells were then suspended in 75% filtered seawater:double distilled water and Synechococcus cells were incubated at 25°C, E. coli at 34°C. A stock solution of 20% Ficoll (Sigma Chemical Co., Saint Louis, MO) was prepared in 75% seawater and the viscosity of a range of stock Ficoll:seawater dilutions was measured with a Brookfield viscometer at 25°C and 34°C. Stock Ficoll was added to cell suspensions of Synechococcus or E. coli to yield the desired final viscosity. Wet mounts were prepared and video recorded immediately by phase contrast video microscopy. Speed of motility and the percent of cells remaining motile were determined by manual analysis of video recorded experiments, as described below.

Analysis of swimming motility: Wet mount cell suspensions recorded by phase contrast video microscopy (Zeiss inverted microscope IM 35, 40x neofluar objective, Dage-MTI video camera, Panasonic AG6300 video recorder, Panasonic WV-5410 b/w monitor) were used for experiments analyzed manually by tracing cell bodies on acetate overlays at 1/6-second intervals (Waterbury et al., 1985; Appendix II).

Dark field microscopy as detailed by Willey et al., 1987 (Chapter 3) rather than phase contrast microscopy was used to achieve the high level of contrast needed for computer-assisted motion analysis of video recordings (Motion Analysis Corp., Santa Rosa, CA), as described by Sundberg et al. (1985).

Results

Isolation and growth: Motile Synechococcus strains have been isolated from a variety of depths from within the euphotic zone of the Sargasso Sea, the temperate south and tropical Atlantic Ocean and the Caribbean Sea (Table 1). In addition, swimming Synechococcus cells were observed in concentrated natural seawater samples examined in wet mounts by epifluorescence microscopy on shipboard. As described by Waterbury et al. (1985; Appendix II), both motile and nonmotile strains are obligate photoautotrophs. In addition, they possess the same relative abundance of the chromophores phycourobilin and phycoerythrobilin in the accessory light harvesting pigment phycoerythrin as other open ocean isolates (Olson et al., in press). All oceanic strains, regardless of the presence or absence of motility, are obligately marine. A minimum of 185 mM NaCl was needed for growth of the motile Synechococcus strains WH 8103, WH 8112, and WH 8113, as determined by growth experiments in medium AN containing NaCl that ranged from 30 to 500 mM. Other parameters for optimum growth are listed in Appendix I.

Motile Synechococcus strains grew very poorly, if at all on solid substrates. Initial attempts to grow the four axenic motile strains on 0.2 to 0.4% super clean agar (Difco; Waterbury et al., 1986) resulted in either no growth or growth rates that were too slow to avoid fungal contamination. Several approaches were taken to improve growth on solid substrates including substituting agar with gelatin (2, 4 and 5%) or gelrite (0.1% supplemented with 0.1mM $MgCl_2$ $6H_2O$), as well as preparation with sterilized (filtered or autoclaved) spent liquid medium SN from mid-logarithmic or senescent motile Synechococcus cultures. Plates were prepared as pour plates or streaked after solidification. None of these procedures improved growth on plates for any of the four axenic motile strains.

Electron microscopy: Examination by TEM revealed no evidence of flagella (Figure 1A,B). A variety of conditions were used to prepare negatively stained cell suspensions of the four axenic motile Synechococcus strains to eliminate the possibility that flagella were being destroyed by any one kind of preparation. The peritrichous

Table 1

Motile *Synechococcus* strains: Isolation data

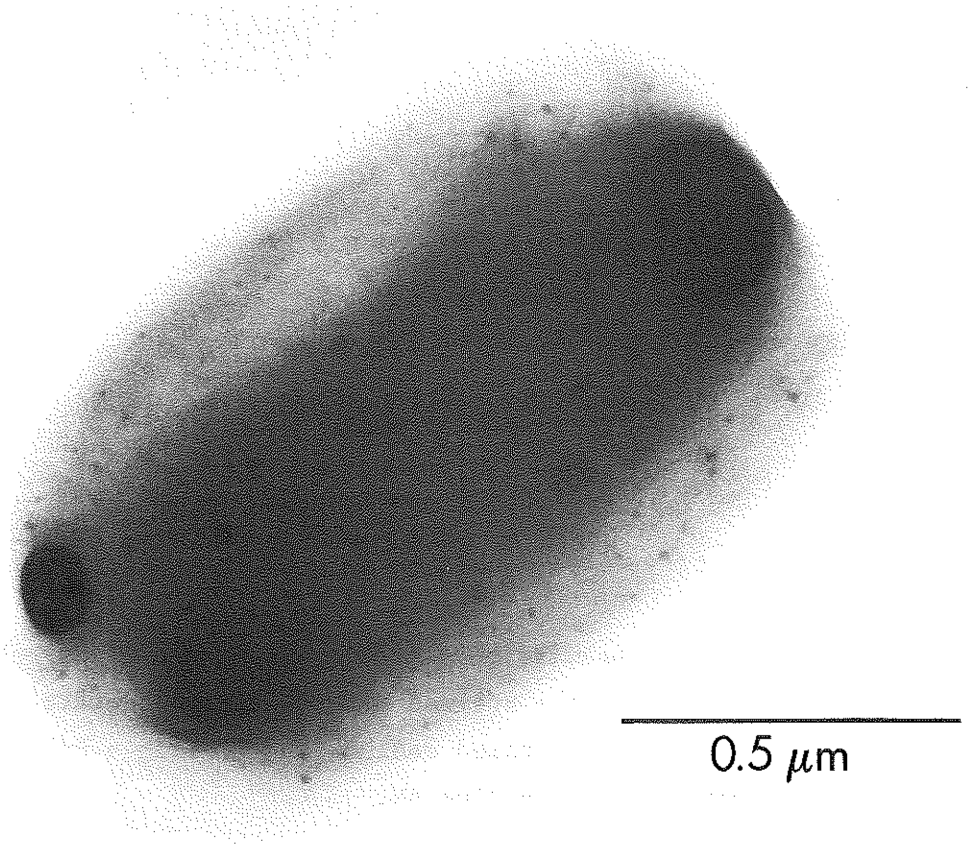
Strain	Location	Date	Depth
		Axenic	
WH8011	34°N, 65°W	June 1980	25 m
WH8103	28°N, 67°W	March 1981	surface
WH8112	36°N, 66°W	October 1981	20 m
WH8113	36°N, 66°W	October 1981	60 m
		Nonaxenic	
WH8401	39°N, 49°W	March 1984	10 m
WH8406	30°N, 77°W	December 1984	50 m
RO-1	19°N, 67°W	May 1986	surface
RO-3	36°N, 70°W	May 1986	surface
RO-2	19°N, 67°W	June 1986	surface
RO-5	32°N, 69°W	June 1986	surface
P13-VBRT	39°N, 62°W	September 1986	surface
P14-20m	38°N, 60°W	September 1986	20 m
P22-15mBulk	35°N, 54°W	September 1986	15 m
P25-20mBulk	32°N, 53°W	September 1986	20 m
P60-0Bulk	19°N, 24°W	September 1986	surface
P61-25mdim	18°N, 23°W	September 1986	25 m
P64-10mdim	17°N, 21°W	September 1986	10 m
P64-10mBRT	17°N, 21°W	September 1986	10m
P64-10mBulk	17°N, 21°W	September 1986	10m
P64-30mBulk	17°N, 21°W	September 1986	30 m

Motile *Synechococcus* strains: Isolation data
(continued)

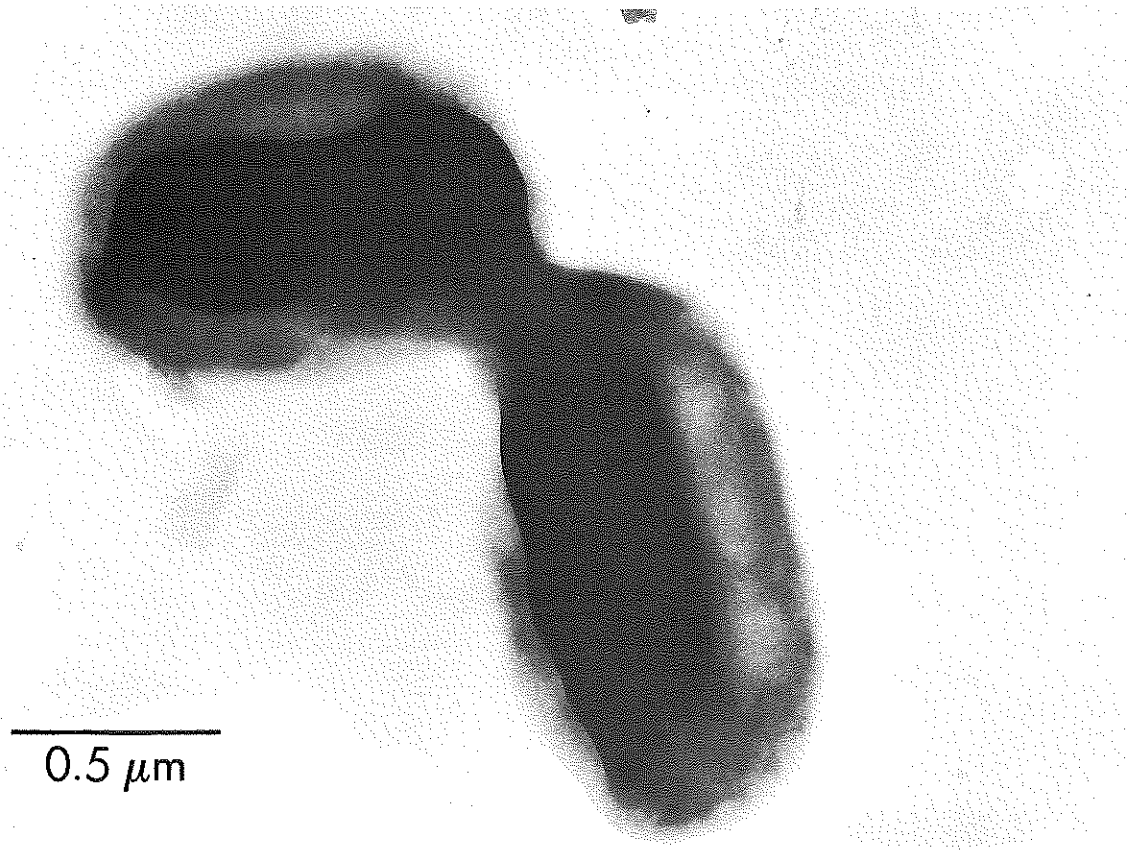
Strain	Location	Date	Depth
		Nonaxenic	
B15-Syn	38°N, 60°W	September 1986	surface
B23-Bulk	34°N, 55°W	September 1986	surface
B26-Bulk	32°N, 52°W	September 1986	surface
B29-Bulk	30°N, 48°W	September 1986	surface
B37-Syn	27°N, 40°W	September 1986	surface
B44-Bulk	24°N, 34°W	September 1986	surface
B45-Bulk	24°N, 33°W	September 1986	surface
B62-Bulk	18°N, 22°W	September 1986	surface
N2S-14D	39°N, 65°W	September 1986	surface

Figure 1: Transmission electron micrograph of Synechococcus strain WH 8103, negatively stained with 1% uranyl acetate. A) From a culture grown in seawater-based medium SN. B) From a culture grown in artificial seawater-based medium AN.

1a



1b

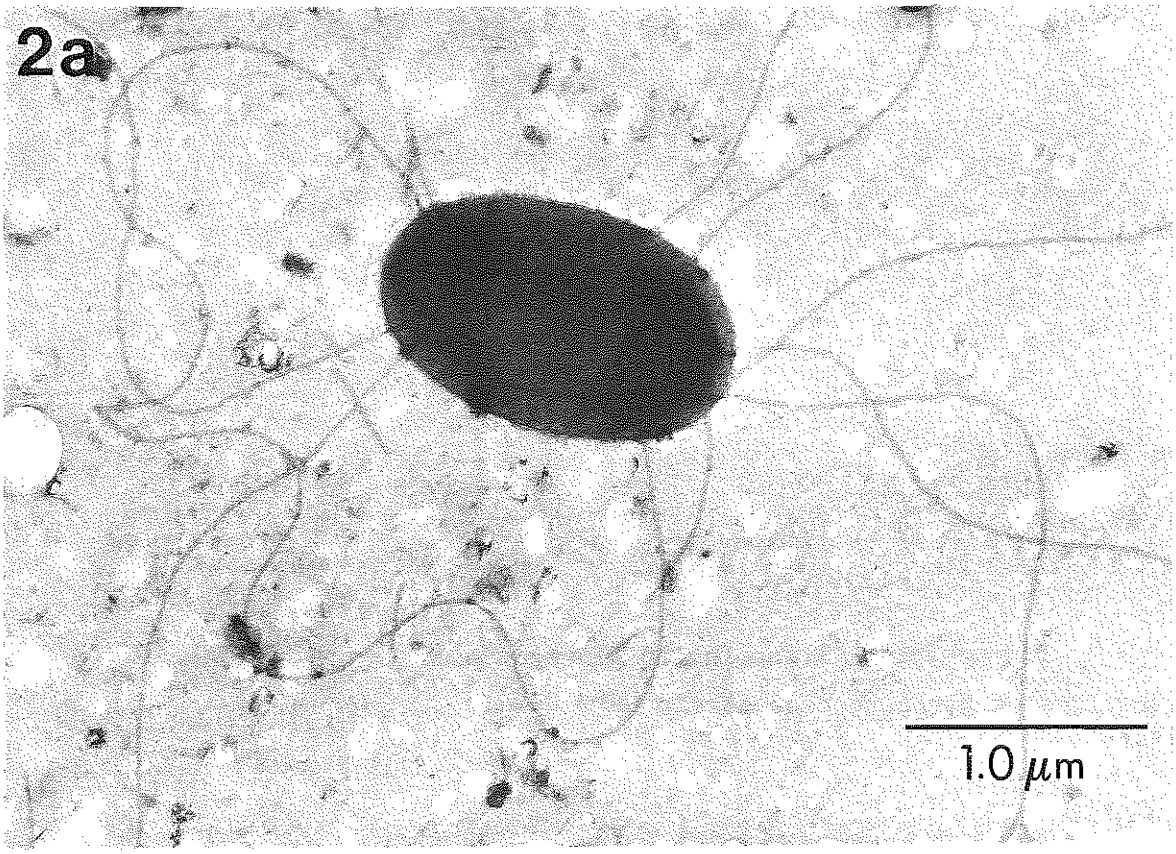


flagella of Escherichia coli H102 (Figure 2A) and the sheathed polar flagellum of Vibrio parahaemolyticus (Figure 2B) were clearly evident when cells were prepared under identical conditions.

Flagella or other ultrastructural features that might propel the cell were not observed in thin sections examined by TEM (Appendix II, Figure 1B; Figure 3A,B). Cell preparations were stained with the acid polysaccharide-specific dye ruthenium red to enhance visualization of the cell wall in motile (Figures 4A,B and 5) and nonmotile (Figure 6A,B) strains. This resulted in higher levels of contrast, but no specific cell wall-associated feature that might be involved in swimming motility could be discerned. The electron density of the cell wall of the motile strains had a peculiar pattern that was particularly evident in cells sectioned tangentially (Figure 5), where it resembled an orange peel. This was absent in the nonmotile strain WH 7803 (Figure 6A,B), however a similar pattern was reported in ruthenium red-stained thin sectioned cell preparations of the unicellular coastal cyanobacterium Synechocystis CB3 (Lounatmaa et al., 1980).

Specimens were prepared for freeze fracture and etch by the quick-freezing or "freeze-slam" method since this technique omits the use of chemical fixatives and cryoprotectants, thereby avoiding artifacts produced by these compounds. Freeze fractured (Figure 7A,B) or etched (Figures 8 and 9A,B) preparations of Synechococcus WH 8113 lacked flagella or any other cell wall or membrane-associated appendage. Fractures along concave and convex surfaces (Figure 7A,B) were densely populated with particles comparable to those observed in other bacteria (Remsen and Watson, 1972) and cyanobacteria (Golecki, 1977; Golecki, 1979; Budel and Rhiel, 1985). These particles lacked the rosette pattern of studs seen in freeze fracture preparations of Aquaspirillum serpens (Couton and Murray, 1978), which were later termed the "force generating units" (Block and Berg, 1984) required to drive motility. The pattern of globules observed when cells were etched (Figure 8) and the visualization of thylakoid membrane and carboxysomes in deeply etched preparations (Figure 9A,B) was similar to that observed in other cyanobacteria (Smarda, 1979; Lountamaa et al., 1980). Frequently, extracellular capsular-like material was

Figure 2: Transmission electron micrographs of flagellated cells negatively stained with 1% uranyl acetate. A) *Escherichia coli* strain H102 and B) *Vibrio parahaemolyticus*.


2a
1.0 μm

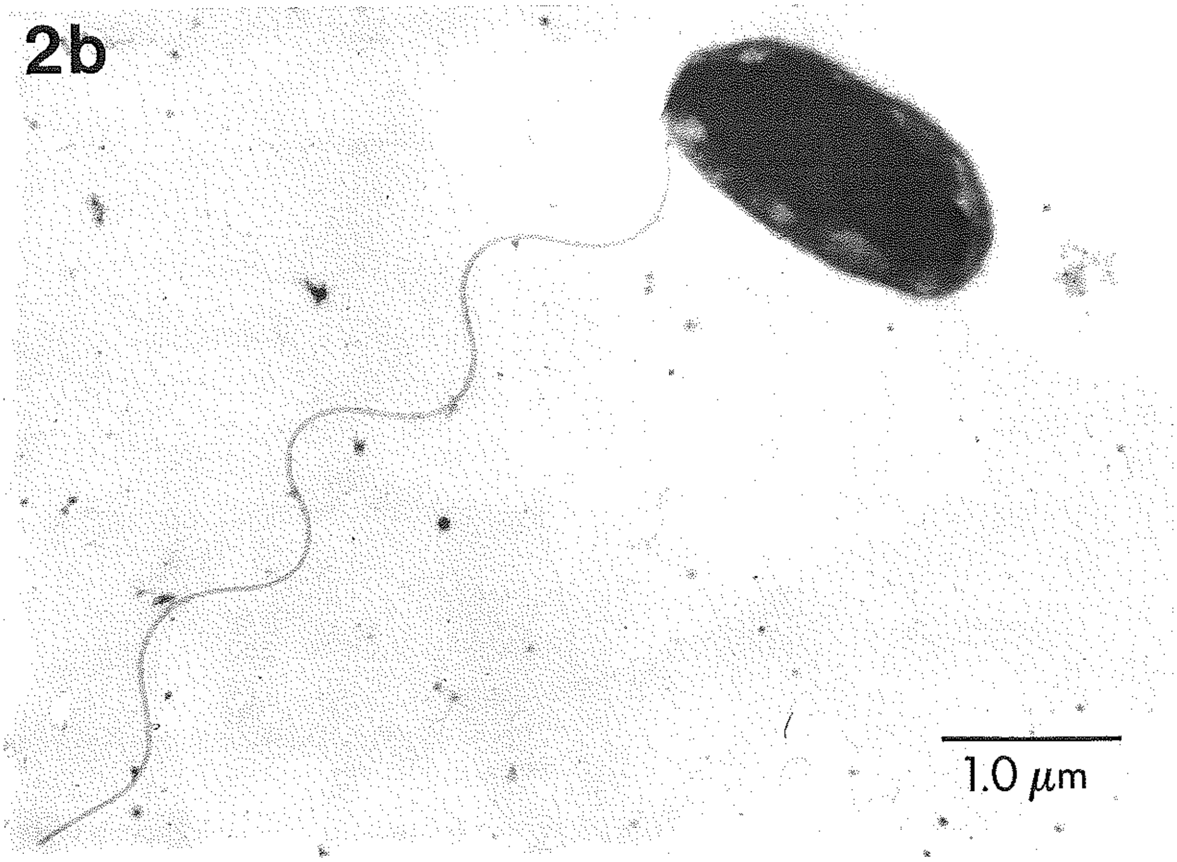

2b
1.0 μm

Figure 3A&B: Transmission electron micrographs of thin sections of *Synechococcus* strain WH 8103.

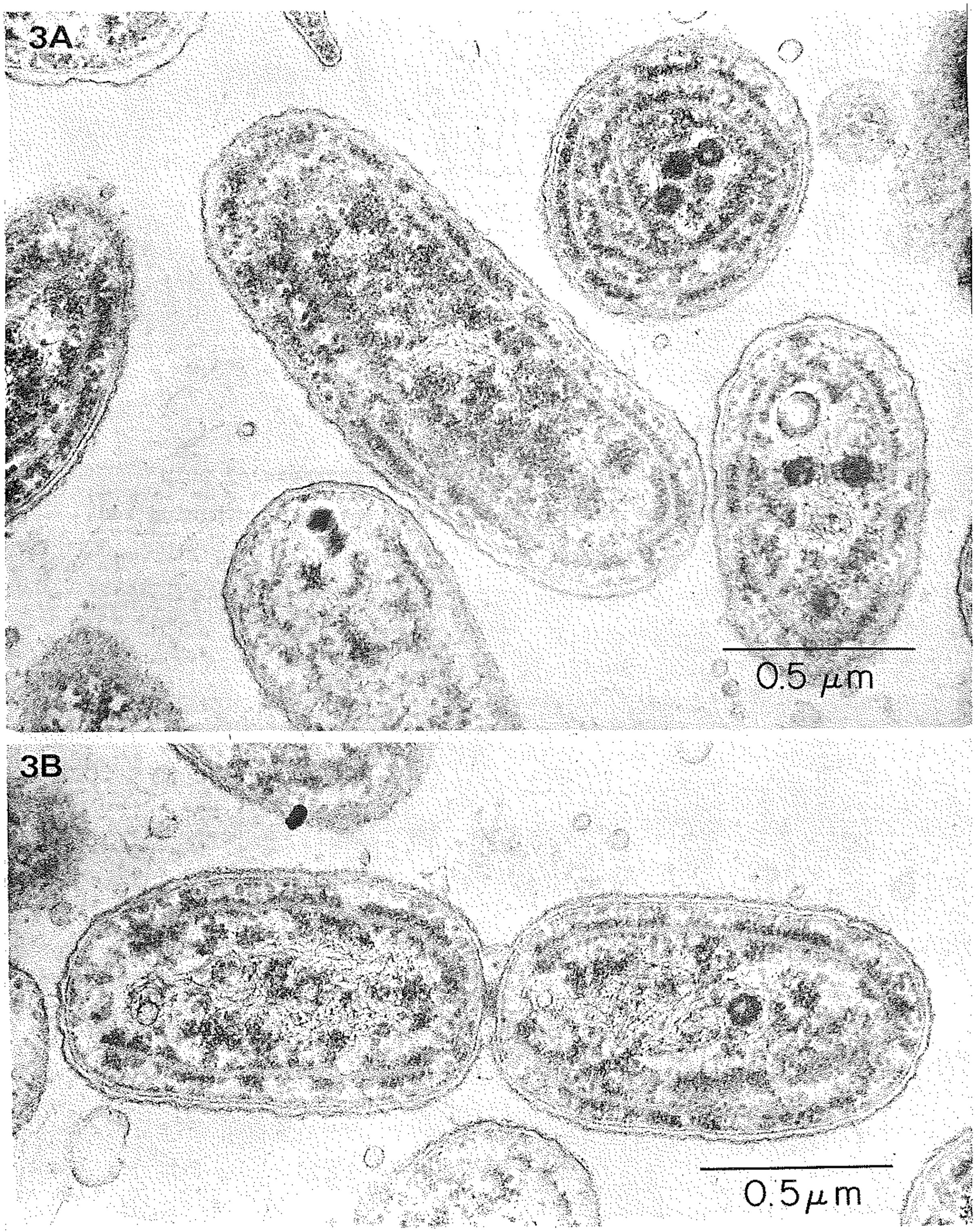
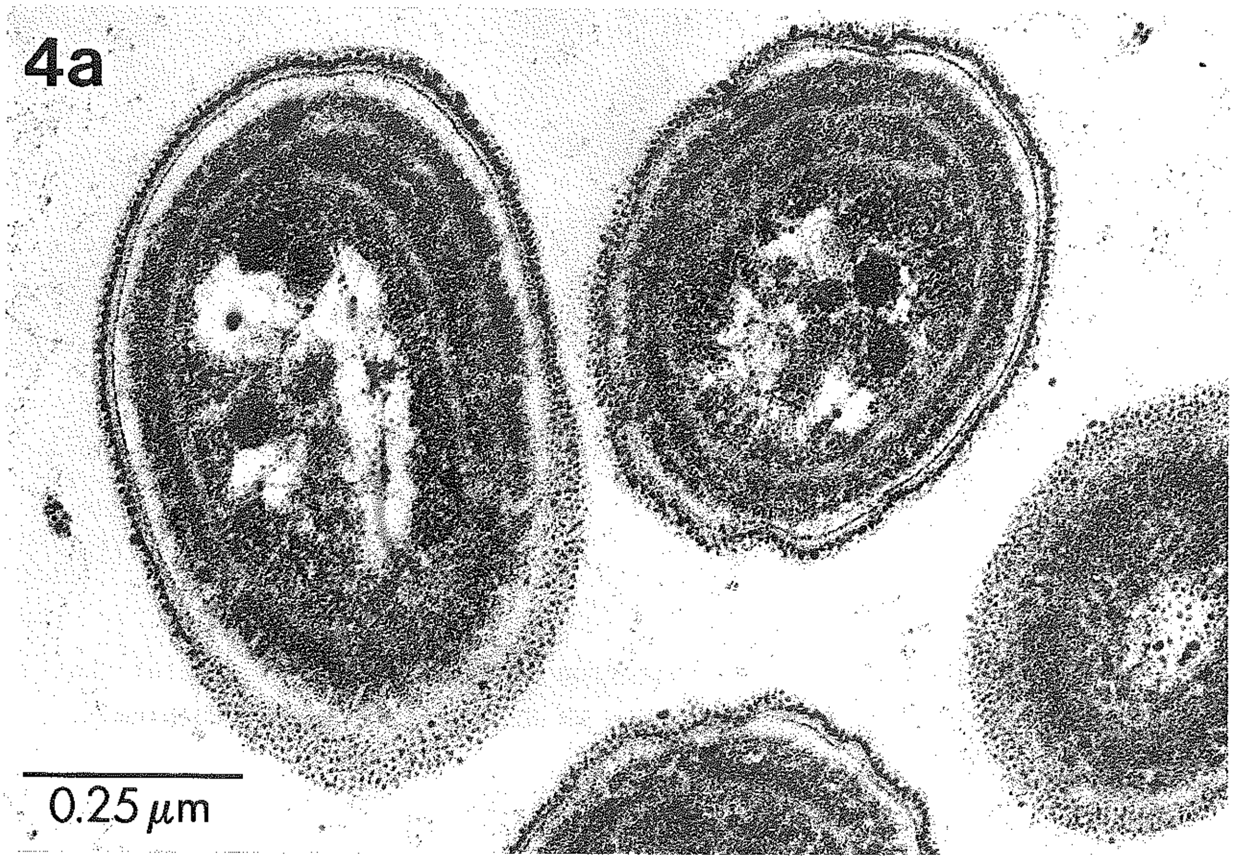

3A
0.5 μm
3B
0.5 μm

Figure 4A&B: Transmission electron micrographs of thin sections of *Synechococcus* strain WH 8113 stained with ruthenium red.


4a
0.25 μm

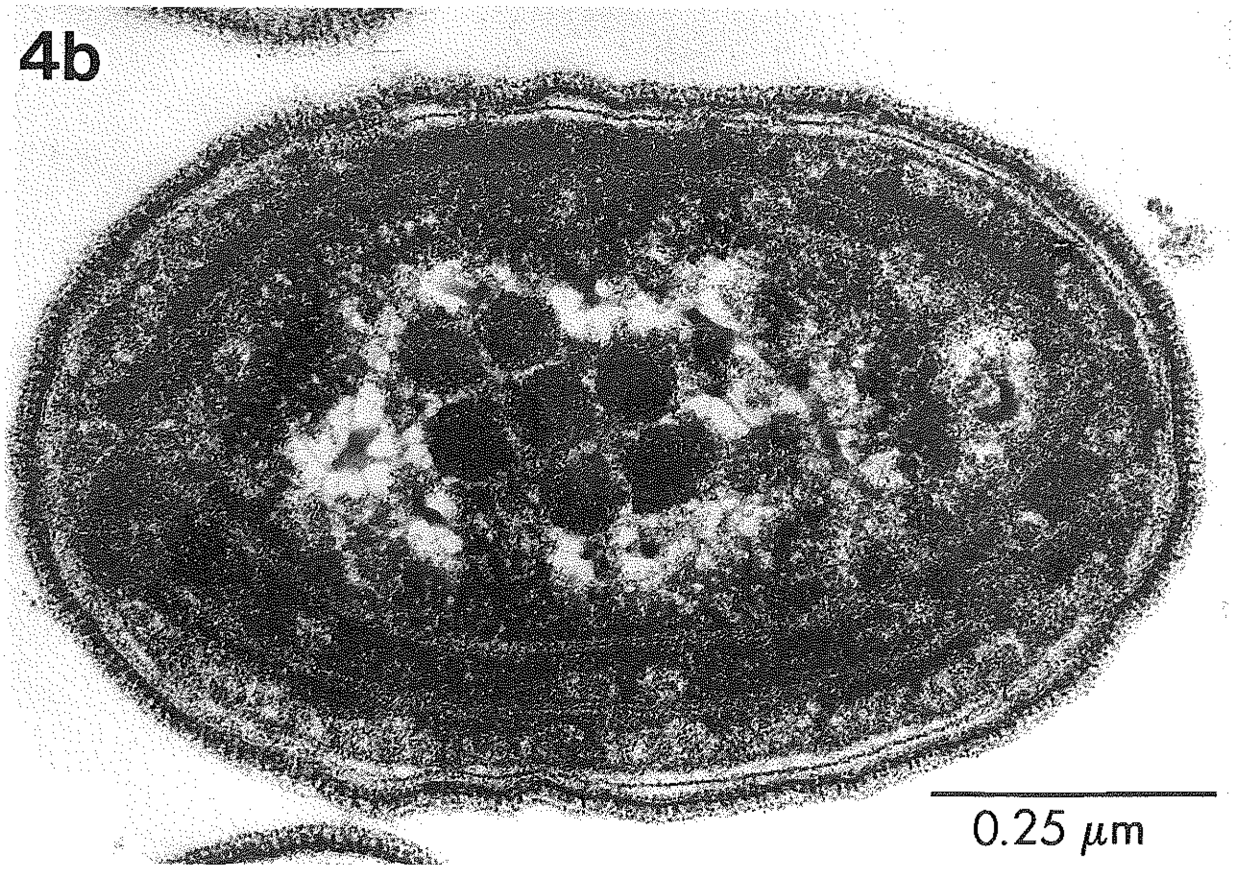

4b
0.25 μm

Figure 5: Transmission electron micrograph of a tangential thin section of Synechococcus strain WH 8113 stained with ruthenium red.

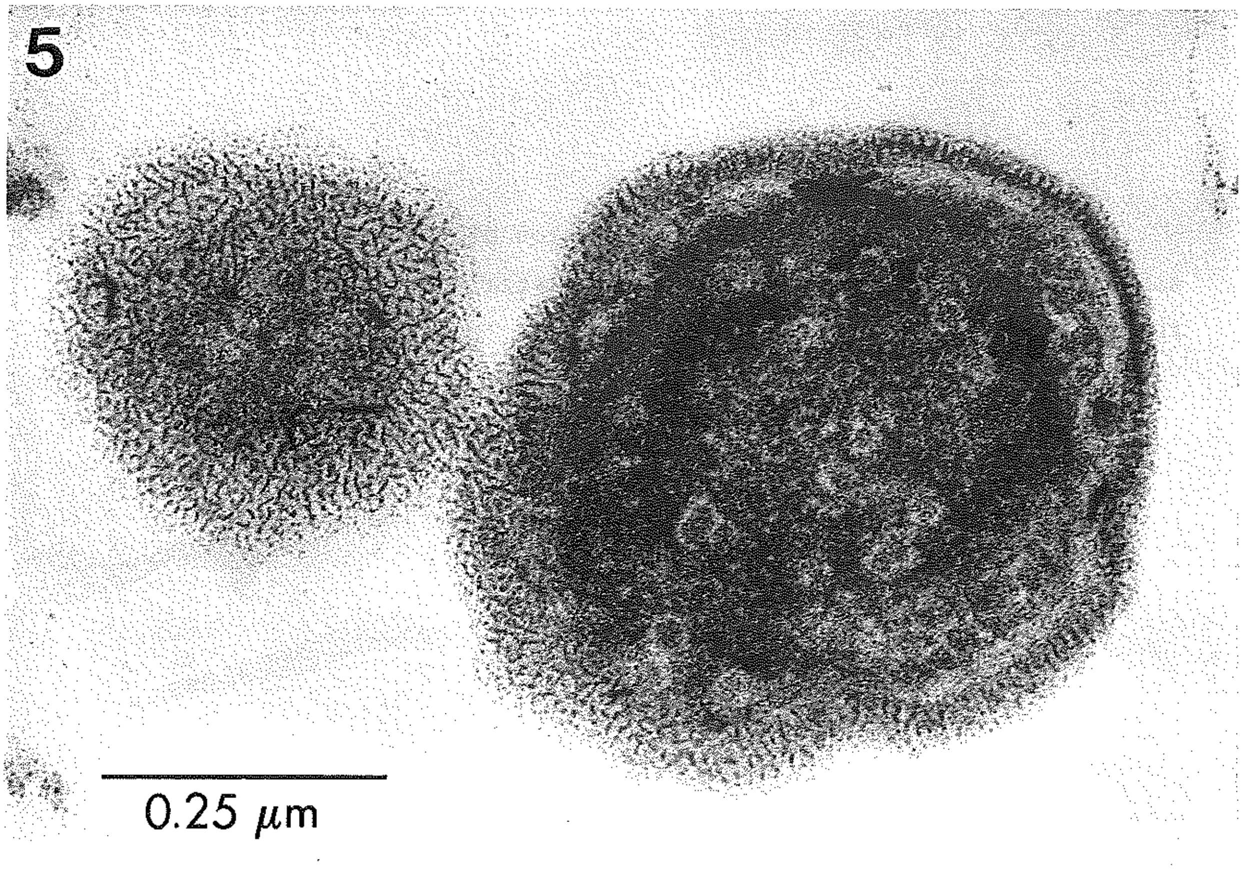
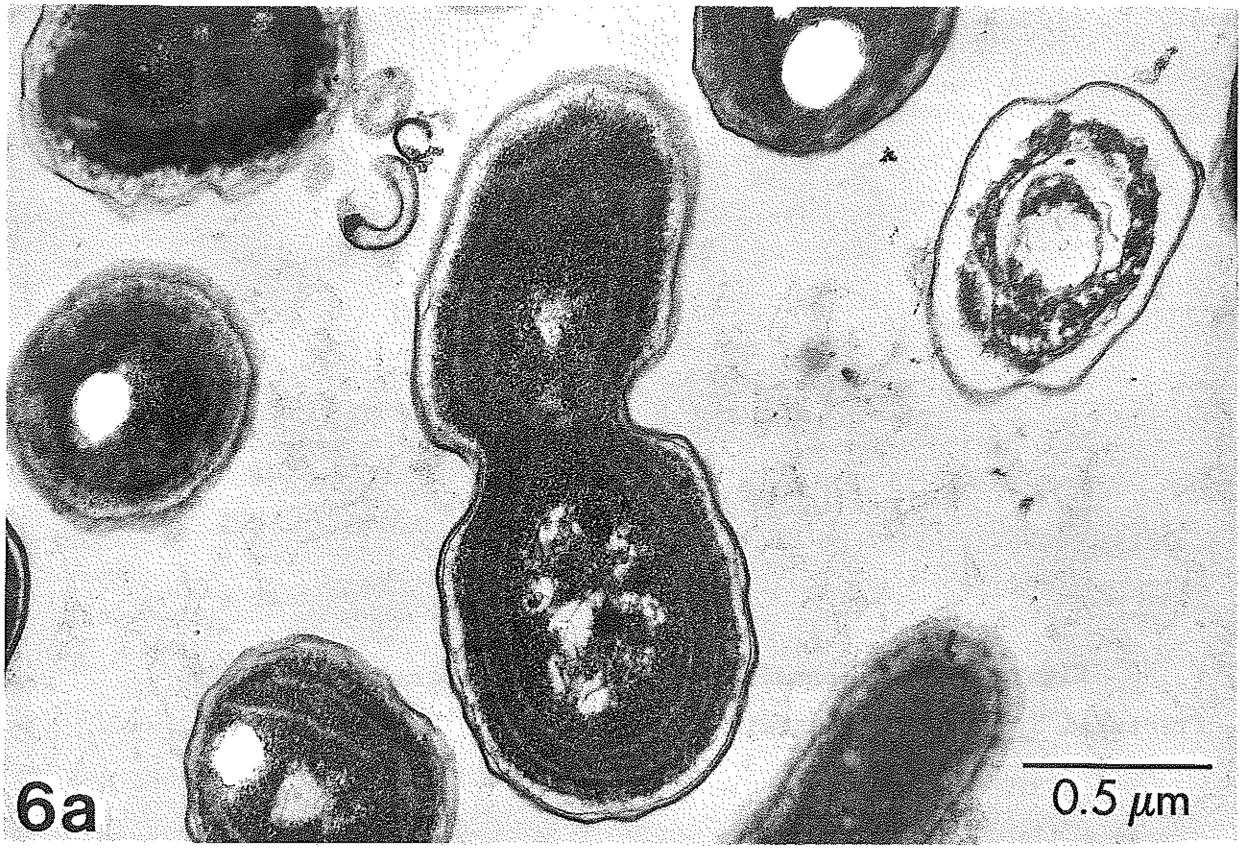

5
0.25 μm

Figure 6A&B: Transmission electron micrographs of thin sections of the nonmotile *Synechococcus* strain WH 7803 stained with ruthenium red.


6a

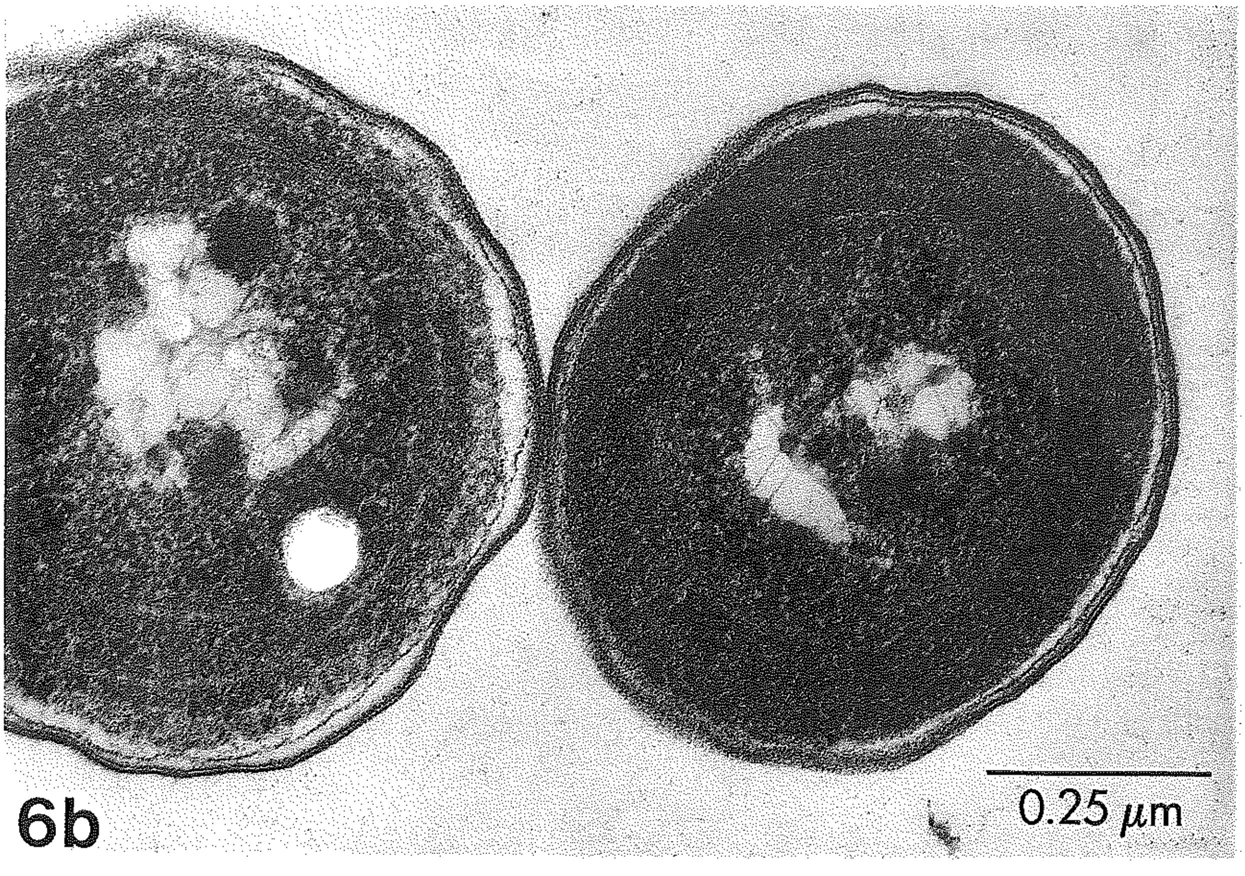
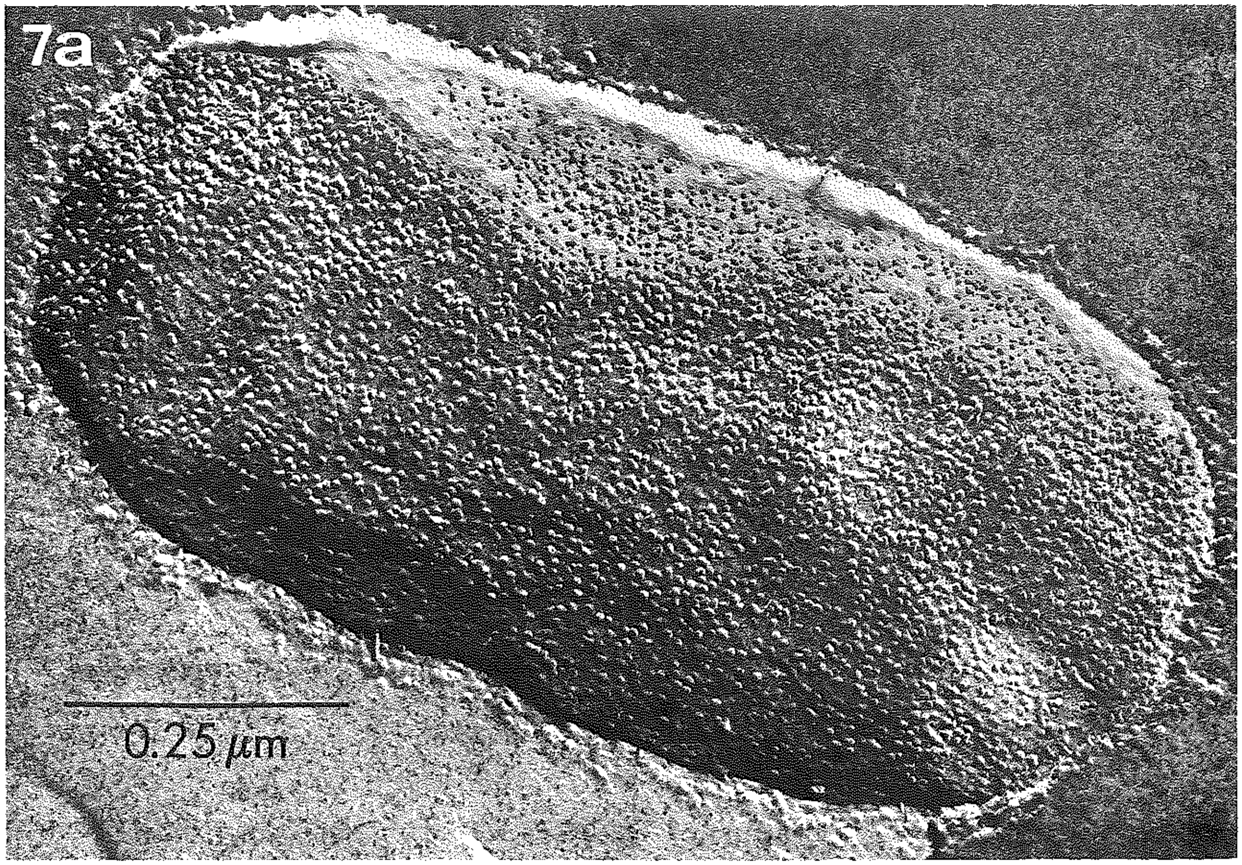

6b

Figure 7A&B: Transmission electron micrographs of freeze fractured *Synechococcus* strain WH 8113.


7a
0.25 μm

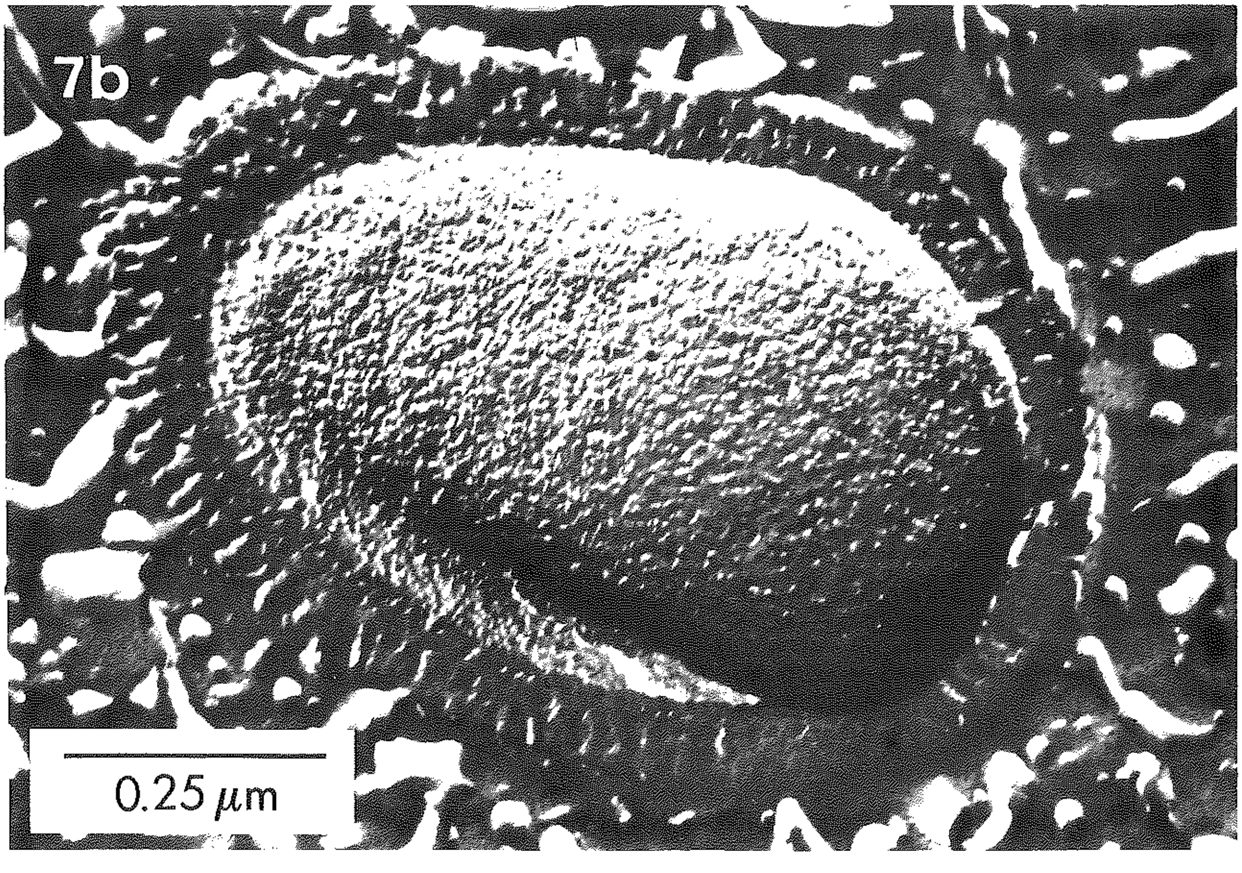

7b
0.25 μm

Figure 8: Transmission electron micrograph of freeze fractured and etched *Synechococcus* strain WH 8113.

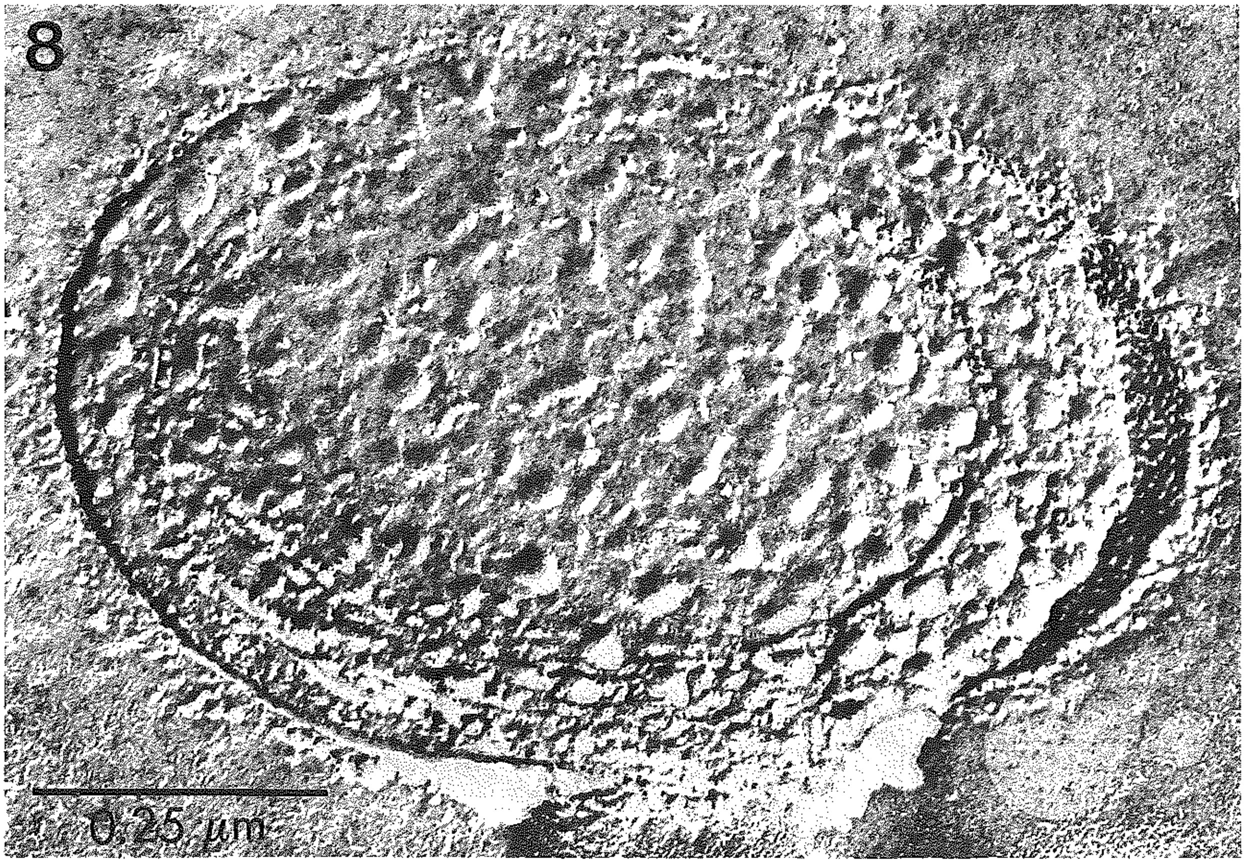
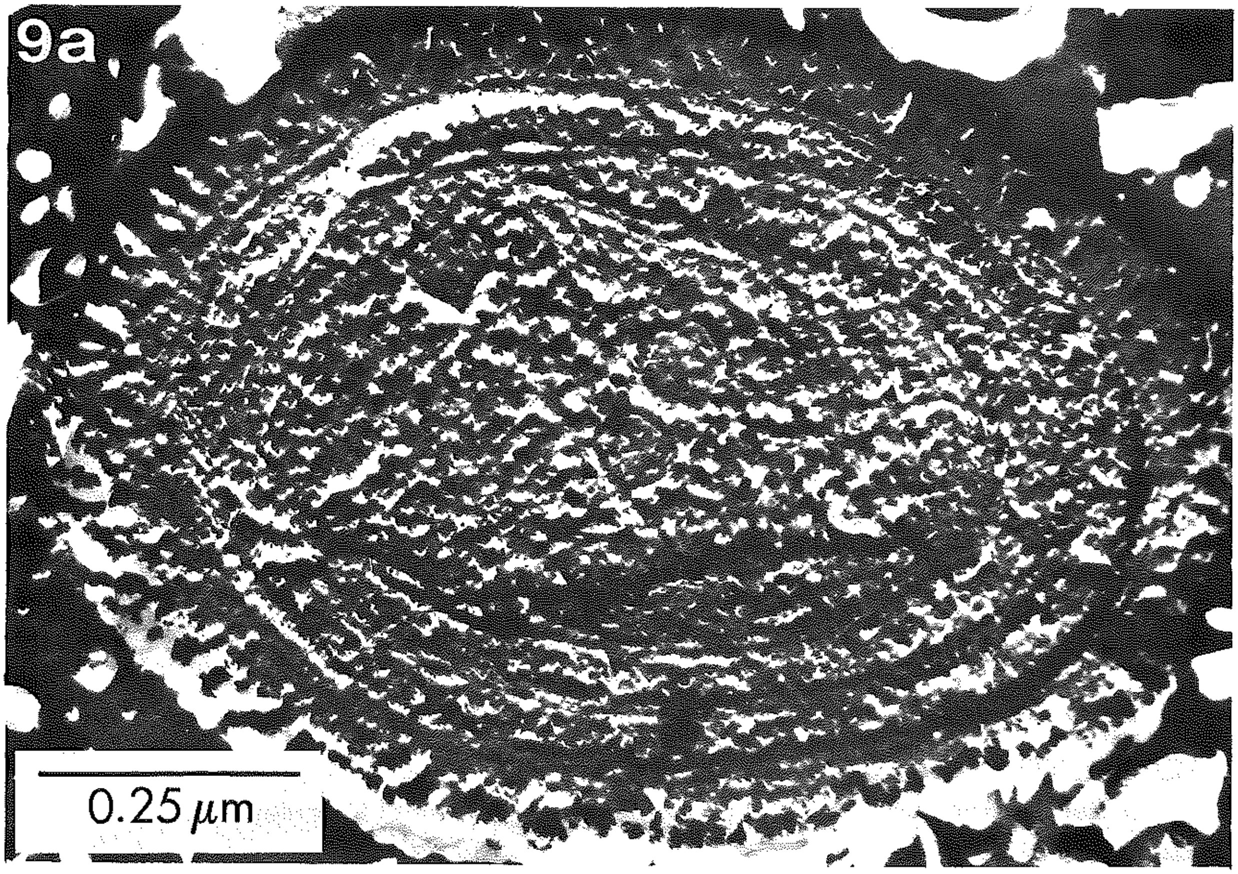
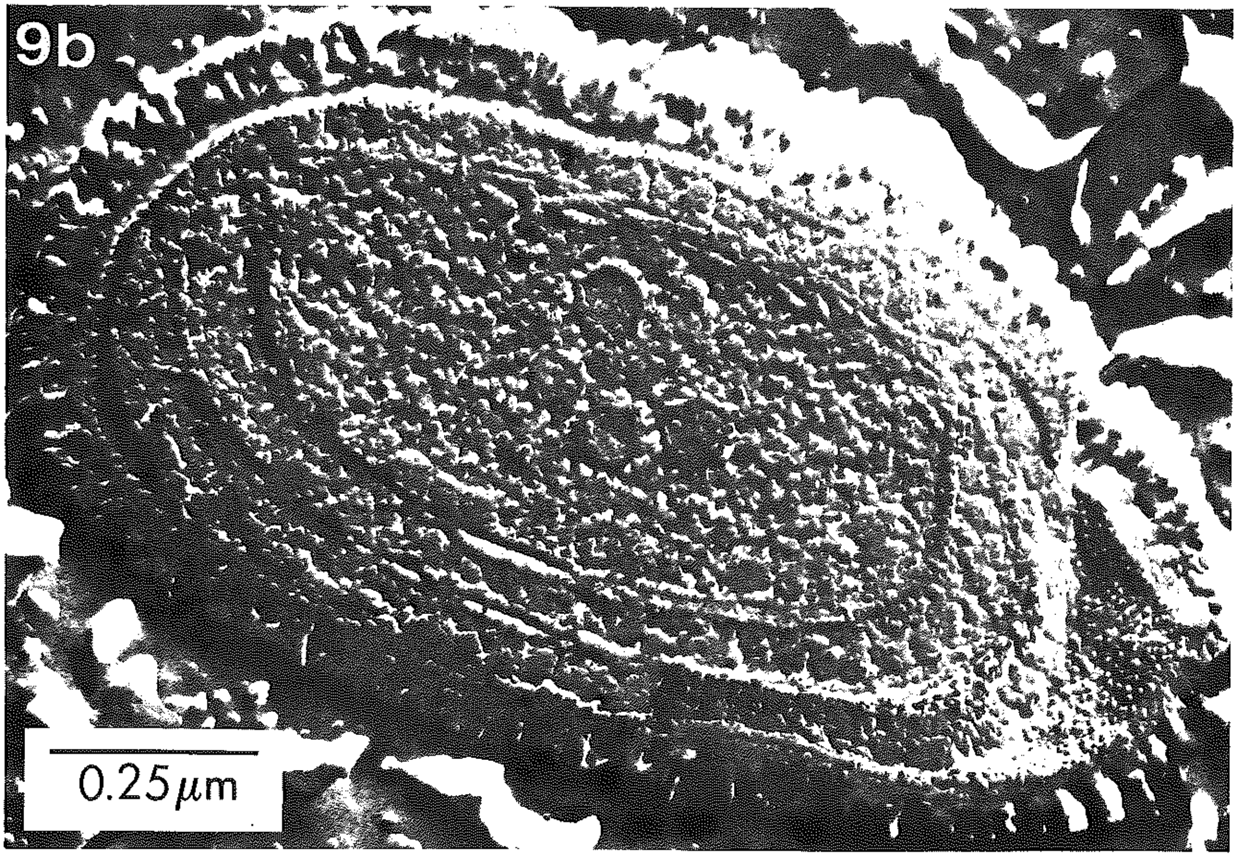

8
0.25 μm

Figure 9A&B: Transmission electron micrographs of freeze fractured and deeply etched *Synechococcus* strain WH 8113.

9a



9b



observed (Figures 7B and 9A,B).

High-intensity dark field microscopy: Synechococcus WH 8103 was examined by high-intensity dark field microscopy in the laboratory of Dr. Robert Macnab at Yale University. Portions of the spectrum of the high intensity illumination were eliminated, thereby preventing photoinhibition in this strain. Flagella or other extracellular structures were not seen in preparations of actively swimming cells, either as attached appendages or unattached in the medium. The high intesity illumination required by this form of microscopy produces a halo around the periphery of cell bodies making it impossible to see a short or closely appended flagella when attached to cells. However, preparations of flagellated cells commonly have detached flagellar filaments in the medium, so that such structures are still observed. The absence of free filaments or filament-like strucures did not completely rule out the possibility that short, presumably chemically labile structure(s) held close to the cell body might be present in Synechococcus.

Motility-dependent amplitude spectra: No motility-dependent amplitude spectra in images of actively swimming Synechococcus WH 8113 cells were observed by Dr. Berg. The high resolution of this technique and the ability to examine cells directly from the growth medium without further preparation makes the detection and measurement of the rotational frequency of even very short cellular projections possible. Since it had been established that Synechococcus motility involved both thrust and torque prior to the application of this technique (Waterbury et al., 1985; Appendix II), any extracellular projections not previously visualized should have been revealed by this method (H. C. Berg, personal communication). However, the spectra did not contain any peaks that could be attributed to either cell wobble or vibration, indicating cells lacked even short extracellular appendages that might not have been visualized by electron or high-intensity dark field microscopy.

Bead-cell suspension experiments: Cell suspensions were examined by phase contrast microscopy for evidence of any specific microcurrents associated with swimming motility. Experiments of this type furnished the data on which a model for gliding motility in Cytophaga sp. strain U67 is based (Lapidus and Berg, 1982).

With the exception of the hydroxylate polybead monodisperse (0.23 - 0.25 um) and the polystyrene beads (0.38 um), all beads tested either clumped and/or settled out of the cell suspension so that no cell-bead interactions could be observed. Although the hydroxylate beads stayed in suspension, no coherrent pattern of bead circulation was observed around the Synechococcus cell body. In contrast to the majority of beads that remained in suspension and exhibited only Brownian motion, a polystyrene bead adhered to an occasional cell and served as a fixed reference point as the cell body spun in a corkscrew-like fashion concomitant with translocation (Figure 10). This confirmed previous observations that both thrust and torque were involved in Synechococcus swimming motility.

Shearing experiments: Cells were subjected to shearing forces in a Waring blender to determine if swimming motility could be arrested by the removal of flagella or other extracellular processes. As discussed in Appendix II, motile Synechococcus strains could be blended for as long as 15 minutes without entirely eliminating swimming motility, while motility in flagellated bacteria was completely arrested within 10 to 15 seconds.

Cell fractionation procedures: Three approaches were taken in the analysis of cellular constituents in an effort to identify a protein(s) or localize a structure(s) unique to motile strains. These included: preparation of spheroplasts and cell wall-enriched fractions from motile (WH 8103 and WH 8113) and nonmotile (WH 7803 and WH 8018) strains, and a flagellar hook-basal body complex (HBB) isolation procedure applied to motile strain Synechococcus WH 8113. No unusual or suggestive features were observed in either the negatively stained preparations examined by TEM or the gel electrophoretic pattern of cell material collected by any of these techniques. However, it was of interest to note that the electrophoretic patterns of the cell wall-enriched fractions of the two motile strains were remarkably similar, while those for the two nonmotile strains were quite different from the motile strains as well as from one another (Figure 11).

Figure 10: Schematic of a Synechococcus cell A) with an attached polystyrene bead, as visualized by phase contrast microscopy. Arrows indicate direction of translocation. B) attached to the microscope slide and rotating about one pole in direction indicated by arrow.

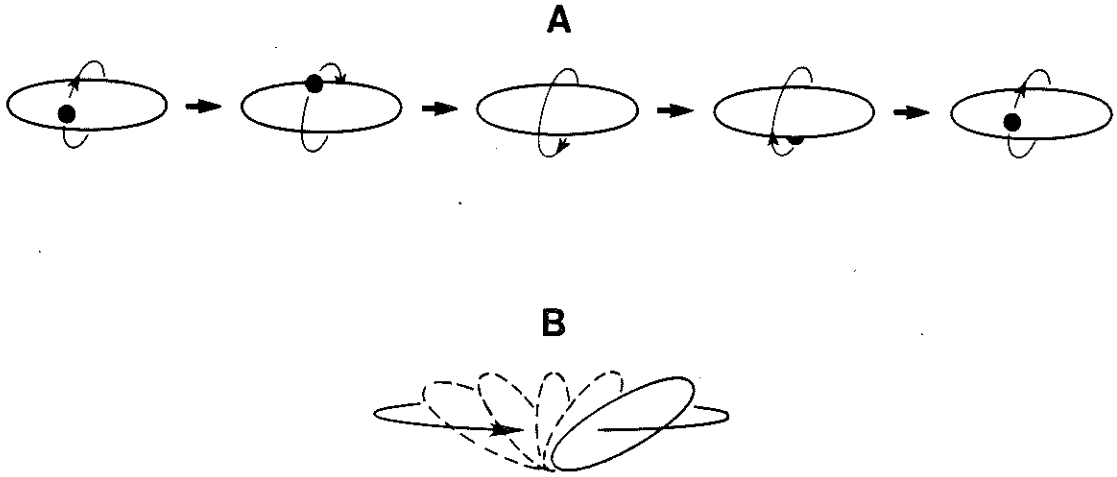
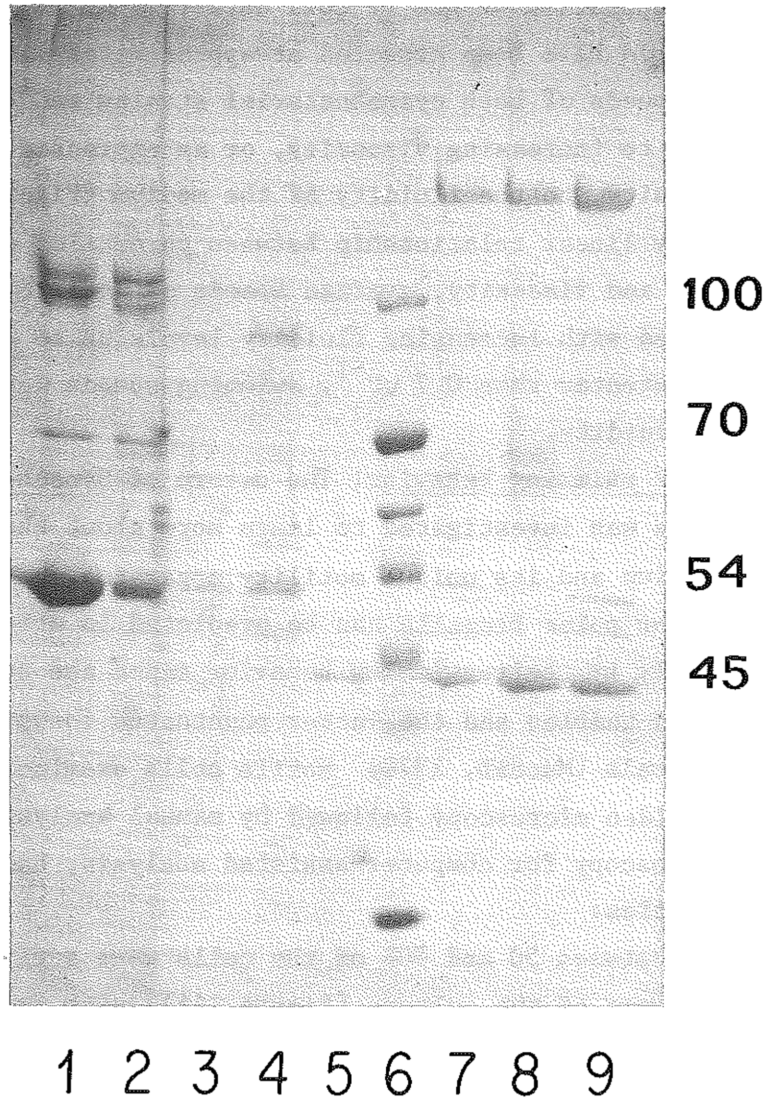

A
B

Figure 11: Polyacrylimide electrophoretic gel of Synechococcus cell-wall enriched fraction prepared as described by Resch and Gibson (1983). Lanes 1 and 2) Nonmotile strain WH 7803, Lanes 3 and 4) Nonmotile strain WH 8018, Lanes 5 and 7) Motile strain WH 8113, Lanes 8 and 9) Motile strain 8103, Lane 6) Molecular weight standard. Molecular weights (Kdalton) are denoted on the right hand margin.


100
70
54
45
1
2
3
4
5
6
7
8
9

Viscosity experiments: The relationship between motility and viscosity in the two Synechococcus strains and E. coli H102 was quite similar. Both strains demonstrated a decrease in the percent of cells remaining motile as viscosity increased (Figure 12). Motility was arrested in strain WH 8103 at 5 cP and at 6 to 6.5 cP in strain WH 8113, while extrapolation of the E. coli data (Figure 12) indicated that motility would have been arrested at approximately 10 cP.

Swimming speeds of both cyanobacterial strains and E. coli had a similar repsonse to increasing viscosity, or as expressed as the inverse of viscosity, to the fluidity of the medium (Figure 13). Unlike the nearly linear relationship between percent of cells remaining motile and viscosity, average speeds of motility in all bacteria increased with increasing fluidity levels up to 0.6 - 0.7 cP^{-1}; at values greater than 0.7 cP^{-1}, swimming speeds were independent of fluidity.

Analysis of swimming behavior: The nature of Synechococcus swimming patterns was investigated to learn more about the behavior of this cyanobacterium and its unique motility apparatus. The presence of peritrichous or polar flagella can be predicted in other swimming procaryotes simply by their swimming behavior since the former exhibits runs and tumbles and the latter punctuates forward movement with brief reversals (Macnab, 1978) Motile cells examined by either phase contrast video microscopy followed by manual analysis, or dark field video microscopy for computer-assisted analysis, had the following properties:

1. Between 50 and 80% of the cells were actively motile, depending on the age of the culture (Figure 14A), with fewer cells displaying motility as the culture entered the stationary phase of growth.

2. Swimming speeds ranged from 5 to 40 um s^{-1}, with an average of 13 to 15 um s^{-1} (Figure 14B). When only motile cells were considered, swimming speed was independent of culture age and light intensities at which cells were grown.

3. There was no predictable or repeatable pattern of motility in swimming paths of Synechococcus WH 8113 such as runs and

Figure 12: Percent of cells displaying swimming motility in response to increasing viscosity of surrounding medium. *Synechococcus* strain WH 8103 (x ,—), *Synechococcus* strain WH 8113 (● ,—), and *E. coli* strain H102 (○ ,---).

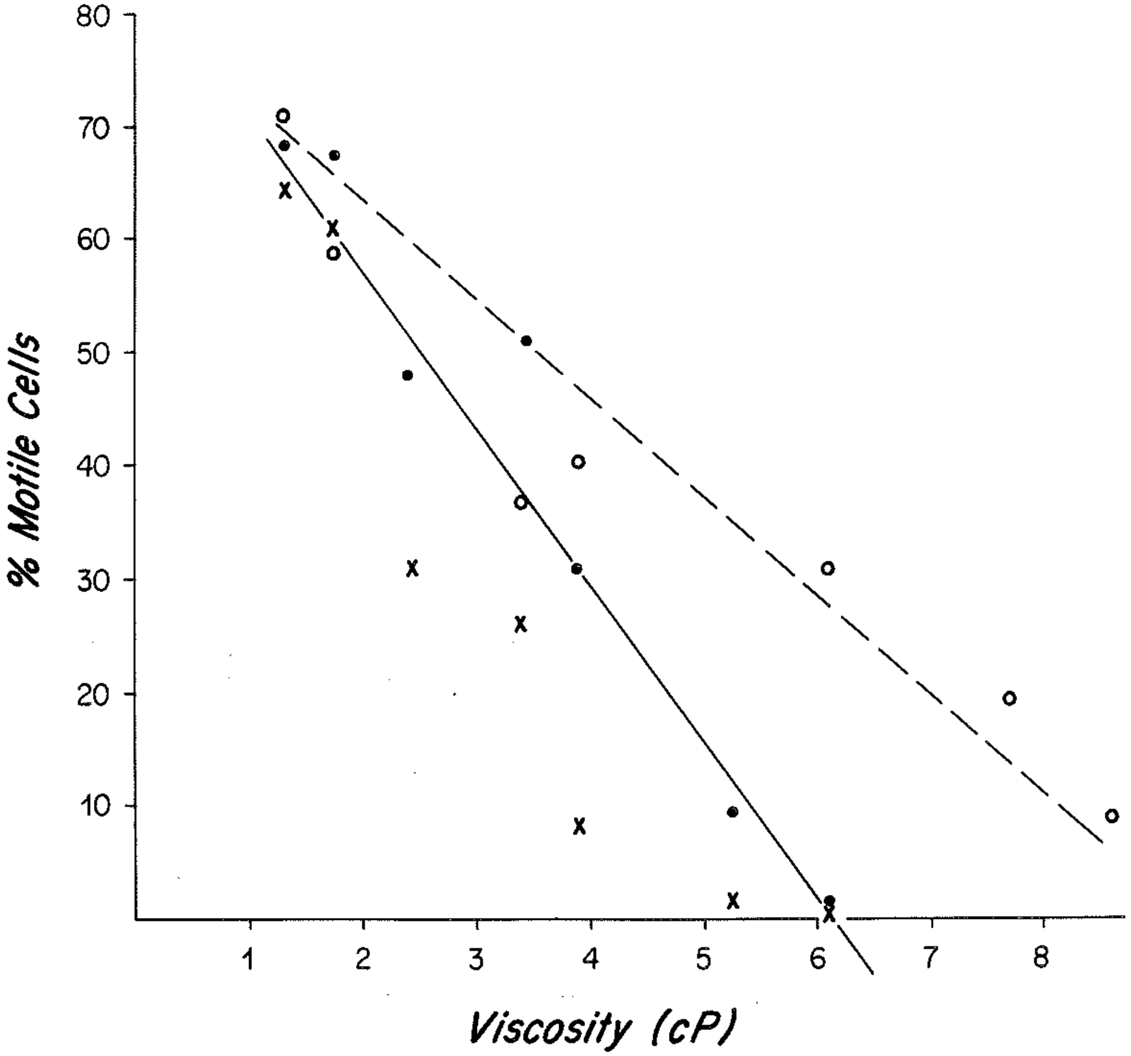

80
70
60
50
40
30
20
10
% Motile Cells
1
2
3
4
5
6
7
8
Viscosity (cP)

Figure 13: Average swimming speed of swimming motility in response to changes in fluidity of surrounding medium. *Synechococcus* strain WH 8103 (✕ ,—), *Synechococcus* strain WH 8113 (● ,—), and *E. coli* strain H102 (○ ,--).

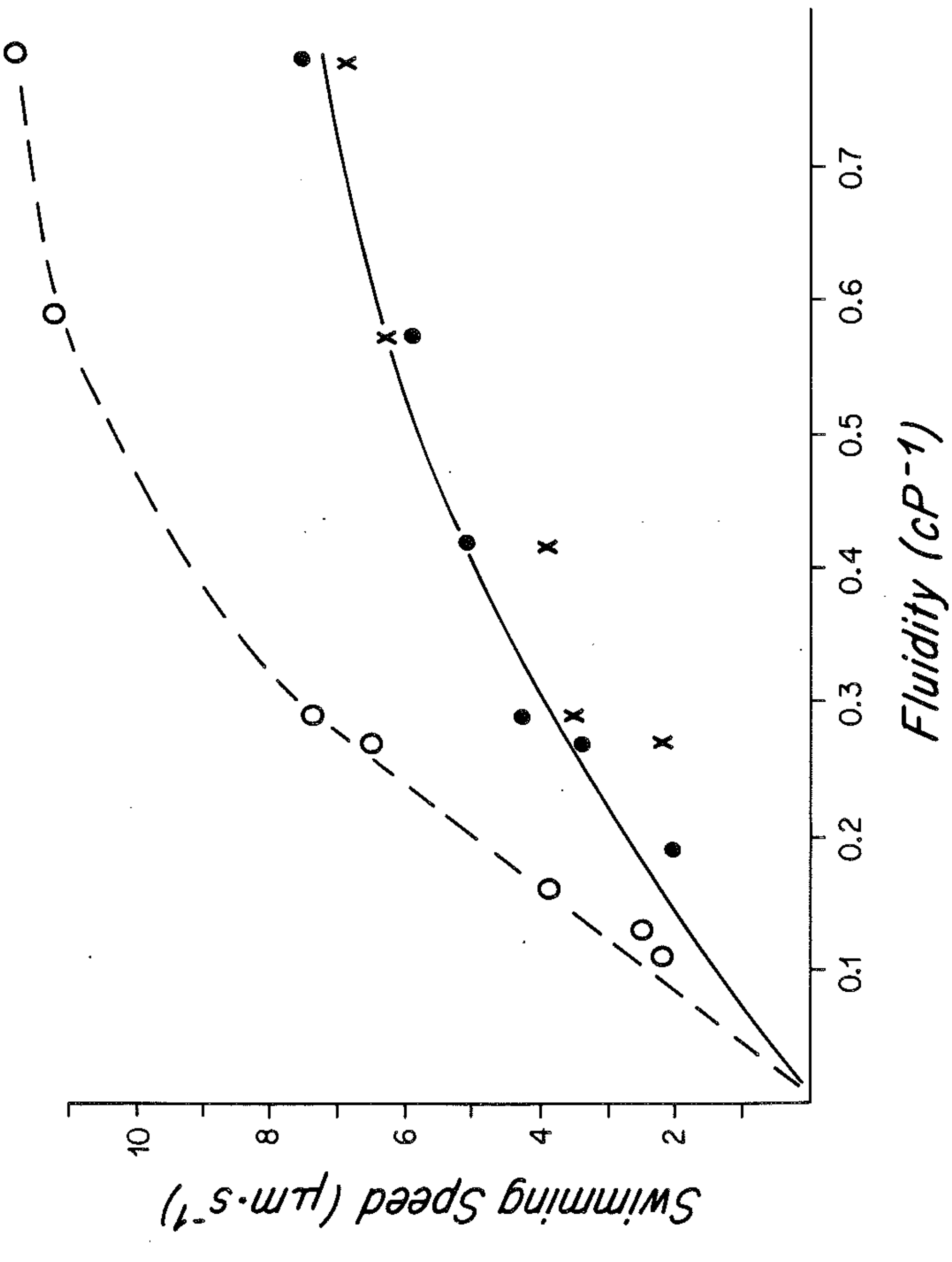
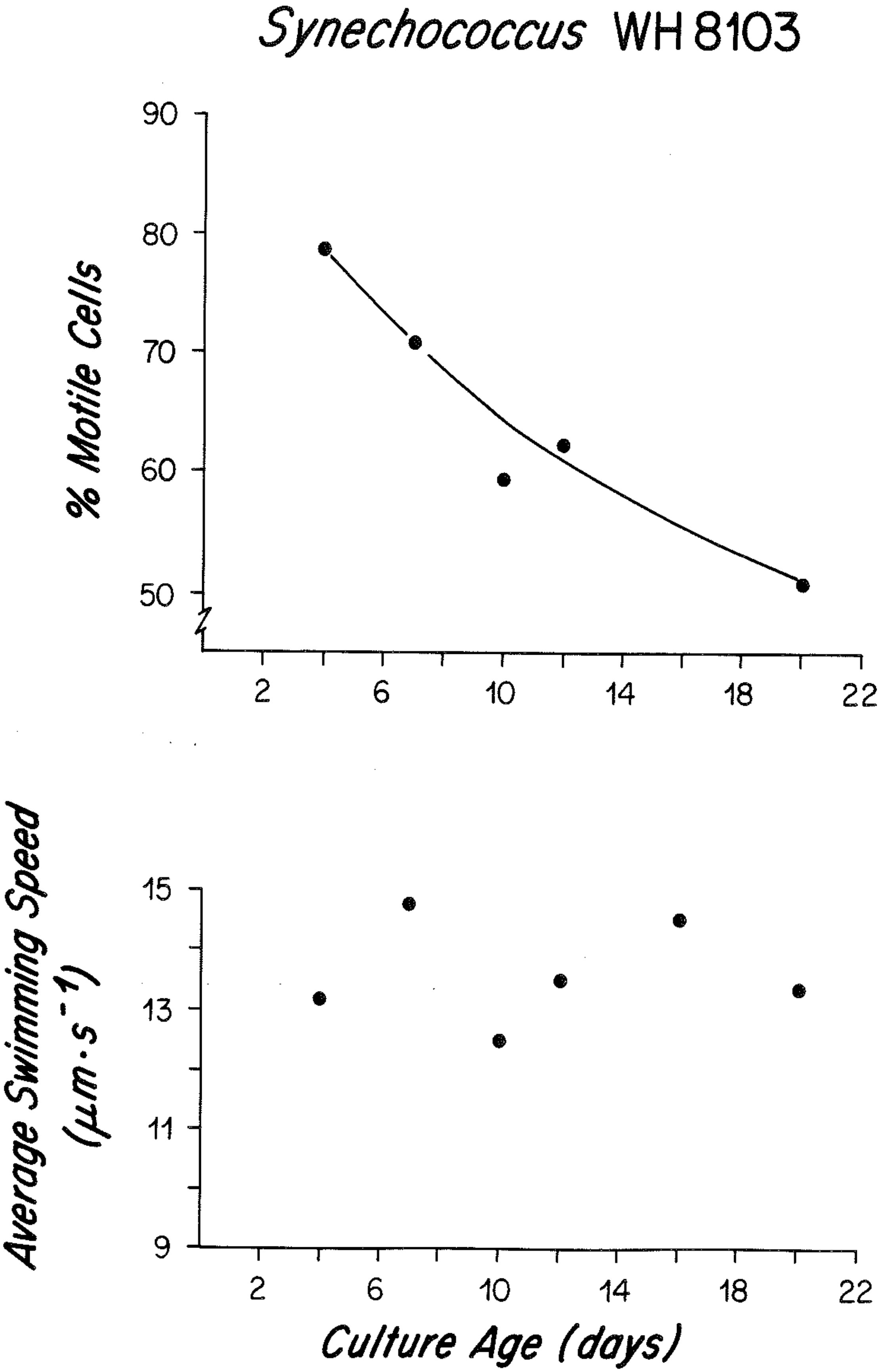

Swimming Speed (μm·s⁻¹)
10
8
6
4
2
Fluidity (cP-1)
0.1
0.2
0.3
0.4
0.5
0.6
0.7

Figure 14: Swimming motility of Synechococcus strain WH 8103 observed with increasing age of culture. A) Percent of cells displaying motility B) Average swimming speed of only those cells displaying motility.


Synechococcus WH 8103
% Motile Cells
90
80
70
60
50
2
6
10
14
18
22
Average Swimming Speed
(μm·s⁻¹)
15
13
11
9
2
6
10
14
18
22
Culture Age (days)

tumbles or forward-backward movements (Figure 15). Only cell morphology appeared to affect swimming behavior (Appendix II, Figure 2), since long rod-shaped cells swam in relatively straight paths whereas more coccoid cells tended to loop and spiral.

4. Individual cells were sometimes seen to rotate end over end at a rate of 3 to 5 revolutions per second.

5. Analysis of cells rotating about one pole when foruitously attached to the coverslip or slide and viewed in one plane of focus (Figure 10B), demonstrated that:

a. The rate of rotation was variable (0.25 - 2.5 rev s^{-1}), with an average rate of 1.0 rev s^{-1}.

b. 50% of the cells spun clockwise, 50% spun counterclockwise, but unlike flagellated bacteria, Synechococcus was never seen to change direction of rotation.

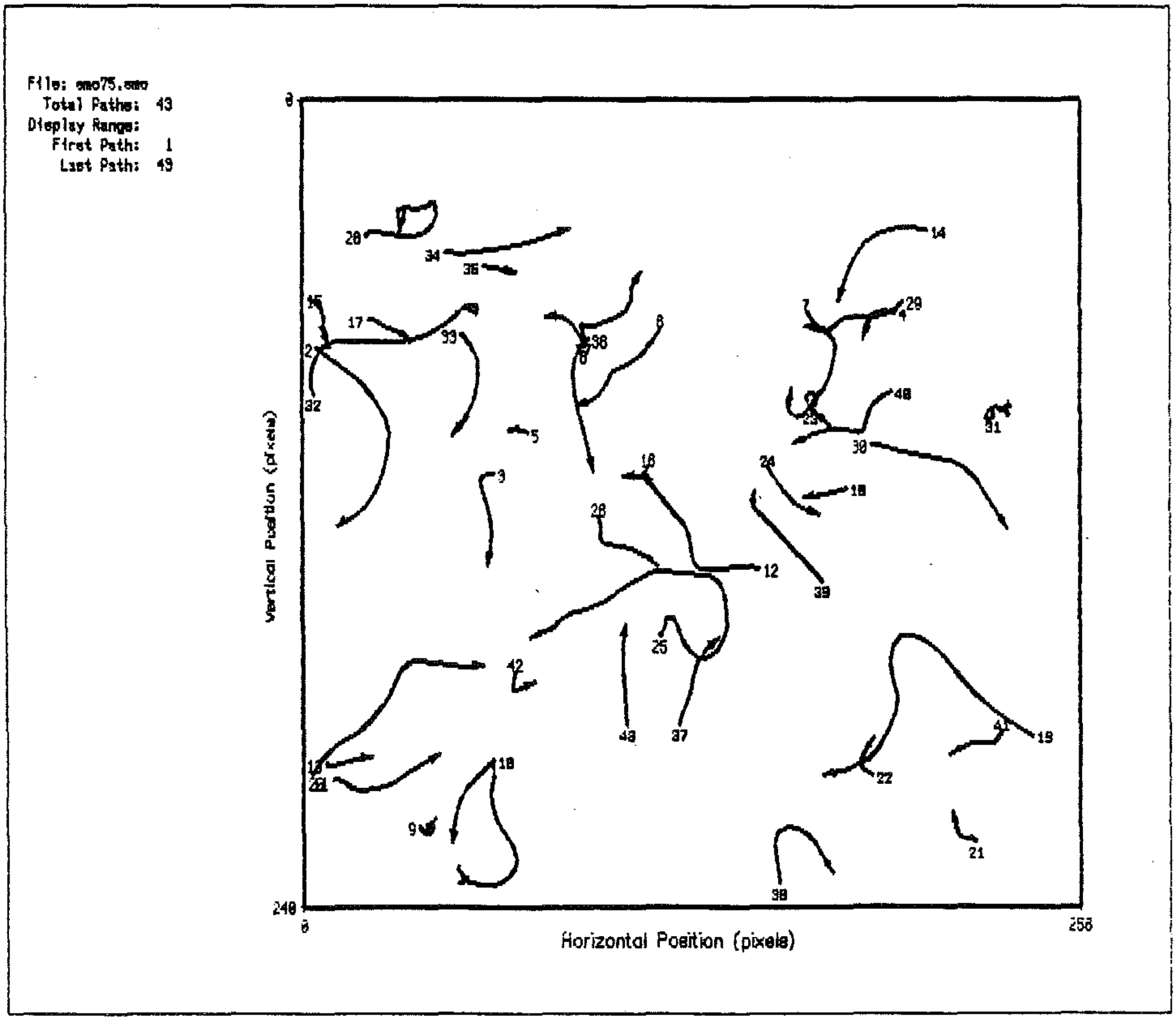
c. There was no significant difference in spinning rates or directions among cells at different phases of growth, between the three strains examined (WH 8011, WH 8103 and WH 8113), or when cells were suspended in artificial seawater buffer rather than seawater-based medium.

d. Rarely, a cell was seen to stop rotating and either exhibit vibration due to Brownian motion or swim away.

e. Dividing cells spun either in tandem or with the bottom cell stationary and the upper cell rotating about it. Such cells frequently completed division while spinning and the upper cell swam away, while the lower cell remained attached and continued to spin or was stationary.

f. There was no evidence that motile Synechococcus was capable of gliding motility since attached cells were never seen to move along the surface.

Figure 15: Computer-generated picture of swimming paths of _Synechococcus_ strain WH 8113 during a 10 sec interval. 1 um = 1.14 pixel.



```
File: emo75.emo
  Total Paths:  43
Display Range:
    First Path:   1
     Last Path:  43
```

Discussion

Analysis of the morphology, ultrastructure, biochemical and behavioral aspects of the swimming motility displayed by some oceanic Synechococcus isolates has demonstrated that this motility is mechanistically unique, since it is not driven by bacterial flagella or any other identifiable extracellular appendage. No extacellular structures were visualized in a variety of preparations by TEM or by high-intensity dark field microscopy in the laboratory of Dr. Robert Macnab. Although the possibility of short, chemically fragile appendages could not be ruled out by these methods, the lack of a discernible cell wobble or vibration, as tested by Dr. Howard Berg, indicated that motile Synechococcus did not possess such structures. This is corroborated by the absence of discrete circulation of microspheres around the cell body. TEM and gel electrophoretic analysis of spheroplasts, cell wall-enriched fractions from motile and nonmotile strains, and the cell material collected following the application of a flagellar hook-basal body complex isolation technique to a motile Synechecoccus strain provided no evidence of a structure or protein unique to the motile strains. Cells tolerated shearing forces and continued to swim long after motility in flagellated cells was arrested due to deflagellation. Finally, since swimming patterns are indicative of the type of flagellation in other swimming bacteria, the random swimming paths associated with Synechococcus motility was consistent with the lack of bacterial flagella.

The widespread distribution of motile Synechococcus strains indicates that this novel form of procaryotic motility may represent an important behaviorial characteristic of this cyanobacterium. Although these isoloates shared a number of important physiological and morphological traits with nonmotile strains, the results of cell fractionation procedures suggested that it is unlikely that motile strains are simple genetic variants of nonmotile strains (Figure 11).

Unlike other cyanobacteria, which are capable only of surface-associated gliding at average speeds of 1 to 3 um s^{-1}, motile Synechococcus strains were unable to move across solid substrates.

Attached cells were never seen to creep along the glass surface. Indeed, any motility associated with such cells required detachment followed by swimming.

Although swimming in these *Synechococcus* strains was executed without flagella, two important similarities to flagellar-mediated motility were revealed. First, *Synechococcus* motility involved both thrust (forward movement) and torque (cell rotation). The presence of torque was easily observed by the rotation of a cell body when attached at one pole to the coverslip or slide. Although direction of rotation in such cells was never seen to reverse, a fundamental feature of tethered, rotating flagellated cells, the frequency of *Synechococcus* cells spinning CW versus CCW was evenly divided. This would be predicted if the cell body were rotating as a single unit and the motility apparatus were incapable of reversing direction of rotation. In this case, half the cells would be expected to attach at the "front" end and spin in one direction, while the other half of the cells would attach at the "rear" end and spin in the opposite direction.

The other significant feature shared by flagellated bacteria and motile *Synechococcus* was the relationship between cell motility and medium fluidity. This has been examined in a number of flagellated bacteria, including those with polar, bipolar and peritrichous flagella (Schneider and Doetsch, 1974; Berg and Turner, 1979; Manson et al., 1980; Lowe et al., 1987) as well as in endoflagellated spirochetes (Kaiser and Doetsch, 1975; Greenberg and Canale-Parola, 1977, Berg and Turner, 1979). When tested in a uniformly viscous medium, such as a Ficoll-based solution, the angular velocities of tethered flagellated cells is directly proportional to fluidity (Berg and Turner, 1979; Manson et al., 1980), indicating the torque of the motor is constant and independent of dynamic load. In addition, the bundle frequency (indicative of the speed of the flagellar motor relative to the cell body) and swimming speeds of *Streptococcus* sp. and *E. coli* increase with fluidity up to about 0.6 cP^{-1}; at greater fluidity values these motility parameters remain unchanged (Lowe et al., 1987). The results obtained with *E. coli* H102 and motile

Synechococcus strains WH 8103 and WH 8113 in this study (Figure 13) are in good agreement with these data suggesting that the motility apparatus in this cyanobacterium also operated at constant torque, ie. the work done by the motility apparatus per unit time was constant.

When a motile cell exhibiting both thrust and torque moves at a constant velocity, these forces must be at equilibrium so that the net force acting on the cell is zero (Berg, 1983). The small size of a bacterium imparts a low Reynolds number (Purcell, 1977), thus the thrust associated with cell translocation is balanced by the resultant viscous drag. Although this might be a means by which the thrust involved in Synechococcus motility was held at equilibrium, it is not clear how this thrust was generated. As a first approximation, two lines of evidence suggest that the motility apparatus in this cyanobacterium might be analogous to the cytoplasmic membrane-bound rotary motor that drives flagellar-mediated motility: torque was coupled to translocation and this torque was constant. However, the mechanism by which torque was maintained and balanced is not known. In flagellar-mediated swimming, the torque generated by filament rotation is at equilibrium with that produced by counterrotation of the cell body (Berg, 1983). In spirochetes, viscous shear at the surface of the external sheath allows it to rotate about the counterrotating periplasmic cylinder so that torque is balanced (Berg, 1976). If Synechococcus were to use the circumferential viscous shear at the surface of the cell body to sustain rotation, the simplest model for balancing torque might be one analogous to that of spirochetes, invoking a counterrotating inner cell wall or membrane, however such a hypothesis remains conjecture.

In searching for flagella in motile strains of marine Synechococcus, it has been confirmed that these isolates are not only unique among cyanobacteria, since they display swimming rather than gliding motility, but more importantly, represent a novel form of procaryotic motility. Although the structural mechanism responsible for the observed motility awaits elicidation, lack of bacterial flagella will remain a fundamental feature of this form of motility.

CHAPTER 3

THE ENERGETICS OF *SYNECHOCOCCUS* SWIMMING MOTILITY

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Sodium-Coupled Motility in a Swimming Cyanobacterium†

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The energetics of motility in *Synechococcus* strain WH8113 were studied to understand the unique nonflagellar swimming of this cyanobacterium. There was a specific sodium requirement for motility such that cells were immotile below 10 mM external sodium and cell speed increased with increasing sodium levels above 10 mM to a maximum of about 15 μm/s at 150 to 250 mM sodium. The sodium motive force increased similarly with increasing external sodium from −120 to −165 mV, but other energetic parameters including proton motive force, electrical potential, the proton diffusion gradient, and the sodium diffusion gradient did not show such a correlation. Over a range of external sodium concentrations, cell speed was greater in alkaline environments than in neutral or acidic environments. Monensin and carbonyl cyanide *m*-chlorophenylhydrazone inhibited motility and affected components of sodium motive force but did not affect ATP levels. Cells were motile when incubated with 3-(3,4-dichlorophenyl)-1,1-dimethylurea and arsenate, which decreased cellular ATP to about 2% of control values. The results of this investigation are consistent with the conclusion that the direct source of energy for *Synechococcus* motility is a sodium motive force and that below a threshold of about −100 mV, cells are immotile.

Among procaryotes there are two principal types of motility: swimming through liquids and surface-associated gliding. Swimming, common to many eubacteria and archaebacteria, is mediated by the rotation of flagellar filaments, resulting in swimming speeds ranging from 10 to 100 μm/s (19). The structure and function of bacterial flagella are reasonably well understood (18, 19); however, although there are a variety of models for gliding, the cellular apparatus responsible has not been identified (2, 15, 16, 23).

Motility systems in most procaryotes are driven by a proton motive force (6, 7, 9, 17, 20, 21). The reported exceptions are the flagellated alkalophilic *Bacillus* strain YN-1 (10, 11) and a marine vibrio (3, 4), both of which couple motility to a sodium motive force. In addition, it has been suggested that sodium motive force provides the energy for the gliding motility in the marine cyanobacterium *Oscillatoria brevis* (3). The examples of sodium motive force-dependent motility may be considered analogous to those of proton motive force-dependent motility; the former couples force generation to sodium translocation, and the latter couples it to protons. To date, only eucaryotic motility systems have been shown to utilize the energy from ATP hydrolysis directly (18).

Recently several strains of a unicellular cyanobacterium belonging to the genus *Synechococcus* (as defined by Rippka et al. [24]) were isolated from the open ocean and observed to be capable of swimming motility. These strains are novel in two respects: they are the first cyanobacteria reported to display swimming, and their motility does not appear to involve bacterial flagella. In fact, no organelle of motility has yet been discovered (28). Fundamental to an understanding of any motility system is the determination of the source of energy required to drive that system. In this paper we report an investigation of the energetics of the apparently novel motility exhibited by *Synechococcus* strain WH8113.

MATERIALS AND METHODS

Bacterial strain and culture conditions. The organism used was *Synechococcus* strain WH8113 (28). The culture medium consisted of 10 mM $NaNO_3$, 100 μM $Na_2CO_3 \cdot H_2O$, 100 μM K_2HPO_4, 10 μM disodium EDTA, vitamin B_{12} (0.1 μg/ml) and trace metals (22) (1 ml/liter of medium) in 75% aged, filtered seawater–25% double-distilled water. Cultures were grown in constant light (10 microeinsteins/m^2 per s) at 25°C.

Preparation of cell suspensions. Cells in a mid-logarithmic phase of growth were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C. The cells were then suspended in a salts solution containing 30 mM $MgSO_4 \cdot 7H_2O$, 20 mM $MgCl_2 \cdot 6H_2O$, 10 mM $CaCl_2 \cdot 2H_2O$, 10 mM KCl, 2 mM $NaHCO_3$, and the desired concentration of NaCl. Unless otherwise specified, the NaCl concentration was 185 mM and the pH was adjusted to 8.4 with HCl or KOH. In these suspensions, 60 to 80% of the cells were motile for at least 4 h at 25°C. For motility analyses and measurements of intracellular ATP levels, cell densities were 1×10^8 to 4×10^8 cells per ml. For measurements of intracellular volume, pH, and sodium concentrations or measurements of membrane potential, cell suspensions were washed once in the salts solution described above with the indicated sodium concentration and pH and adjusted to 0.5 mg of protein per ml (approximately 5×10^{10} cells per ml). In all cases, cell suspensions were incubated at a light intensity of 20 to 30 microeinsteins/m^2 per s.

Analysis of motility. Cells suspended in a small drop were placed on a cover slip and observed with an inverted darkfield microscope fitted with a 40× neofluar objective (Carl Zeiss, Inc.). The microscope was coupled to a high-resolution video camera (closed circuit video camera model TC 1005; RCA), and images were recorded with a PV8950 video recorder (Panasonic). Cell motility was then analyzed by automated motion analysis (Motion Analysis Systems,

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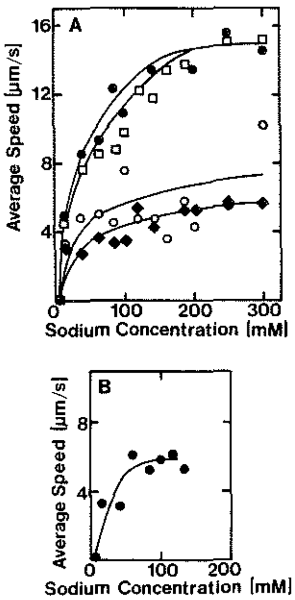

FIG. 1. Effect of extracellular sodium chloride on the swimming speed of *Synechococcus* strain WH8113. (A) Cells were suspended in salts solution containing the indicated concentrations of sodium chloride at pH 6.0 (◆), pH 7.0 (○), pH 8.4 (●), or pH 9.0 (□). (B) Response of cells suspended in salts solution without added sodium chloride to the addition of increasing concentrations of sodium chloride at pH 8.3. Sodium chloride is given as final concentration.

Inc., Santa Rosa, Calif.) in a manner similar to that described by Sundberg et al. (26).

Measurement of intracellular volume. Intracellular volume was measured as described previously (12), except that a receiving layer was omitted and cells were double-labeled with [^{3}H]water (1 μCi/ml of cell suspension; specific activity, 1 mCi/g) and [^{14}C]sorbitol (0.1 μCi/ml of cell suspension; specific activity, 346 mCi/mmol). A 200-μl portion of cell suspension was centrifuged through silicone oil, and the amount of each radiolabel in the buffer above the silicone oil and in the pellet below was determined by standard scintillation counting techniques. Intracellular volumes were calculated as described by Rottenberg (25). Volumes were determined for each experimental condition used (six replicates per experiment) and in fact were similar in all cases (3.0 μl/mg of protein; standard error, 0.09 μl/mg).

Intracellular pH measurements and calculation of ΔpH. Intracellular pH was determined by the same procedure used in the measurement of intracellular volume, except that the partitioning of [^{14}C]benzoic acid (0.3 μCi/ml of cell suspension; specific activity, 21.2 mCi/mmol) was measured. When inhibitors were included, cells were pretreated for 5 min at the indicated concentration prior to addition of [^{14}C]benzoic acid. Intracellular pH was calculated after correcting for label associated with the extracellular matrix as described by Rottenberg (25). ΔpH was calculated as the difference between internal and external pH.

Measurement of $\Delta\Psi$. The probe [^{3}H]triphenylphosphonium ([^{3}H]TPP$^+$, 1 μCi/ml of cell suspension; 35.5 mCi/mmol) was used to measure the transmembrane electrical potential ($\Delta\Psi$). Techniques similar to those described above were used to incubate cells in the presence of probe for 30 min and separate them from the suspending buffer by centrifugation; however, the centrifugation was not through silicone oil. Because TPP$^+$ tends to stick to the walls of polycarbonate centrifuge tubes, centrifugation was in glass capillary test tubes machined to fit inside 1.5-ml microcentrifuge tubes. The probe concentration in the cell-free supernatant fluid was measured, and the data were used to calculate cellular probe concentration. Nonspecific probe binding, found to be negligible, was determined by measuring [^{3}H]TPP$^+$ associated with cells treated with 5% butanol as described by Kashket et al. (14). The $\Delta\Psi$ was calculated by using the Nernst equation (25).

Determination of ΔpNa$^+$. After equilibration in the indicated buffer, 300 μl of cell suspension was centrifuged through 200 μl of silicone oil. For each sample the extracellular and intracellular sodium concentrations were determined by the following procedure. Supernatant fluid (5 μl) and the pellet remaining in the bottom of the microcentrifuge tube (sliced off with a razor blade) were incubated for 10 min at 100°C in separate acid-rinsed screw-cap borosilicate tubes each containing 1 ml of 0.1 N HNO_2. The tubes were then cooled, volumes were brought to 5 ml with double-distilled water, and sodium was measured by atomic absorption spectrophotometry (model 403 spectrophotometer; The Perkin-Elmer Corp., Norwalk, Conn.). Control experiments demonstrated that the tips of the microcentrifuge tubes and the silicone oil contributed insignificantly to the measured sodium levels. After correction for extracellular sodium contamination in the pellet, the measured internal and external sodium concentrations were used to calculate the transmembrane sodium gradient (ΔpNa$^+$) by using the Nernst equation (25).

Measurement of cellular ATP. ATP was extracted from cells by previously described techniques (9), and the extracted ATP was measured with firefly luciferase (Analytical Luminescence Laboratories, Inc., San Diego, Calif.). Luminescence was monitored with an ATP photometer (model TD-20e; Turner Designs, Mountain View, Calif.). Cellular ATP was normalized to chlorophyll *a* as previously described (24).

Chemicals. All of the radiolabeled compounds were purchased from New England Nuclear Corp., Boston, Mass. The inhibitor of oxygenic photosynthesis, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and the ionophores monensin, valinomycin, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical Co., St. Louis, Mo., and *p*-trifluoromethoxyphenylhydrozone (FCCP) was purchased from ICN Biochemicals, Cleveland, Ohio. The ionophores were dissolved in ethanol, and in cell suspensions containing these chemicals the ethanol concentration did not exceed 0.5%. This concentration of ethanol did not affect motility or the bioenergetic parameters measured. Furthermore, the ionophore concentrations used did not appreciably affect photosynthesis, as indicated by measuring rates of oxygen evolution. Oxygen concentrations in cell suspensions were monitored by using a Clark oxygen electrode (9).

RESULTS

Effects of sodium and pH on motility. The motility of *Synechococcus* strain WH8113 was affected by both external sodium chloride concentration and external pH (Fig. 1A).

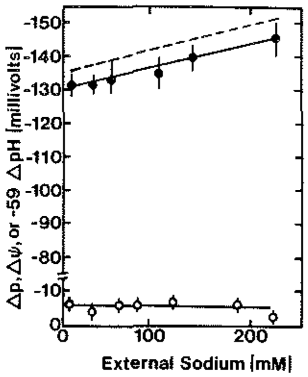

FIG. 2. Effect of external sodium chloride concentration on ΔΨ (●), −59ΔpH (O), and proton motive force (---) in *Synechococcus* strain WH8113. For ΔΨ and ΔpH, each point represents the average of three separate experiments, each with six replicates, and the marker bars represent standard errors. The proton motive force is the sum of ΔΨ and −59ΔpH.

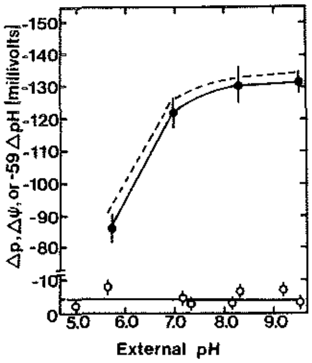

FIG. 3. Effect of external pH on ΔΨ (●), −59ΔpH (O), and proton motive force (---) in *Synechococcus* strain WH8113. For ΔΨ and −59ΔpH, each point represents the average of three separate experiments, each with six replicates, and the marker bars represent the standard errors. The proton motive force is the sum of ΔΨ and −59ΔpH.

The average speed of cells was directly related to sodium chloride concentration over a pH range of 6.0 to 9.0. Cell motility was more vigorous at high pH than at neutral or low pH, over a range of external sodium chloride concentrations (Fig. 1A). Furthermore, when sodium chloride was added to suspensions of immotile cells incubated in a solution containing approximately 5 mM sodium, motility was partially restored (Fig. 1B). Motility was observed within 20 s of sodium addition, and average cell speed remained constant over a 30-min period. Partial restoration of motility by sodium chloride addition indicated that the motility apparatus and cell integrity were not irreversibly damaged by short exposure to very low concentrations of sodium chloride.

The specificity of the sodium chloride requirement was determined by ion replacement experiments. When sodium chloride was replaced with potassium chloride, cesium chloride, rubidium chloride, lithium chloride, ammonium chloride, calcium chloride, or magnesium chloride at 60 or 120 mM, cells were immotile. Cell motility in the presence of sodium nitrate or sodium sulfate (120 mM) was indistinguishable from motility in the presence of sodium chloride (120 to 300 mM). These results indicate that there is a specific sodium requirement for motility.

Relationship between extracellular sodium chloride concentration, pH, and proton motive force. The two components of proton motive force, ΔpH and ΔΨ were measured under various external NaCl concentrations (Fig. 2) and pHs (Fig. 3). The ΔpH was independent of external sodium chloride concentration and remained less than −10 mV over the range of external sodium chloride concentrations tested. The ΔΨ increased from about −130 mV at 5 mM external sodium chloride to −140 mV at 200 mM. The resulting proton motive force varied from about −140 mV to −150 mV as the external sodium chloride was increased (Fig. 2).

The internal pH reflected the external pH such that ΔpH was negligible (ca. 0.1 pH unit) over a pH range of 5.1 to 9.3, at an external sodium chloride concentration of 185 mM (Fig. 3). The inability to maintain a constant internal pH over a range of external pH values is not unique to *Synechococcus* strain WH8113; recent investigations have shown a similar pattern for certain other bacteria (5, 8). Under alkaline conditions (pH 7.5 to 9.5) ΔΨ was maintained at −130 ± 5 mV, but decreased under more acidic conditions (Fig. 3). The lack of a significant ΔpH resulted in a proton motive force that was composed almost entirely of ΔΨ, regardless of external sodium chloride concentration or pH.

ΔpNa$^+$ and sodium motive force. For *Synechococcus* strain WH8113 there was a linear relationship between extracellular and intracellular sodium concentrations; however, the increases in internal sodium levels were lower than the increases in external concentrations (Fig. 4). Thus ΔpNa$^+$ became larger as external sodium concentrations were increased, to a maximum of −25 ± 5 mV at about 150 mM external sodium (Fig. 5). Because ΔΨ remained relatively constant over the range of sodium concentrations tested (Fig. 2), the sodium motive force, the sum of ΔpNa$^+$ and ΔΨ, increased with increasing external sodium (Fig. 5).

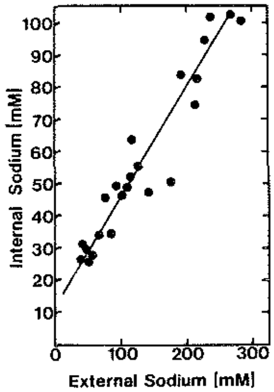

FIG. 4. Relationship between external sodium concentrations and internal concentrations. Each point represents the mean of six replicates, and the slope was generated by a linear regression analysis ($r^2 = 0.91$).

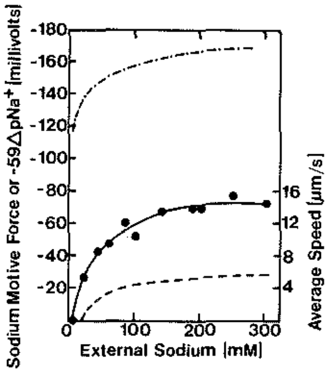

FIG. 5. Relationship between external sodium, ΔpNa^+, sodium motive force, and motility of *Synechococcus* strain WH8113. Sodium motive force (-·-·-) $-59\Delta pNa^+$ (---), and motility (●) are given as a function of external sodium concentration. $-59\Delta pNa^+$ was calculated from the linear regression in Fig. 4. Sodium motive force was calculated from the $\Delta\Psi$ values in Fig. 2 and the $-59\Delta pNa^+$ values in this figure.

Relationship between motility and cellular ATP pools. The role of ATP in *Synechococcus* motility was evaluated in several ways. When cells were treated with DCMU and sodium arsenate (60 mM) following growth in a medium without added phosphate, ATP levels were approximately 2% of the control level. Nevertheless, the cells were vigorously motile (Table 1). When cells were suspended in the absence of added sodium chloride, ATP levels were similar to those in cells at higher sodium chloride concentration, yet motility was absent (Table 1) Both the proton ionophore CCCP and the ionophore monensin, which effects an electroneutral exchange of sodium ions and protons, abolished motility but had no appreciable effect on intracellular ATP pools (Table 1). From these data, it is clear that motility is not correlated with intracellular ATP levels.

Although CCCP and monensin did not alter intracellular ATP levels, these ionophores did affect components of the sodium motive force. This suggested a relationship between sodium motive force and motility (see Discussion). CCCP reduced $\Delta\Psi$ and ΔpNa^+, and hence the total sodium motive force, roughly by one-half. Monensin only slightly affected $\Delta\Psi$ but essentially collapsed ΔpNa^+ (Table 2). Results with FCCP were virtually identical to those with CCCP. Valinomycin in the presence of 200 mM KCl and gramicidin had no effect on $\Delta\Psi$, ΔpNa^+, or motility (data not shown).

DISCUSSION

This report indicates that a sodium motive force is the source of energy directly coupled to *Synechococcus* motility. Of several possible energy sources considered, only sodium motive force correlated well with motility. It seems unlikely that the energy from ATP hydrolysis is coupled to motility directly. When treated with arsenate and DCMU (to block photosystem II), cells contained little ATP but remained motile. Motility was arrested in the presence of CCCP or monensin or in the absence of sodium chloride; however, there was no significant decrease in intracellular ATP levels (Table 1).

Motility in most other bacteria that have been studied is driven by a proton motive force (6, 7, 9, 17, 20, 21); however, this does not appear to be the case for *Synechococcus* strain WH8113. Motility in this cyanobacterium exhibited a specific requirement for sodium. At external sodium concentrations less than 10 mM, cells were immotile and swimming speed increased with increasing extracellular sodium until a maximum was attained at 150 to 250 mM sodium (Fig. 1). This is in contrast to the proton motive force and its components, $\Delta\Psi$ and ΔpH, which remained relatively constant over the range of extracellular sodium concentrations tested (Fig. 2). The ΔpH was minor under all conditions (Fig. 2 and 3), and as for certain other bacteria (5, 8), internal pH varied with external pH. It is likely that this is of little consequence for *Synechococcus* strain WH8113, since the marine environment is well buffered at approximately pH 8.3.

The presence of a specific sodium requirement for motility and the lack of any correlation between swimming speed and intracellular ATP pools (Table 1) or proton motive force (Fig. 2) suggested that a sodium motive force might drive *Synechococcus* motility. Indeed, sodium motive force with respect to external sodium concentration was closely correlated to the average swimming speed (Fig. 5). Although $\Delta\Psi$ was the major component of the sodium motive force, its increase with increasing external sodium concentration was slight and did not correlate with changes in swimming speed (Fig. 2 and 5).

As an alternative to sodium motive force, could ΔpNa^+ specifically be the energy source for motility? We believe this to be unlikely for the following reasons. At low external sodium concentrations, cells are motile but ΔpNa^+ is minimal. For example, at 20 mM sodium the average cell speed was nearly one-third maximum (Fig. 1), but ΔpNa^+ was negligible (Fig. 5). In the presence of CCCP cells were immotile, yet ΔpNa^+ was relatively high (−12 mV). CCCP substantially dissipated $\Delta\Psi$ (Table 2), and this may have

TABLE 1. Effects of sodium chloride and metabolic inhibitors on ATP pools and motility in *Synechococcus* strain WH8113

Addition to cell suspension[a]	ATP concn (nmol/mg of chlorophyll *a*)	% Motile
Sodium chloride (185 mM)	4.8	70
None	4.8	0
Sodium arsenate (60 mM) + sodium chloride (125 mM) + DCMU[b]	0.1	70
CCCP (20 μM) + sodium chloride (185 mM)[c]	3.6	0
Monensin (15 μM) + sodium chloride (185 mM)[d]	4.5	0

[a] Cells were suspended in pH 8.4 salts solution (described in Materials and Methods) containing the concentration of sodium chloride indicated.

[b] The growth medium for the cells used in these experiments was prepared as described in Materials and Methods, except that K_2HPO_4 was omitted.

[c] Data were obtained after an incubation period of 3 min in the presence of CCCP.

[d] Data were obtained after an incubation period of 2 min in the presence of monensin.

TABLE 2. Effects of CCCP and monensin on sodium motive force, $\Delta\Psi$, and ΔpNa^+

Inhibitor	$\Delta\Psi$ (mV)	ΔpNa^+ (mV)	Sodium motive force (mV)
None	−140	−21	−160
CCCP (20 μM)	−75	−12	−87
Monensin (15 μM)	−135	−4	−140

effected a decrease in sodium motive force to a level below the threshold for motility.

The best interpretation of the data seems to be that the sodium motive force is the energy source for *Synechococcus* motility and that there is a threshold value below which cells are immotile. The best-studied sodium motive force-driven motility system, that of the flagellated alkalophile *Bacillus* strain YN-1 (10, 11), also has a high threshold value for motility (ca. −100 mV). This is much higher than the threshold values of proton motive force for the motility of other flagellated bacteria. It was suggested that this may be related to the difference in proton and sodium ion sizes (11). Our results are in general agreement with those for *Bacillus* strain YN-1. There was a specific sodium requirement for motility, changes in ATP levels and motility did not correlate, cells were more vigorously motile at pH values above neutrality, motility did not correlate with proton motive force, and monensin inhibited motility. The monensin effect does not have a clear bioenergetic basis (Table 2). Perhaps, as suggested for *Bacillus* YN-1, monensin inhibits motility by affecting the flux of sodium (11), perhaps it interacts with a component of the motility apparatus directly, or perhaps it affects motility by altering intracellular pH. For *Bacillus* strain YN-1, however, valinomycin and potassium chloride were used to dissipate $\Delta\Psi$ independently of ΔpNa^+. This enabled an evaluation of the role of ΔpNa^+ and $\Delta\Psi$ in this gram-positive bacterium, and it provided substantial evidence that sodium motive force was coupled directly to motility (10). Unfortunately, as is the case with many gram-negative bacteria (13), valinomycin was inactive in *Synechococcus* strains.

The motile *Synechococcus* strains are members of the marine bacterioplankton (27); as such they exist in an alkaline environment with a relatively high sodium concentration (ca. 500 mM). Thus it is not surprising that, as for other alkalophilic bacteria, motility is sodium dependent (3, 4, 10, 11). The most probable source of energy for *Synechococcus* motility is the sodium motive force, although the relative contribution of the two components of the sodium motive force, the sodium diffusion gradient (ΔpNa^+) and the electrical potential ($\Delta\Psi$) remains unclear. The role of sodium motive force in *Synechococcus* motility indicates that the device for conversion of metabolic energy into mechanical work is located in the cytoplasmic membrane of this cyanobacterium, as is the case in flagellated bacteria (1).

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CHAPTER 4

FUNCTIONAL SIGNIFICANCE OF MOTILTY IN SWIMMING STRAINS OF *SYNECHOCOCCUS*

Introduction

Oligotrophic environments, by definition are limited in the availablity of one or more essential nutrients. Under such conditions, microoganisms capable of a variety of specifically adapted physiological and ecological strategies possess a selective advantage (Chet and Mitchell, 1976a; Harder and Dijkhuizen, 1983; Pilgram and Williams, 1976; Lauffenburger et al., 1982; Poindexter, 1987). Among motile microbes, the ability to respond to changes in light, nutrient and oxygen concentrations permits such organisms to position themselves in regions best suited to their energy and nutrient requirements (Macnab and Koshland, 1972; Purcell, 1977; Castenholz, 1982).

For motile marine bacteria, the nature and utility of any behavioral response is limited by the physical constraints of an environment dominated by large scale mixing driven by wind and thermohaline processes (Pickard, 1963). In addition, for a bacterium the size of Synechococcus, inertial forces are negligable and motility is dominated by viscous forces. This is quantitatively expressed by the Reynolds number, which is the ratio of inertial to viscous forces for any object in motion through its fluid medium. For a 1 to 2 um bacterium, the Reynolds number is ca 10^{-5} (Purcell, 1977).

Within the marine ecosystem, the potential for chemotactic responses has been examined in several heterotrophic bacteria and in the eucaryotic green alga Dunaliella tertiolecta. Bell and Mitchell (1972) were the first to demonstrate that freshly enriched coastal bacterial isolates display positive chemotaxis toward extracts from the algae Skeletonema costatum, Cyclotella nana and D. tertiolecta. Later studies focus on bacteria grown in axenic cultures responding to specific chemical cues. Examples include amino acid and sugar chemotaxis in a coastal pseudomonad (Chet and Mitchell, 1976b) and Vibrio Ant 300 (Geesey and Morita, 1979). D. tertiolecta, (Sjoblad et al., 1978) is capable of phototactic behavior, as well as chemotaxis to a limited range of attractants includng ammonium, aromatic and sulfur-containing amino acids. Threshold values for the detection and response to these chemoeffectors range from 10^{-8} M for leucine and cysteine chemotaxis in the pseudomonad (Chet and Mitchell, 1976b) to 10^{-3} M for the cysteine response in D. tertiolecta (Sjoblad, 1978). The capillary assay of Adler and Dahl (1967) and Adler (1973) was employed to assess bacterial chemotaxis, while Palleroni's chemotaxis plates were used in the study of D. tertiolecta chemotaxis, thus responses in temporal gradients (Macnab and Koshland, 1972) and individual behavioral responses have not been evaluated.

Within the euphotic zone of tropical and subtropical waters, there is strong evidence that the transport of biologically active nitrogen sources into the system, not the degree of light penetration, drives primary production (Harrison, 1978; Eppley et al., 1979a,b). Therefore, the role of chemotaxis in the marine environment is often considered at the ecological level of nutrient availablity and recycling. Since levels of nitrogenous nutrients are often below the limit of analytical detection (Eppley et al., 1979a,b; McCarthy and Goldman, 1979), it has been suggested that chemotaxis allows motile marine microorganisms to exploit local sources of nutrient enrichment (Silver et al., 1978; Caron et al., 1982; Azam and Ammerman, 1984; Goldman, 1984; Alldredge and Cohen, 1987; Azam and Cho, 1987). Fecal pellets (Alldredge and Cohen, 1987) larger phytoplankers (Azam and Ammerman, 1984; Azam and Cho, 1987) and amorphous particles of complex biological origin, termed microaggregates (Goldman, 1984) or marine snow (Silver et al., 1978; Caron et al., 1982) are potential sites for elevated nutrient concentrations and turnover rates.

Quantification by direct counts of microorganisms associated with preserved marine snow particles demonstrates that such particles are predominantly colonized by motile bacteria and flagellated protozoa. Both photosynthetic and heterotrophic forms may exceed the concentration of the same organisms in the ambient seawater by as much as four orders of magnitude (Silver et al., 1978; Caron et al., 1982). The NH_3, NO_2 and NO_3^- concentration within and "immediately surrounding" coastal microaggregates range from 3 to 300 times greater than that of the ambient seawater (Shanks and Trent, 1979). Despite diffusion and advection, the biological activity within marine snow particles and fecal pellets is sufficient to maintain enriched microzones with steep pH and oxygen gradients, as measured with microelectrodes (Alldredge and Cohen, 1987).

At the level of the individual marine microorganism changes in cell behavior and morphology have been observed in response to such zones of nutrient enhancement. For example, when cells of the coastal strain Vibrio DW1 encounter a solid substrate under starvation conditions, they adhere (Dawson et al., 1981) and immediately increase oxygen consumption and heat production as reflected by enhanced metabolic rates (Kjelleberg et al., 1983). It has been suggested that this results from the onset of exoenzyme synthesis needed to degrade the particulate organic matter of which a nutrient replete particle is composed (Azam and Cho, 1987). Vibrio parahaemolyticus displays both behavioral and structural adaptation following attachment to a solid substrate. The ability to detect changes in viscosity allows these cells to undergo morphological differentiation from single polarly flagellated cells to peritrichously sheathed flagellated cells capable of swarming (Belas et al., 1986).

Rather than adhering to marine snow and fecal pellets, it has been postulated that some microbial communities within the oligotrophic marine environment are formed as clusters around microscale sources of sustained nutrient input, such as larger phytoplankton cells (Azam and Ammerman, 1984; Goldman, 1984, Azam and Cho, 1987). The presence of bacteria around such a nutrient source has never been directly observed or empirically tested. The notion of a "phycosphere" was first introduced by Bell and Mitchell (1972) who based it on the chemotaxis of mixed marine isolates toward algal exudates and considered this analogous to the rhizospheres which form microenvironments for soil bacteria. Based on several assumptions regarding cell sizes, nutrient concentrations and diffusion rates, Azam and Ammerman (1984) have calculated that such a phycosphere around a spherical algal cell 10 um in diameter would encompass a 100 um radius.

The potential importance of such small scale nutrient patches has been examined at the physiological level through the uptake kinetics of $^{15}NH_4^+$ in coastal and oceanic clones of the diatom _Thalassiosira pseudonana_ (McCarthy and Goldman 1979). Under conditions of nutrient deprivation, only the latter is able to rapidly modify the rate of $^{15}NH_4^+$ uptake in response to pulses of nitrogen input. This suggests that only the oceanic clone, not the clone from relativey enriched coastal waters could efficiently exploit transient zones of nutrient enrichment.

Motile and nonmotile open ocean strains of _Synechococcus_ inhabit the euphotic zones of the world's oligotrophic oceans (Waterbury et al., 1986). Currently, more than half the open ocean _Synechococcus_ strains in the Woods Hole cyanobacterial culture collection display swimming motility and differ from other motile marine procaryotes in several regards. First, open ocean _Synechococcus_ isolates can metabolize only a limited number of organic compounds (Waterbury et al., 1986). In addition, this unique form of motility couples lack of flagella or a flagellar-like appartus with random patterns of translocation. Unlike peritrichously or polarly flagellated bacteria, which display distinct swimming behaviors both before, during and after chemical stimulation (Macnab and Koshland, 1972; Macnab, 1978), no predictable pattern of motility has been observed under any condition in the motile _Synechococcus_ strains.

In culture, optimum growth of these isolates is maintained at low light intensities (10 - 30 uE m^{-2} s^{-1}) in a seawater-basal salts medium containing nitrate in millimolar concentrations (Waterbury et al., 1986). This is in sharp contrast to the oligotrophic ocean where nitrogen limiting conditions prevail. Thus, of the two most essential requirements for growth, light and combined nitrogen, the availability of the latter appears to be more likely limit _Synechococcus_ growth. The ability of motile _Synechococcus_ to actively respond to envrionmental stimuli would almost certainly be of ecological importance.

Yet among other, gliding forms of cyanobacteria the only behavioral adaptations observed are photoresponses, which have been termed photokinetic, phototactic or photophobic reactions. Standard definitions of these behaviors, as detailed by Diehn et al. (1977) are i) photokinesis is a modification in the speed of motility in response to changes in light intensity, ii) phototaxis describes movement either towards (positive) or away (negative) from a light source, and iii) photophobic or shock reactions are transient and rapidly induced alterations in motility in response to a sudden change in light intensity. Phototaxis and photophobic responses are the most common types of photosensory behaviors observed in cyanobacteria (Castenholz, 1982).

When tested, motile Synechococcus strains did not alter their patterns of swimming in response to changes in light intensity or when placed in a light gradient. However blind well chemotactic chamber experiments demonstrated that Synechococcus WH8113 was capable of positive chemotaxis in spatial gradients of a limited number of nitrogenous compounds, including ammonium, nitrate, urea, glycine and alanine. Threshold values for the detection of these compounds were quite low and ranged from 10^{-10} M to 10^{-9} M, indicating these responses could be ecologically relevant.

Thus, the investigation of the potential for tactic responses in this cyanobacterium was intended to not only elucidate the functional significance of this unusual form of procaryotic motility, but when viewed in the context of the physiology and ecology of open ocean Synechococcus, it may provide a conceptual basis for further development of the current models of microenvironments and their role in marine microbial food webs.

Materials and Methods

Bacterial strains and culture conditions Synechococcus WH 8103, WH 8011 and WH 8113 were used in the photoresponse experiments while only Synechococcus WH 8113 was used in the chemotaxis and amino acid uptake experiments. All cultures were in medium SN and incubated under the conditions previously described (Willey et al., 1987).

Preparation of cell suspensions Cells in the mid-logarithmic phase of growth were used without additional preparation for the photoresponse experiments. For chemotaxis and amino acid uptake assays, cells in the early to mid-logarithmic growth phase were harvested by centrifugation at 3000 x g for 10 min at 4^{o}C and washed a total of 4 times in 75% sterile Sargassso seawater:double distilled water. Cell densities for chemotaxis assays were then adjusted to 9.5 x 10^{5} to 1.2 x 10^{6} cells ml^{-1} as determined by direct cell counts (Waterbury et al., 1979). Cells used in amino acid uptake experiments were adjusted a density of 5 x 10^{9} to 1 x 10^{10} cells ml^{-1} (120 - 140 ug protein ml^{-1}).

Photoresponse experiments All photoresponse experiments were performed by examining cell suspensions prepared on wet mount slides and viewed by light microscopy (Zeiss phase microscope with 100x oil immersion objective and 100 W halogen transmitted light). The potential for photokinesis, phototaxis and photophobic reactions was tested on each cell suspension prepared under both white and green light.

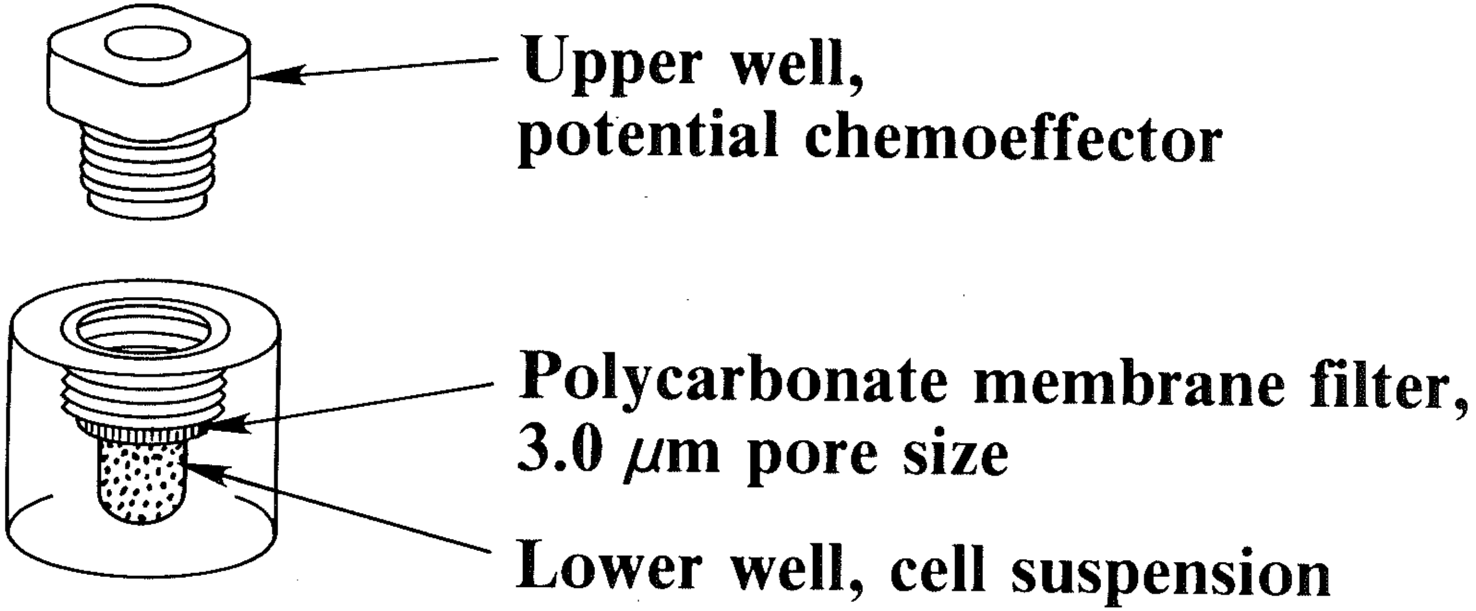
Photokinetic responses were evaluated in two ways. Cells were first subjected to a rapid change in the intensity of the transmitted light source by quickly reducing its brightness as controlled by a rheostat on the illuminator transformer. In an attempt to quantify any modifications in swimming speeds, cell preparations were also examined by dark field microscopy and video recorded, as previously described (Willey et al., 1987, Chapter 3). Motility was then analyzed by the computer-assisted motion analysis system as described by Sundberg et al. (1985). By using dark field microscopy with a green filter over the field diaphram in a darkened room, a discrete column of light was produced. This resulted in the illumination of only a fraction of the microscope slide. Thus only those cells directly within the illuminated field on the microscope slide were at a light level that exceeded that required for saturation of photosynthesis. Experiments were conducted so that cells at one edge of the cover slip could be observed at a high light intensity, while those at the opposite edge experienced a light level (1 - 2 uE m^{-2}

s^{-1}) well below photosynthetic saturation (Kana and Glibert, 1987b). Because illumination at a low light intensities diminished the level of contrast, thereby precluding video taping, cells were video recorded immediately following incubation at reduced light intensities. This permitted the direct comparison between continuously illuminated cells with cells in the same preparation that had experienced incubation at low light intensities.

The potential for phototaxis was assessed in cells subjected to a light gradient created by limiting the diameter of the field diaphram which provided a sustained light gradient. In addition, cells placed in a gradient produced by dark field illumination as described for photokinetic experiments were observed. Finally, the ability to express photophobic reactions was tested by a combination of these two methods, i.e. rapid changes in light intensity by dimming and brightening the light source and the rapid introduction of a light gradient by quickly opening and closing the field diaphram.

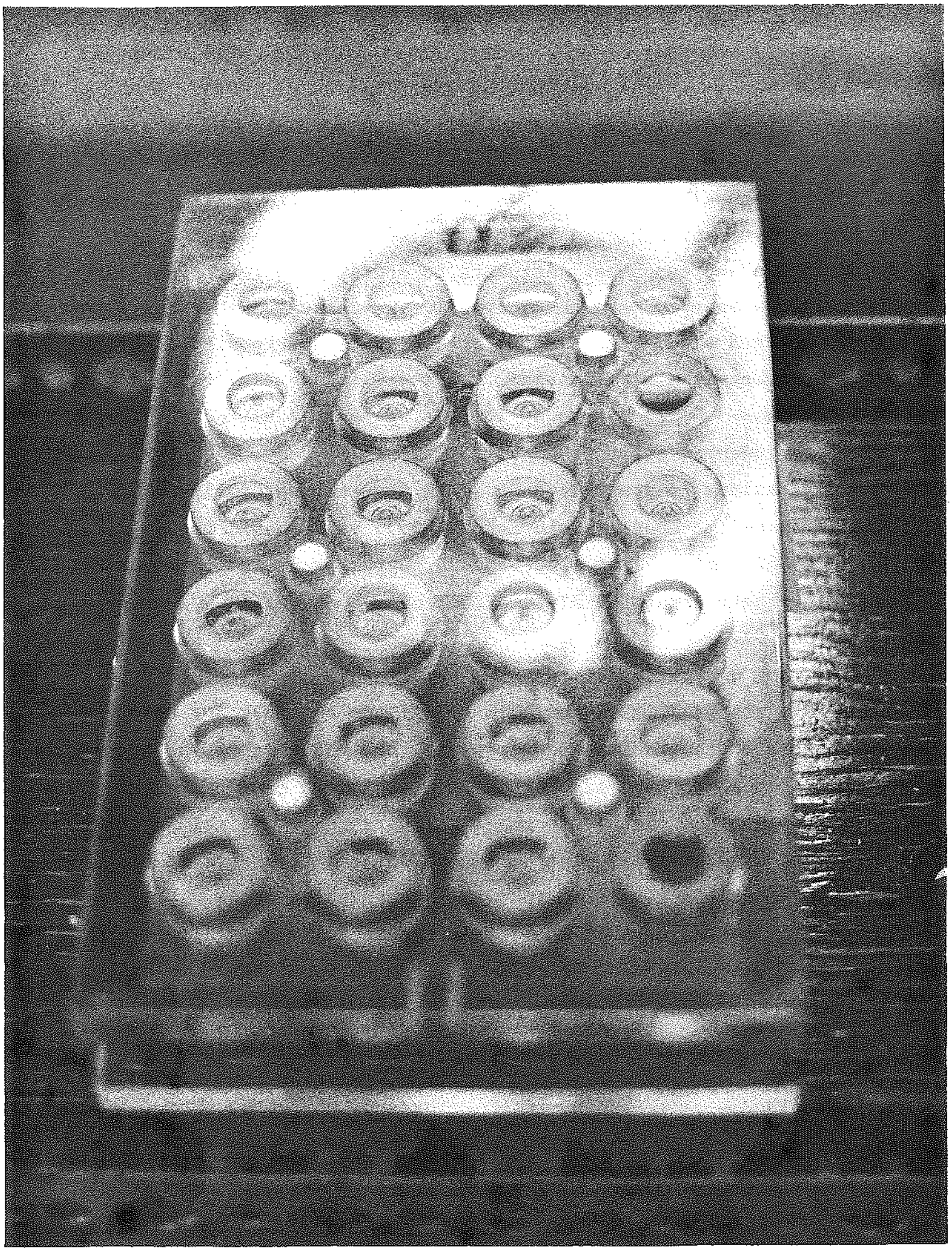
Blind-well chemotaxis chamber experiments Blind-well chemotactic chambers (NeuroProbe, Inc., Cabin John, MD) were used to screen compounds that might elicit a chemotactic response in *Synechococcus* WH 8113. Each of these clear acrylic chambers has a lower and upper well with volumes of 200 ul and 800 ul, respectively, between which a 13 mm polycarbonate filter was inserted (Figure 16A). Twenty-four such chambers were positioned on clear acrylic trays specifically machined for this purpose (Figure 16B). Into each lower well, a 165 ul aliquot of cell suspension was pipetted, over which a polycarbonate filter with a uniform pore size of 3.0 um (Nuclepore, Inc., Pleasanton, CA) was placed, being careful to avoid contact with the cell suspension. The upper well was created by screwing the cap into place, and a spatial gradient was established when 835 ul of SSW containing the designated compound at the indicated concentration was pipetted it. This created a slight miniscus, so that upon sealing with parafilm and another clear acrylic sheet, air bubbles in the upper wells were avoided. Each assay was begun by allowing the cell suspensions in the lower wells to simultaneously make contact with the filter by inverting the tray of chambers and tapping gently allowing

Figure 16A Blind well chemotactic chambers used to test the chemotactic poential of cells suspended in aged, sterile Sargasso Seawater. A spatial chemical gradient created by the presence of chemoeffector in the upper well and chemoresponses were determined by the number of cells crossing the filter, thereby accumulating in the upper well, as described in Materials and Methods.


Upper well,
potential chemoeffector
Polycarbonate membrane filter,
3.0 μm pore size
Lower well, cell suspension

Blind Well Chemotactic Chamber

Figure 16B Tray of blind well chemotactic chambers during experimental incubation. Each tray was machined to hold 24 chambers and was incubated under conditions described in Materials and Methods.



the 35 ul air bubble in the lower well to move away from the filter to the opposite end of the well (Figure 16B).

All cell suspensions and chemoeffector solutions were prepared in 75% aged Sargasso seawater:doubled distilled water (SSW). When cells were suspended in the defined artificial seawater buffer used previously (Willey et al., 1987), experiments demonstrated that more than 50% of the cell population was rendered nonmotile following the four washes by centrifugation required to obtain optimum and reproducible chemoresponses. Attempts to modify this buffer so that motility was preserved were unsuccessful. Sargasso seawater collected in 1980 and aged since that time at room temperature in teflon carboys (to permit nutrient mineralization) was sterilized by autoclaving in 500 ml aliquots. Cells suspended in this seawater retained the levels of motility observed prior to centrifugation. NH_4^+, NO_2 and NO_3^- concentrations in sterilized Sargasso seawater was analyzed by the automated methods of Solorzano (1969), Bendschneider and Robinson (1952) and Wood et al., (1967), respectively and found to be at or below the limits of analytical detection (ca. 0.01 - 0.03 ug atom N liter^{-1}).

For each concentration response experiment, one tray of 22 chambers was prepared which included triplicate chambers for each chemoeffector concentration and 4 seawater control chambers. Chambers were incubated at 25°C at 12 - 15 uE m^{-2} s^{-1} for 65 min. Experiments were stopped by returning chambers to an upright position, removing the upper acrylic sheet and parafilm and transferring the contents of each upper well to individally prelabled tubes. The number of cells that crossed the filter was determined by direct microscopic counts with epifluorescent illumination (Waterbury et al., 1979).

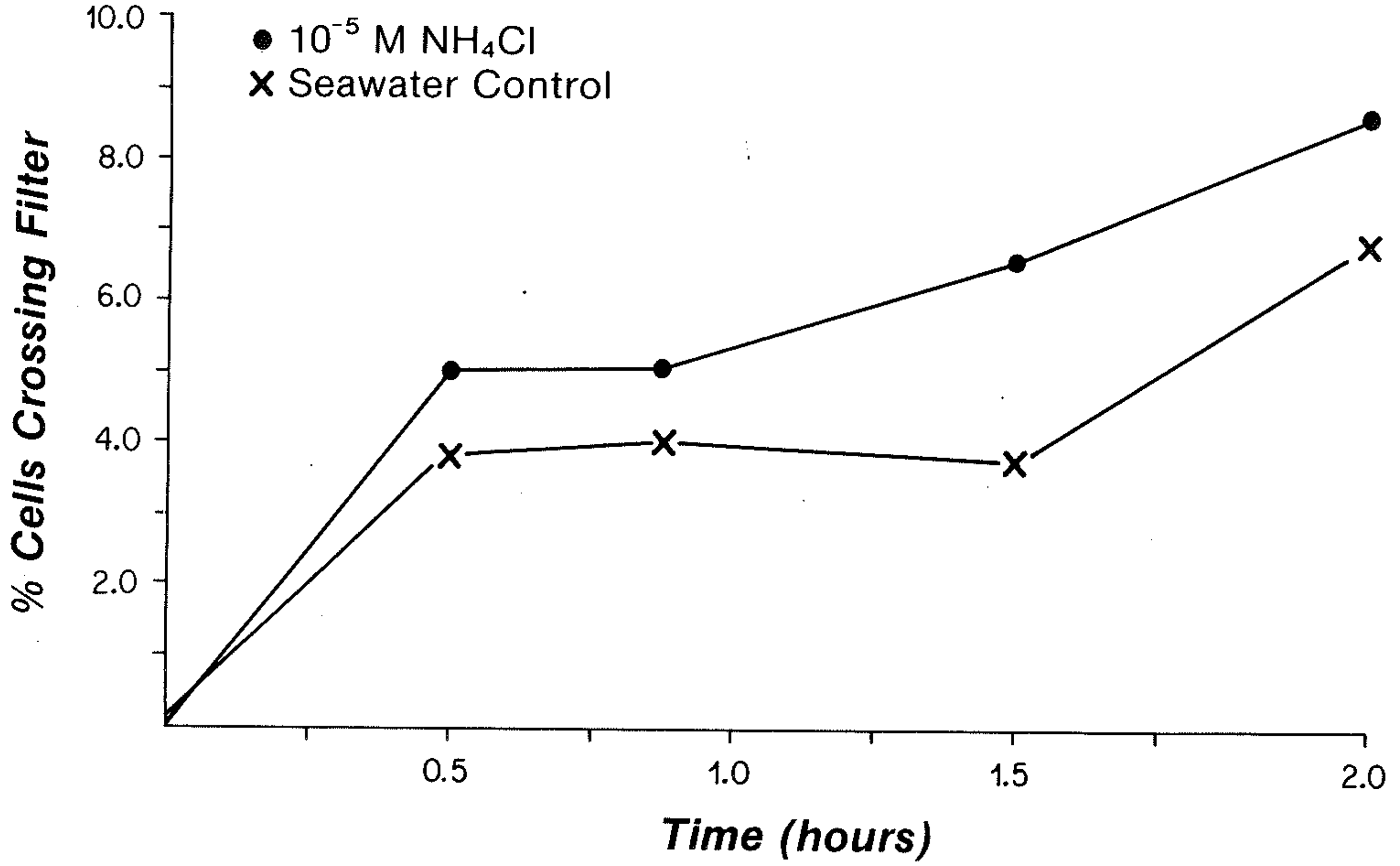
Two types of control chambers, both lacking a chemical gradient were employed. As in experimental chambers, cells in seawater control chambers were suspended in SSW, however the upper well contained only SSW without added chemoeffector. The other type of control chamber had chemoeffector added at the same concentration to both the upper and lower wells. Both types of controls were tested in "control-control" experiments, which were performed when a compound appeared to elicit a positive chemotactic response. In each control-control experiment 9 chambers of each type of control were incubated on the same tray as 4 - 6 chambers with an established gradient of the compound in question at a concentration of 10^{-6} M. In addition, although not specifically designed as control experiments, all those chemoeffectors that failed to elicit a chemotactic response served, in effect, as negative control experiments.

The establishment and stability of a concentration gradient within the chambers was tested by introducing 1 mM $NaNO_2$ into the upper well and SSW either with or without cells into the lower well. During incubation under the described experimental conditions, the contents of both upper and lower wells were separately collected at 15 min intervals for 2 hours and the NO_2 concentration in each well measured by the method of Stickland and Parsons (1972). Regardless of the presence or absence of cells, there was no detectable change in NO_2 concentration in the upper well throughout the 2 hour period. The NO_2 concentration in the lower well reached and remained at 3 - 5 uM following the initial 30 min, indicating the chambers could maintain a steep concentration gradient throughout the experimental incubation period.

A 65 min incubation period was selected based on the observation that following the initial 30 min of incubation, the rate at which cells accumulated in the upper well containing 10^{-5} M NH_4Cl, $NaNO_3$ or urea roughly paralleled that of the seawater control chambers. The results of one such time course for NH_4Cl is shown in Figure 17. The shortest duration that would permit enough cells for accurate cell counts as well as a significant difference between gradient and control chambers was used to minimize consumption of metabolizable substrates being tested and any possible effects containment in such chambers might have on cell physiology and behavior.

The optimum cell density for chamber experiments was determined by monitering the response to 10^{-5} M NH_4Cl and $NaNO_3$ after

Figure 17 Time course experiment designed to optimize the magnitute of chemotactic response. The number of cells crossing the filter into 10^{-5} M NH_4Cl (•) or aged, sterile Sargasso Seawater (×) was determined at 30 minute interavals.


● 10^{-5} M NH_4Cl
X Seawater Control
% Cells Crossing Filter
10.0
8.0
6.0
4.0
2.0
0.5
1.0
1.5
2.0
Time (hours)

filling the lower well with cells ranging in concentration from 1 x 10^5 to 1 x 10^8 cells ml^{-1}. Cell suspensions with less than 5 x 10^5 cells ml^{-1} were impractical because accurate cell counts could not be obtained at such low cell densities. There was a linear relationship between cells crossing the filter and initial cell concentration when the lower well was filled with cell suspensions with densities ranging from 5 x 10^5 to 5 x 10^7 cells ml^{-1}. Greater initial cell densities did not result in an increase in the number of cells accumulating in the upper well. At a cell density greater than 1 x 10^8 cells ml^{-1}, the filter was visibly pigmented at the completion of the incubation period, suggesting that at such high cell densities the filter became clogged.

Cellular uptake of amino acids For each amino acid concentration tested, 1 ml of cell suspension was pipetted into an acid washed 10 ml beaker and slowly mixed by gentle continuous shaking. To prevent light limitation due to high cell density, a circular cool white fluorescent lamp was positioned to generate an irradiance of 100 uE m^{-2} s^{-1} (as determined with a Licor 2 sensor) at the surface of the cell suspension. Each experiment was begun by labelling the cells with the designated concentration of [^{14}C] glycine (0.13 uCi ml^{-1} cell suspension; specific activity 114 mCi $mmol^{-1}$) or [^{14}C] alanine (0.17 uCi ml^{-1} cell suspension; specific activity 171 mCi $mmol^{-1}$). Following rapid, yet thorough mixing (as established by methylene blue dye studies) sample aliquots of 200 ul were rapidly collected by microcentrifugation through a 50 ul layer of silicon oil. The first aliquot was sampled within 15 - 25 seconds after addition of label. The time of sampling was recorded, and when kinetics were calculated, 10 seconds was added to this time, since this was the time required to separate cells from the surrounding medium by microcentrifugation. The amount of radioactivity in the pellet and remaining in the supernatant was determined by standard scintillation counting techniques. Intracellular volumes and the amount of extracellular matrix associated with the pellet were measures as previously described (Willey et al., 1987). Cellular protein cencentration was determined by the method of Lowry (1951).

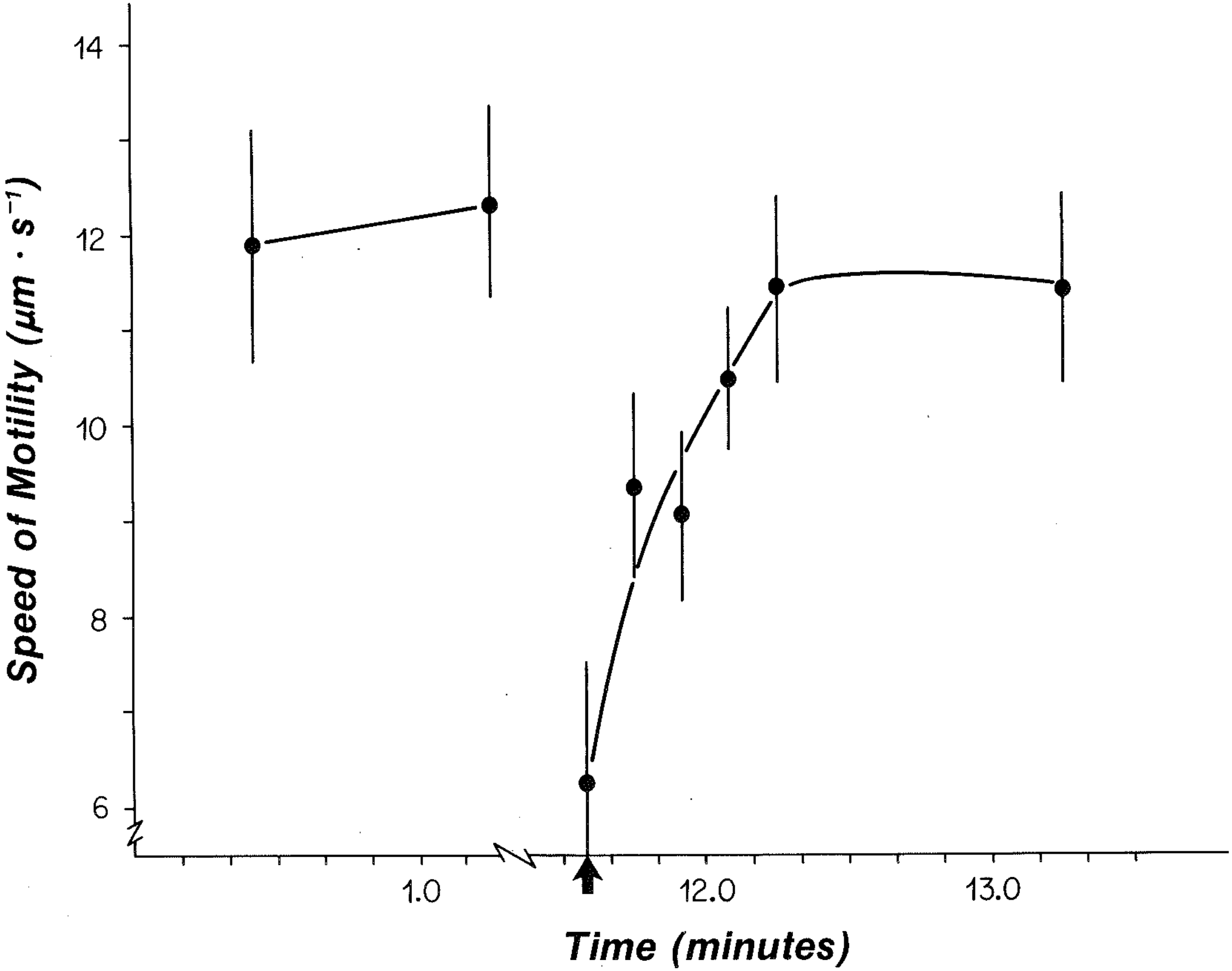
Chemicals Radiolabeled amino acids were purchased from Amersham Radiochemicals, Arlington Heights, IL, and [^{14}C] sorbitol and [^{3}H] H_2O used to measure intracellular volume was supplied by New England Nuclear Corp., Boston, MA. All compounds used for chemotaxis chamber experiments were purchased from Sigma Chemical Co., St. Louis, MO. Solutions of potential chemoeffectors in SSW were prepared on the day used.

Results

Photoresponses Cell suspensions of three motile Synechococcus strains were examined by light microscopy for potential phototactic, photokinetic and photophobic reactions. There was no alteration in cell speed (photokinesis) or photophobic repsonse when cells were subjected to rapid changes in light intensity. Likewise, when placed in a sustained light gradient, neither positive or negative phototaxis was observed since there was no net migration of cells either towards or away from the light source.

When tested under dark field illumination, a photokinetic-like phenomenon was observed following incubation at a light intensity of 1 - 2 uE m^{-2} s^{-1} which is far below the level of photosynthetic saturation in oceanic Synechococcus (Kana and Glibert, 1987b). Computer-assisted motion analysis of cells video recorded after incubation for 10 minutes at this severely limited light intensity revealed that cells exhibited a transient decrease in the speed of motility to about half that of the average control value, with recovery to preincubation swimming speeds within 2 minutes (Figure 18). This relationship between light intensity and swimming speed was not observed when cells were subjected to either rapid changes in light intensity or after incubation in complete darkness for as long as 12 hours (Waterbury et al., 1985). Nor could it be reproduced when cells were incubated at light levels greater than 3 - 4 uE m^{-2} s^{-1}. The observed alteration in swimming speed was similar to that observed in response to incubation in sodium-free buffer with restoration of motility followed by the addition of sodium. Thus,

Figure 18 Recovery of motility following low light incubation was observed in wet mounts of cell suspensions in medium SN examined by dark field video microscopy. For the first minute of incubation, cells were recorded in the area of focused light where they were actively motile. The cell suspension was then incubated for 10 minutes in near darkness which resulted in a decrease in swimming speed from an average of 12 to 6 um s^{-1}. Following the 10 minute low light incubation, cells were again examined in the focused light field (as indicated by arrow) where the recovery of swimming speed was recorded.


Speed of Motility ($\mu m \cdot s^{-1}$)
14
12
10
8
6
1.0
12.0
13.0
Time (minutes)

just as motilty in Synechococcus WH 8113 is sodium-coupled (Willey et al., 1987), it is also light-dependent and this photokinetic-like swimming appeared to be an energetic, not a behavioral response.

Response to spatial chemical gradients Twenty-three different potential chemical stimuli including sugars, amino acids, simple nitrogenous compounds and vitamins were screened in spatial gradients established in blind well chemotaxis chambers. With the exception of the vitamins, all compounds were tested at 6 concentrations ranging from 10^{-10} M to 10^{-5} M, inclusive (Table 2). Relative to control chambers, only NH_4Cl, $NaNO_3$, urea, glycine and β-alanine produced a positive chemoresponse in Synechococcus WH 8113, as measured by a reproducible increase in the number of cells swimming through the filter separating the cell suspension (lower well) from the chemoattractant (upper well, Figures 19 - 23).

Following an incubation period of 65 minutes, 3.5 - 4.5% of the cells in the seawater control chambers passed through the filter from the lower to the upper well. When a positive response was elicited, between 5 - 8% of the cells from the lower well crossed the filter and accumulated in the upper well (Figures 19 - 23).

The minimum concentration at which positive chemotaxis was first detected was extremely low. Threshold values of 10^{-9} M were required for a reproducible response to NH_4Cl and $NaNO_3$ (Figures 19 and 20), and those for urea, glycine and β-alanine fell between 10^{-10} M and 10^{-9} M (Figures 21 - 23). Once above these threshold levels, there was no significant difference in the relative magnitude of the response, over the range of concentrations tested. A measurable positive response was sharply eliminated at 10^{-2} M for both NH_4CL and $NaNO_3$ when concentrations greater that 10^{-5} M were tested.

The validity of the positive results were further confirmed by a series of "control-control" experiments (Figure 24). The 2 types of non-gradient control chambers employed included seawater control chambers, which contained cells suspended in SSW in the lower well and SSW without attractant in the upper well, and control chambers with an equal concentration of attractant in both the cell suspension (lower

Compounds That Failed to Elicit a Chemoresponse in *Synechococcus* WH 8113[a]

Sodium acetate
D-Fructose
D-Glucose
D-Ribose
Sucrose

K_2HPO_4
Thiamine [b]
Vitamin B_{12} [b]

Sodium Glutamate
L-Glutamate
L-Glutamine
L-Aspartic acid
L-Cysteine
L-Methionine
Methylamine
Trimethylamine
$NaNO_2$

a. All compounds were tested on three separate occasions, and with the exception of thiamin and vitamin B_{12}, were tested at 6 concentrations from 10^{-10}M to 10^{-5}M, inclusive.

b. Thiamine and vitamin B_{12} were tested at three concentrations: 10, 1 and 0.1 $\mu g \cdot ml^{-1}$.

TABLE 2

Figure 19 Chemotactic response of cell suspensions of *Synechococcus* WH 8113 to varying concentrations of NH_4Cl. The magnitude of response was measured as the percent of cells crossing the filter in the chemotaxis chamber from the seawater-cell suspension to the seawater enriched with NH_4Cl. The horizontal line represents the mean and the stippled area the standard area of seawater control experiments.

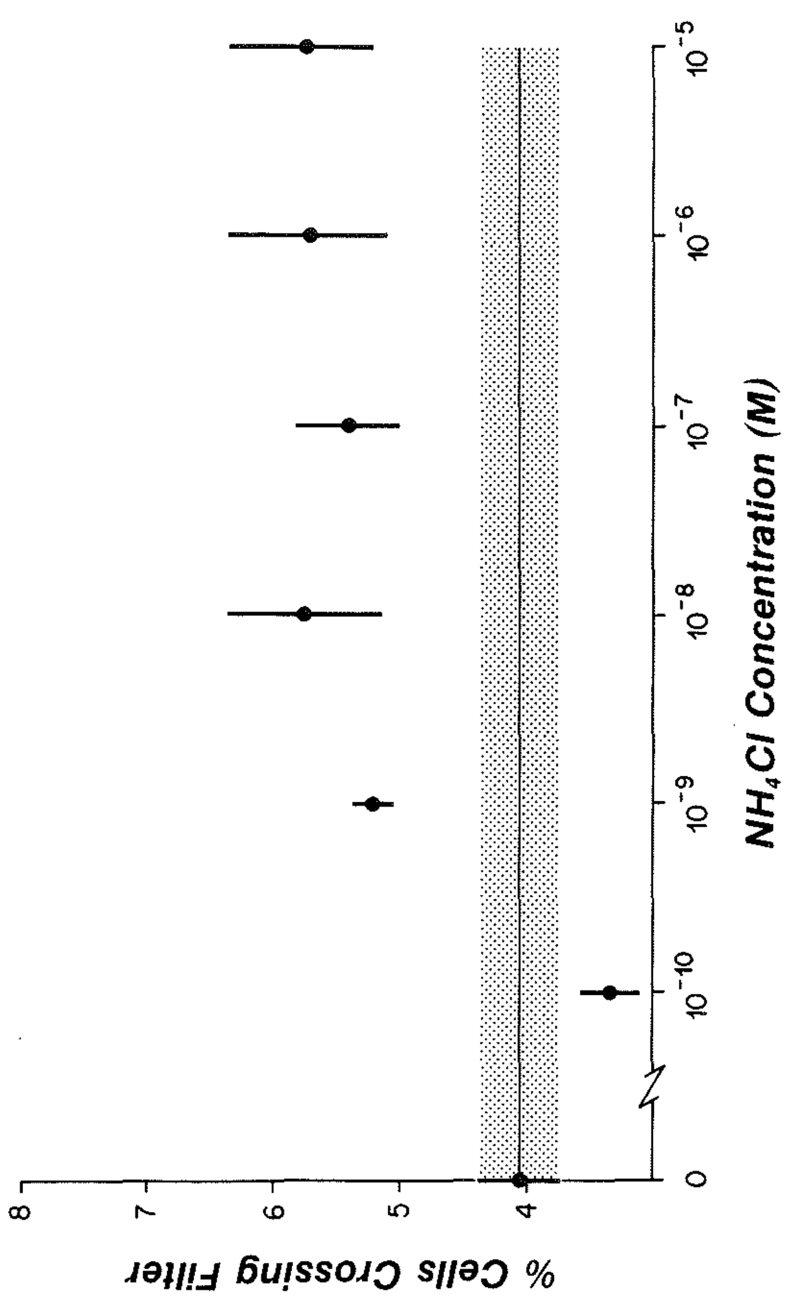

% Cells Crossing Filter
8
7
6
5
4
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
NH_4Cl Concentration (M)

Figure 20 $NaNO_3$ concentration response curve, as for Figure 4, except upper wells contained $NaNO_3$ solutions at the indicated concentrations.

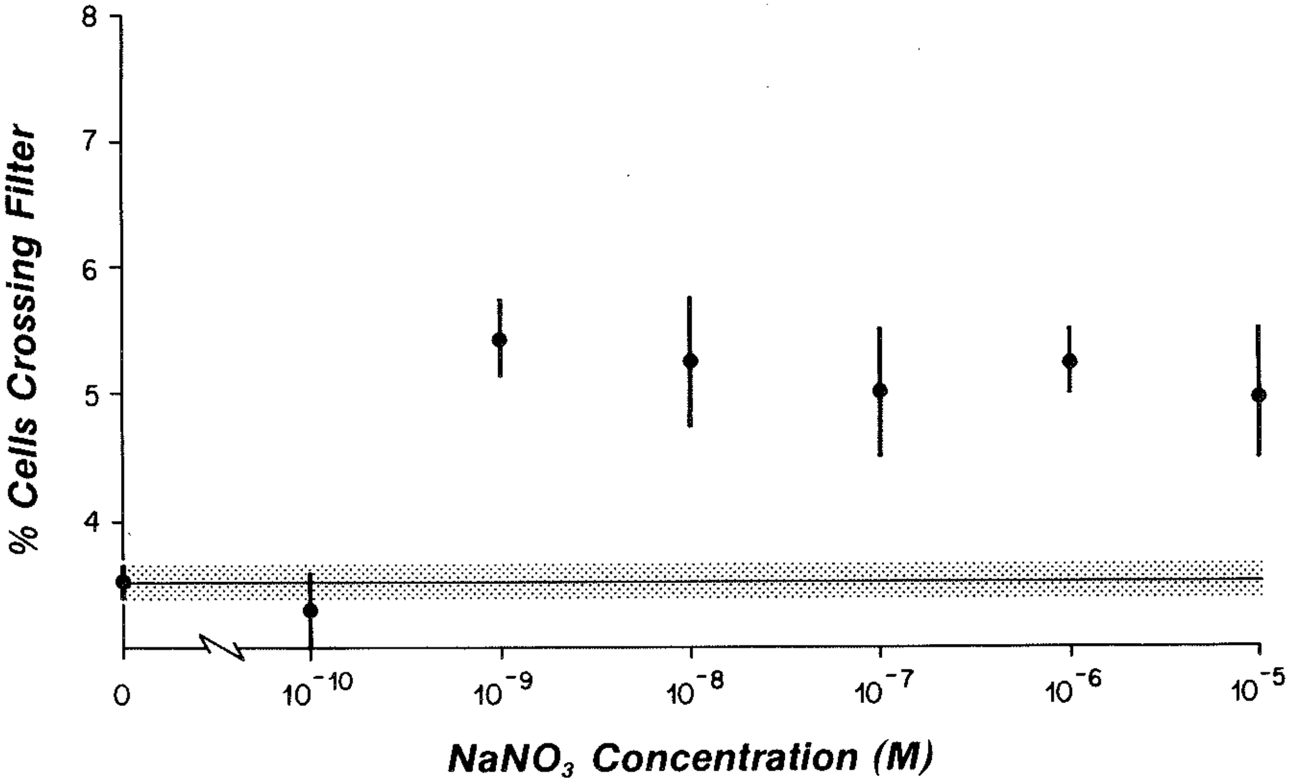

% Cells Crossing Filter
8
7
6
5
4
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
$NaNO_3$ Concentration (M)

Figure 21 Urea concentration response curve, as for Figure 4, except upper wells contained urea solutions at the indicated concentrations.

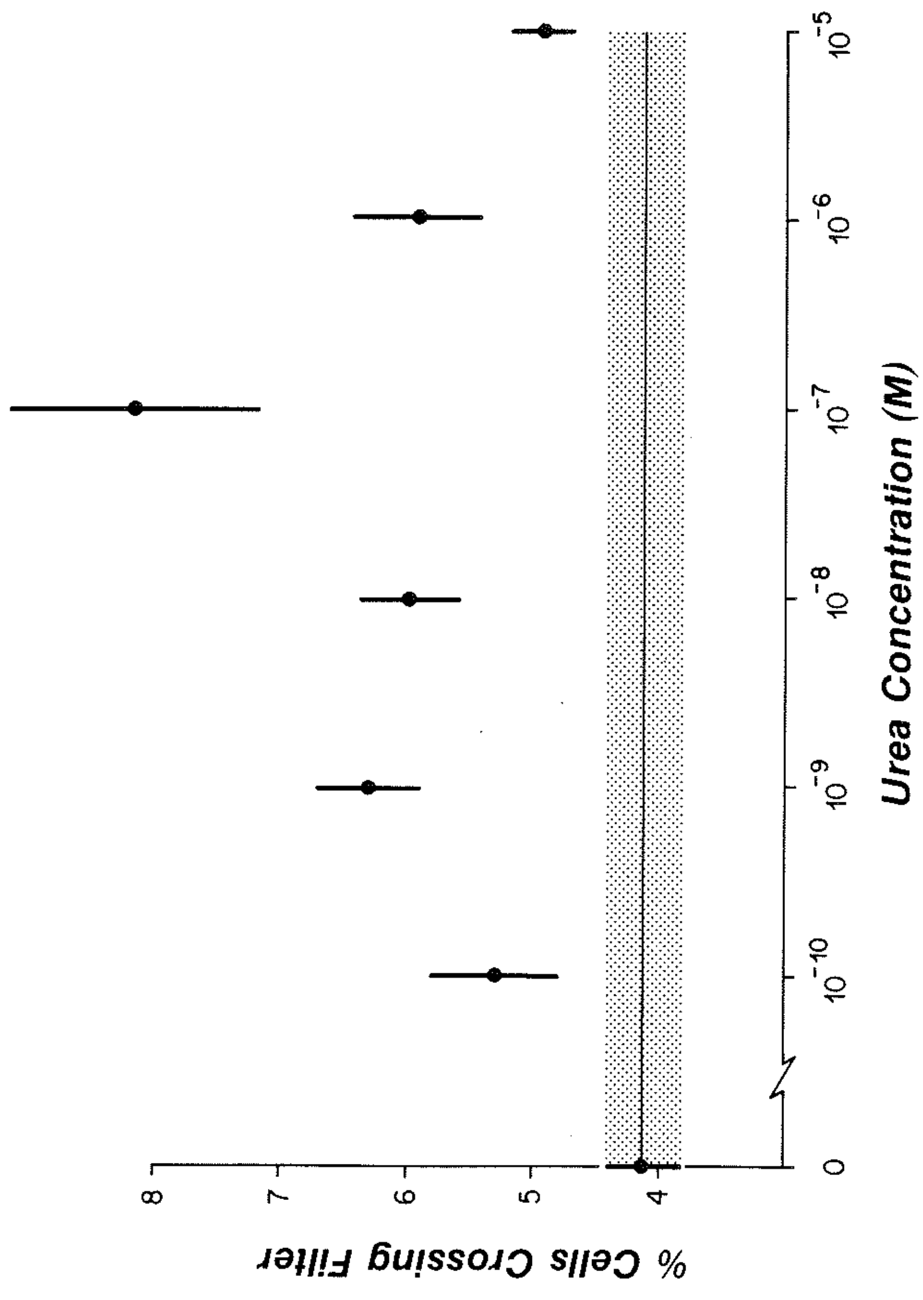

% Cells Crossing Filter
8
7
6
5
4
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
Urea Concentration (M)

Figure 22 Glycine concentration response curve, as for Figure 4, except upper wells contained glycine solutions at the indicated concentrations.

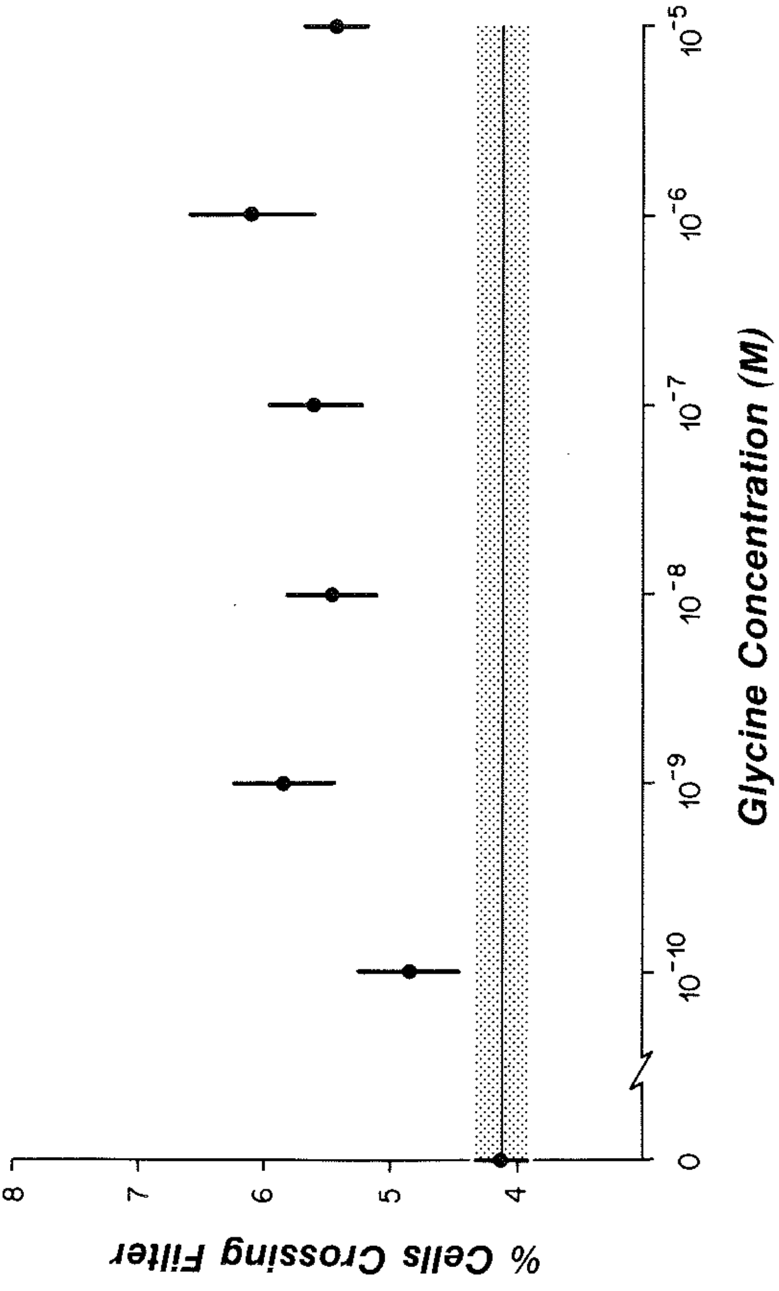

% Cells Crossing Filter
8
7
6
5
4
0
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
Glycine Concentration (M)

Figure 23 β· alanine concentration response curve, as for Figure 4, except upper wells contained β·alanine solutions at the indicated concentrations.

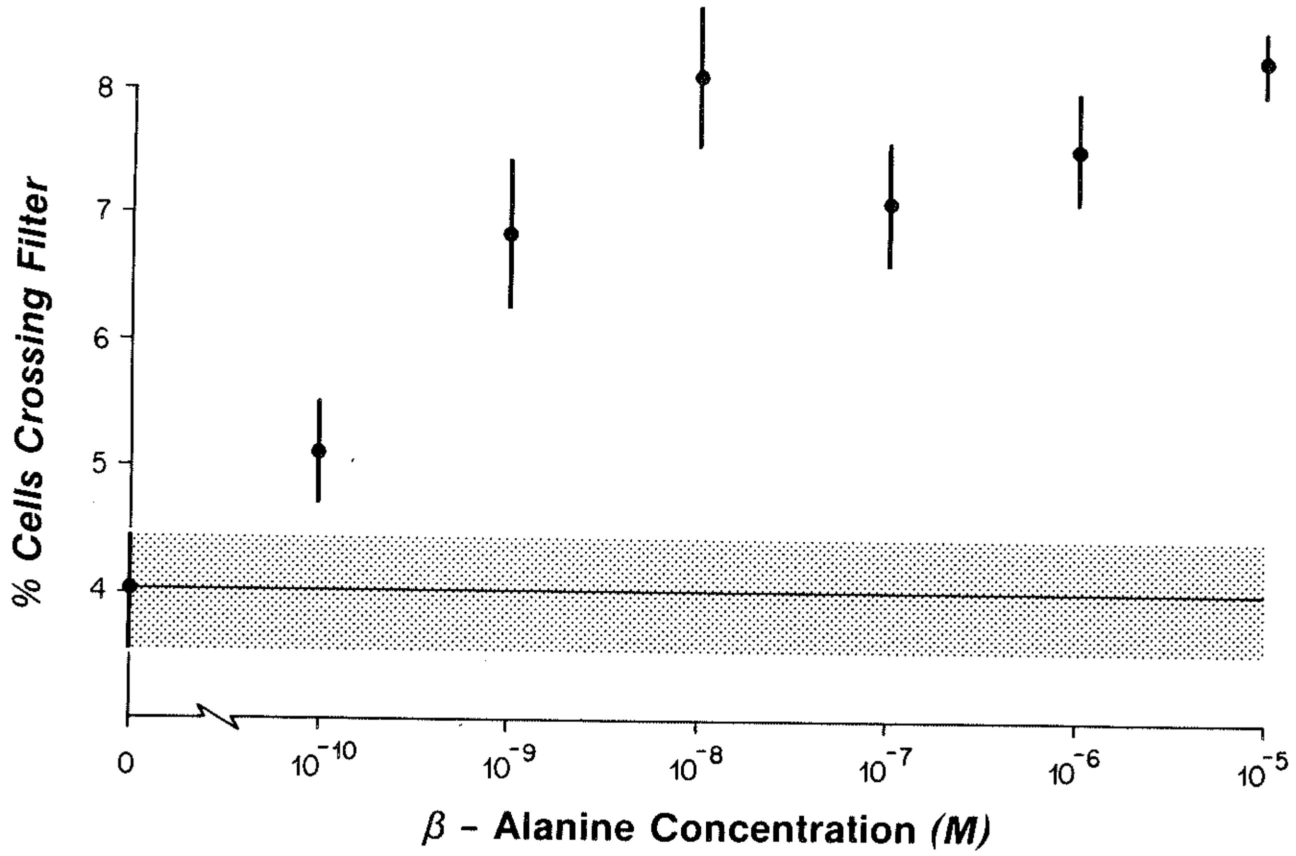
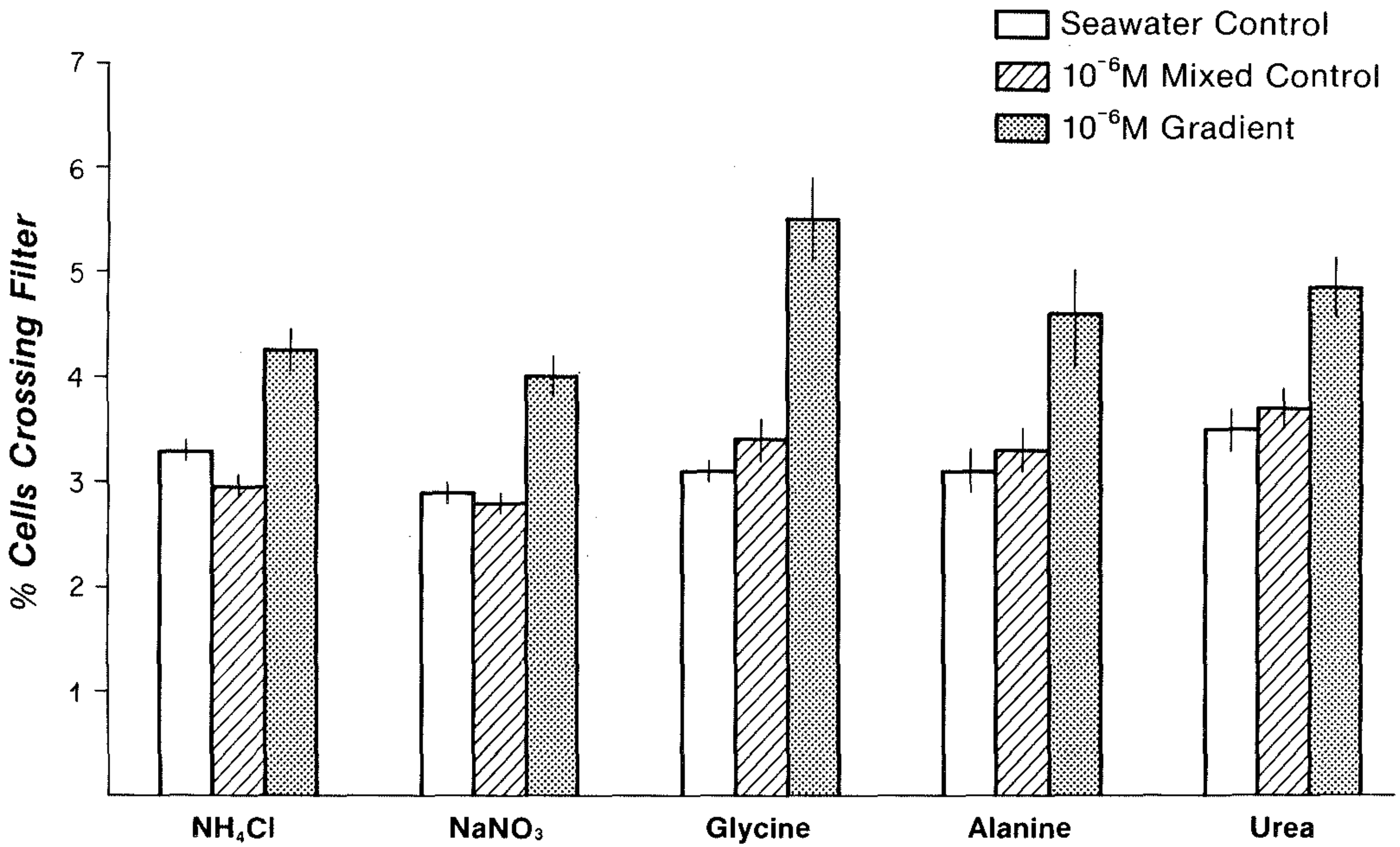

% Cells Crossing Filter
8
7
6
5
4
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
β - Alanine Concentration (M)

Figure 24 The chemotactic response to Synechococcus WH 8113 cell suspensions placed in the lower wells of chemotactic chambers to three experimental setups: Seawater controls, both upper and lower wells contained Sargasso Seawater; Mixed controls, both upper and lower wells contained seawater augmented with 10^{-6} M chemoeffector; Gradients, the lower wells contained seawater and the upper well contained seawater plus 10^{-6} M chemoeffector. Results are expressed as the percentage of cells crossing the filter from the lower to upper wells. Error bars represent standard error.


Seawater Control
10^{-6}M Mixed Control
10^{-6}M Gradient
% Cells Crossing Filter
7
6
5
4
3
2
1
NH_4Cl
$NaNO_3$
Glycine
Alanine
Urea

well) and attractant-SSW solution (upper well). Using cell suspensions that demonstrated a positive chemotactic response, there was no significant difference between these types of control chambers. Thus, such experiments not only substantiated initial findings, but demonstrated that a positive chemotactic response required a spatial gradient of chemoeffector, not just to the presence of the compound.

The magnitude of the chemoresponse relative to the seawater control values was not altered by cell age, as tested by comparing cells in the early to mid-logarithmic phase of growth with those in the early stationary growth phase (Figure 25). However, the absolute number of cells swimming through the filter was diminished in chambers containing the older cells, presumably due to a lower percentage of motile cells in older cultures (as documented in Chapter 2, Figure 14A).

Likewise, there was no change in chemoresponse when cells grown in an excess of nitrate (medium SN) were washed and resuspended in the same medium without combined nitrogen for about one doubling (24 hours). As shown in Figures 26 and 27, neither the threshold concentrations or the magnitude of the response to NH_4Cl or $NaNO_3$ was significantly affected by nitrogen starvation.

Cellular transport of amino acids Although chemotaxis to a specific chemoeffector and its metabolism need not be coupled (Mesibov and Adler, 1972), an effective strategy for survival in an oligotrophic environment might be to augment chemosensory behavior with a high cellular transport and/or metabolic affinity for a chemoattractant. The kinetics of [^{14}C] glycine and [^{14}C] β-alanine transport were measured to determine if the motile cyanobacterium *Synechococcus* WH 8113 was capable of glycine and alanine transport, as well as to test this hypothesis. The k_m for glycine transport was about 1 uM (Figure 28) and for β·alanine, it was between 10 and 15 uM (Figure 29). The V_{max} for transport also differed. For glycine this value was 7.5 nmol mg protein^{-1} min^{-1} and for β·alanine it was 25 nmol mg protein^{-1} min^{-1}. Thus these amino acids appeared to be substrates for *Synechcoccus* WH 8113

Figure 25 Comparison of the effects on chemotaxis on cells harvested from the stationary and mid-logarithmic growth phases of growth in Seawater control chambers, 10^{-4} M NH_4Cl gradient chambers and 10^{-4} M $NaNO_3$ gradient chambers. Results are expressed as the percentage of cells crossing the filter from the lower to upper well. Error bars represent standard error.

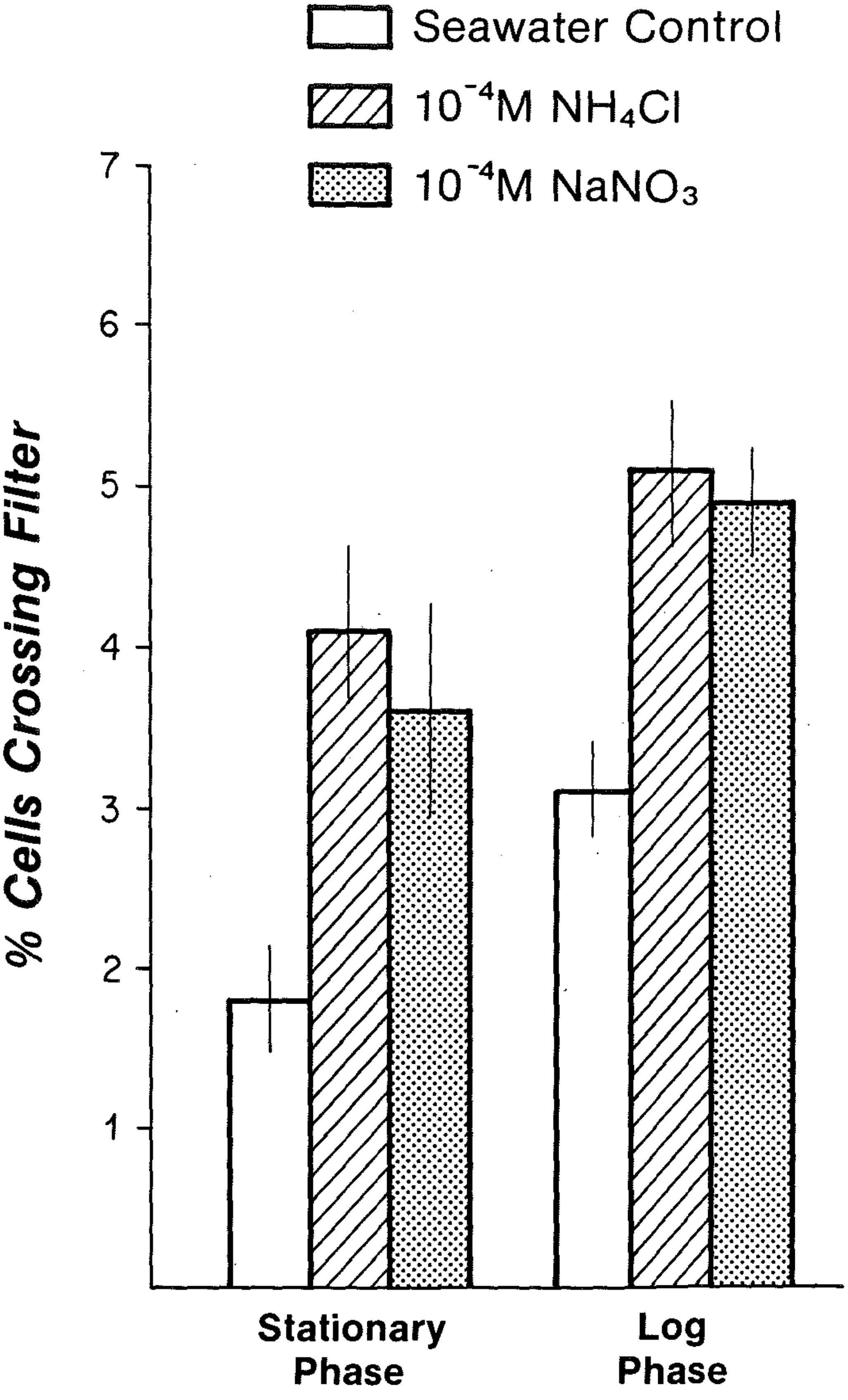

Seawater Control
10^{-4}M NH_4Cl
10^{-4}M $NaNO_3$
% Cells Crossing Filter
7
6
5
4
3
2
1
Stationary Phase
Log Phase

Figure 26 Comparison of nitrogen replete and nitrogen starved cell suspensions to spatial gradients of NH_4Cl. Results are expressed as the percentage of cells crossing the filter from the lower to upper well. Error bars represent standard error.

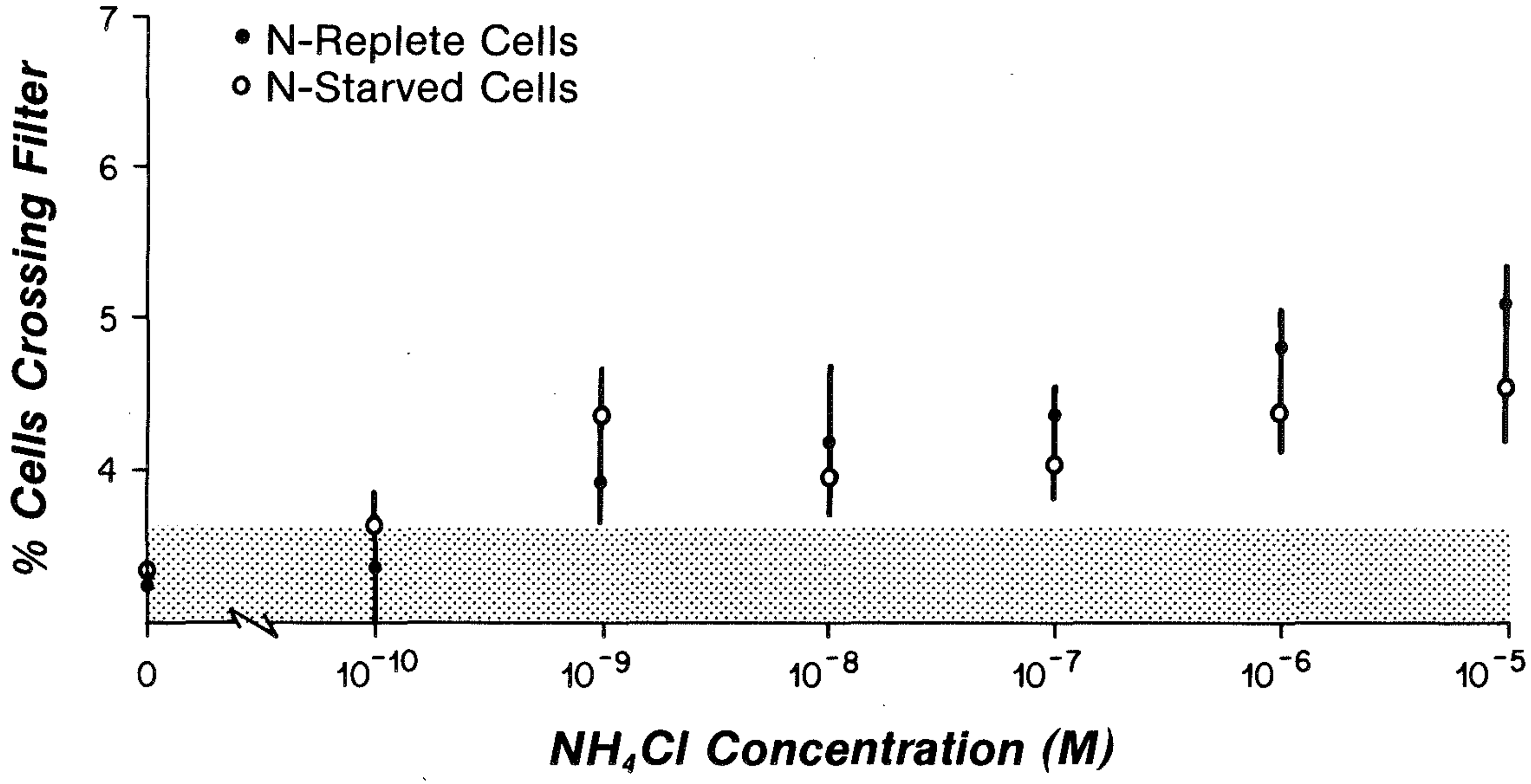
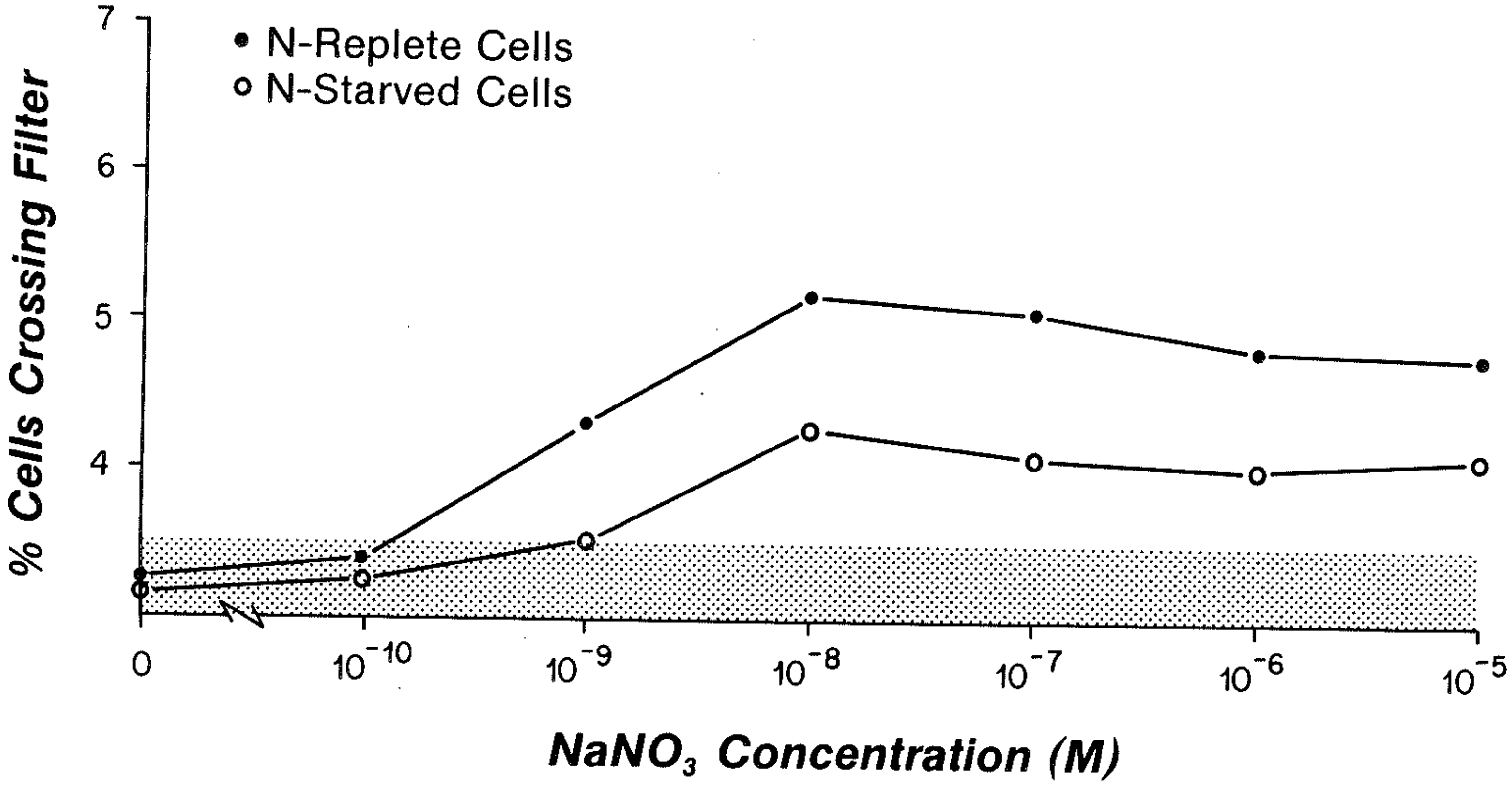
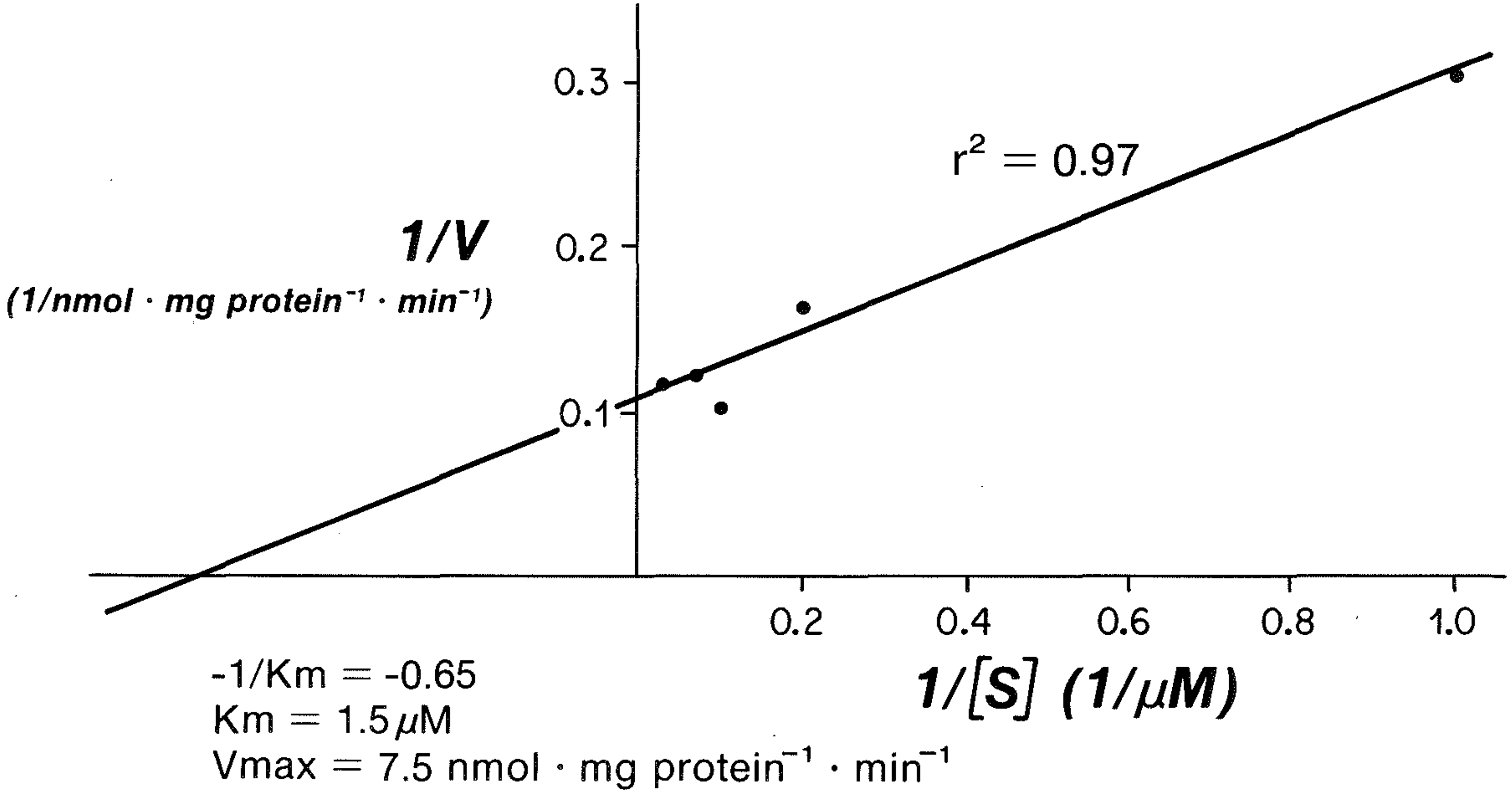

% Cells Crossing Filter
7
6
5
4
• N-Replete Cells
○ N-Starved Cells
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
NH_4Cl Concentration (M)

Figure 27 Effect of nitrogen starvation on chemoresponse to $NaNO_3$ was tested as in Figure 11, except upper wells contained $NaNO_3$ solutions at the indicated concentrations.



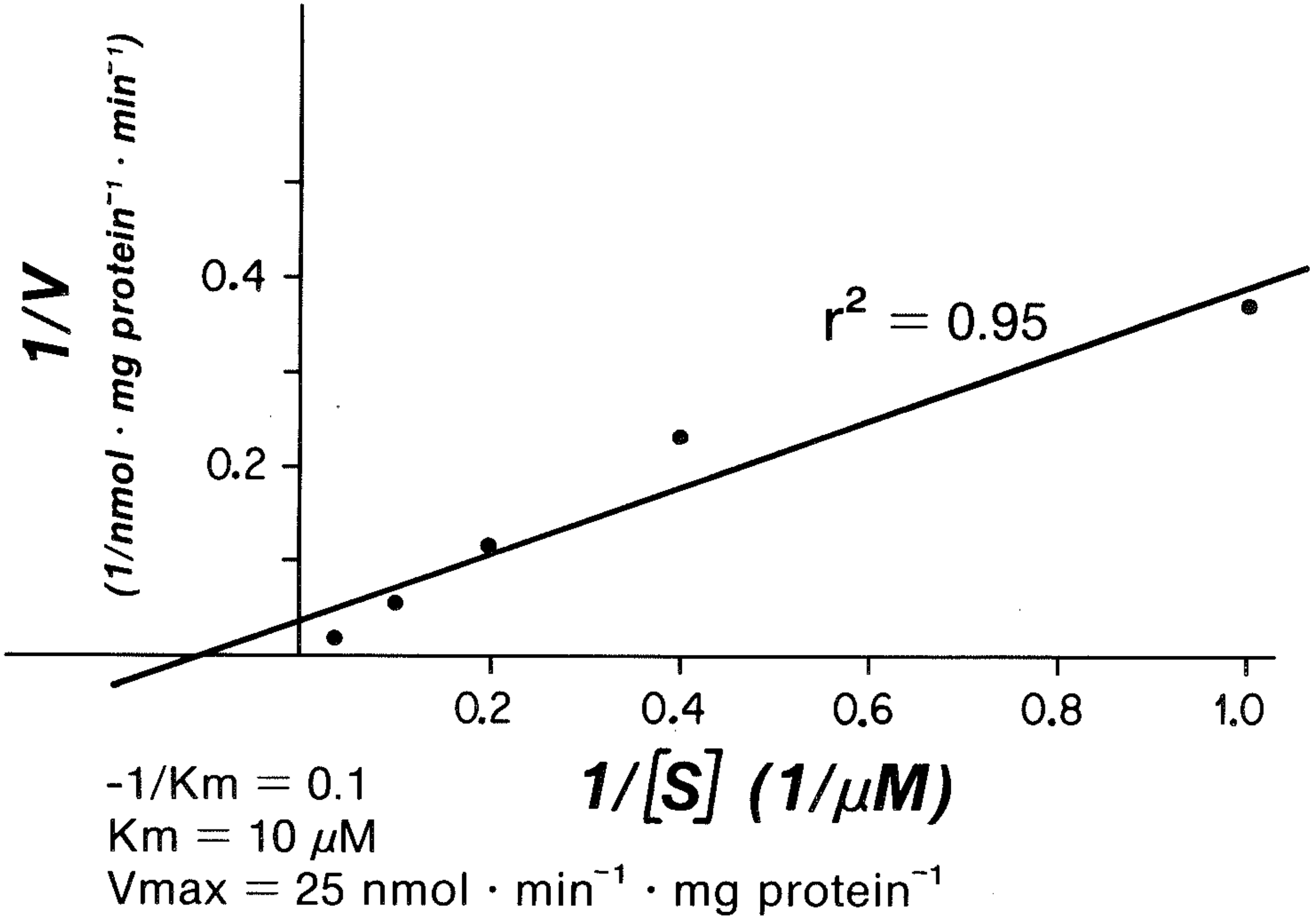
● N-Replete Cells
○ N-Starved Cells
% Cells Crossing Filter
7
6
5
4
0
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
$NaNO_3$ Concentration (M)

Figure 28 Kinetics of glycine uptake. Double reciprocal plot of Synechococcus WH 8113 uptake of [^{14}C] glycine at concentrations of 1 to 30 uM. Cells were incubated and sampled as described in Materials and Methods.



-1/Km = -0.65
Km = 1.5 μM
Vmax = 7.5 nmol · mg protein^{-1} · min^{-1}

Figure 29 Kinetics of β-alanine uptake, as for glycine uptake, except [^{14}C] β-alanine at concentrations from 1 to 30 uM were tested.



$-1/Km = 0.1$
$Km = 10\ \mu M$
$Vmax = 25\ nmol \cdot min^{-1} \cdot mg\ protein^{-1}$

metabolism. However, the relative affinity for these compounds by this photoautotroph, as indicated by these parameters were comparable to those reported for *E. coli* (Kotyk and Janecek, 1975), suggesting that enhanced transport and chemotaxis are not coupled in this instance.

Discussion

Previous studies examining cyanobacterial motility and chemotaxis have been limited to an evaluation of the photoresponses exhibited by filamentous forms capable only of gliding motility (Halfen and Castenholz, 1971; Nultsch and Hader, 1979; Castenholz, 1982; Glagolev, 1984; Hader, 1987). The unicellular motile *Synechococccus* isolates display a unique form of motility (Waterbury et al., 1985), and in addition the results of the current investigation revealed that motile *Synechococcus* is the first and only reported example of a photoautotroph, either eucaryotic or procaryotic to lack photosensory behavior and demonstrate only chemoresponses.

Several lines of evidence indicate that behavioral photoresponses are ecologically unimportant to motile *Synechococcus* while the ability to detect and respond to very low concentrations of simple nitrogenous compounds could confer a survival advantage. When tested for photokinetic, phototactic or photophobic reactions, no behavioral response was observed. Rapid changes in light intensity did not yield a modification in cell speed or a sudden photophobic shock, as displayed by many filamentous cyanobacteria (Castenholz, 1982; Hader, 1987). Phototaxis was also not observed when cells were placed in a light gradient. The only light-induced change in motility appeared to be an energetically-driven transient decrease in swimming speed under photosynthesis-limiting light intensities (Figure 18). This change in swimming speeds was not observed at other light intensities, nor after incubation in the dark, when glycogen reserves maintain cellular energy requirements (Smith, 1982). It has been established that motility in *Synechococcus* WH 8113 is sodium-coupled; the loss and subsequent restoration of control speeds of motility upon suspension in sodium-free buffer followed by the introduction of sodium is analogous to the change in swimming speeds observed following incubation at severely limiting light intensities. Thus, this light-dependent perturbation in motility cannot be construed as a behavioral photosensory response with ecological implications since significant numbers of Synechococcus do not inhabit water with such a low levels of average irradiance (Waterbury et al., 1986), nor did this behavior enhance the relative light regime for the bacterial population.

Moreover, the apparent lack of photoresponses in motile Synechococcus must be considered within the constraints of the physiological requirements of this cyanobacterium as well as in the broader context of the microbial communities of oligotrophic oceans. Open ocean isolates of Synechococcus grew in light intensities as low as 3 uE m^{-2} s^{-1}, and although the growth rate was diminished, motility was unaltered (Chapter 2). Field measurements of Synechococcus [^{14}C] $NaHCO_3$ incorporation in the Sargasso Sea suggest that a cyanobacterial cell would have to swim roughly 20 meters through the water column to obtain an increase in the level of light penetration that would significantly increase its rate of carbon fixation (Waterbury et al. 1986). However, if a Synechococcus cell were to swim this distance in a straight line, ignoring the affects of seawater circulation, at the average speed of 13 um s^{-1}, it would take about 14 days, or almost as many doublings. Two possibilities thus exist in nature. Either the same clone of Synechococcus inhabits a given water column and compensates for varying levels of light penetration by photoadaptation (Kana and Glibert, 1987a,b) rather than motility, or as suggested by Wood (1985), Synechococcus clones differ between strata of the same water column. This has been indicated by comparing the photosynthetic potential of two phycoerythrin-containing Synechococcus clones with two that lack this pigment. Wood (1985) inferred that these clones are specifically adapted for photosynthesis under the light conditions at the depths from which they were isolated.

In contrast, motile Synechococcus WH 8113 displayed positive chemotaxis when placed in spatial gradients established in blind well chemotaxis chambers. This method provided an effective means of screening a variety of compounds, each over a wide range of concentrations. The capillary assay of Adler (1973) has been used extensively for such analyses in many motile microbes (as reviewed by Berg, 1975; Macnab, 1978). One advantage of the capillary method is the greater magnitude of the chemotactic response relative to that for the chemotaxis chamber technique. However, the capillary assay was impractical for the study of Synechococcus chemoresponses because light focusing on the edge of the capillary produced a band of intense light which may have introduced artifact through photoinhibition. This phenomenon has also been observed in attempts to use this technique in chemotaxis assays in photosynthetic bacteria (Armitage, personal communication) and the use of capillary assays has been avoided in the analysis of tactic behavior in other phototrophs as well (Sjoblad et al., 1978).

Several additional experiments were employed to confirm the presence of an observed positive response since the degree of response was lower using the blind well chamber technique than that obtained by capillary assay. Thus, three lines of evidence to support the presence of chemotaxis in motile Synechococcus were observed. Triplicate chambers were used in each concentration response experiment, which was repeated as many as 6 to 8 times, yielding reproducible positive results for gradients of NH_4Cl, $NaNO_3$, urea, glycine and β·alanine. Control-control experiments were performed in which two types of control chambers lacking chemoeffector gradients verified the tactic potential of Synchococcus when placed in a spatial gradient (Figure 24). In addition, the 17 compounds that failed to elicit a response also served as negative controls (Table 2).

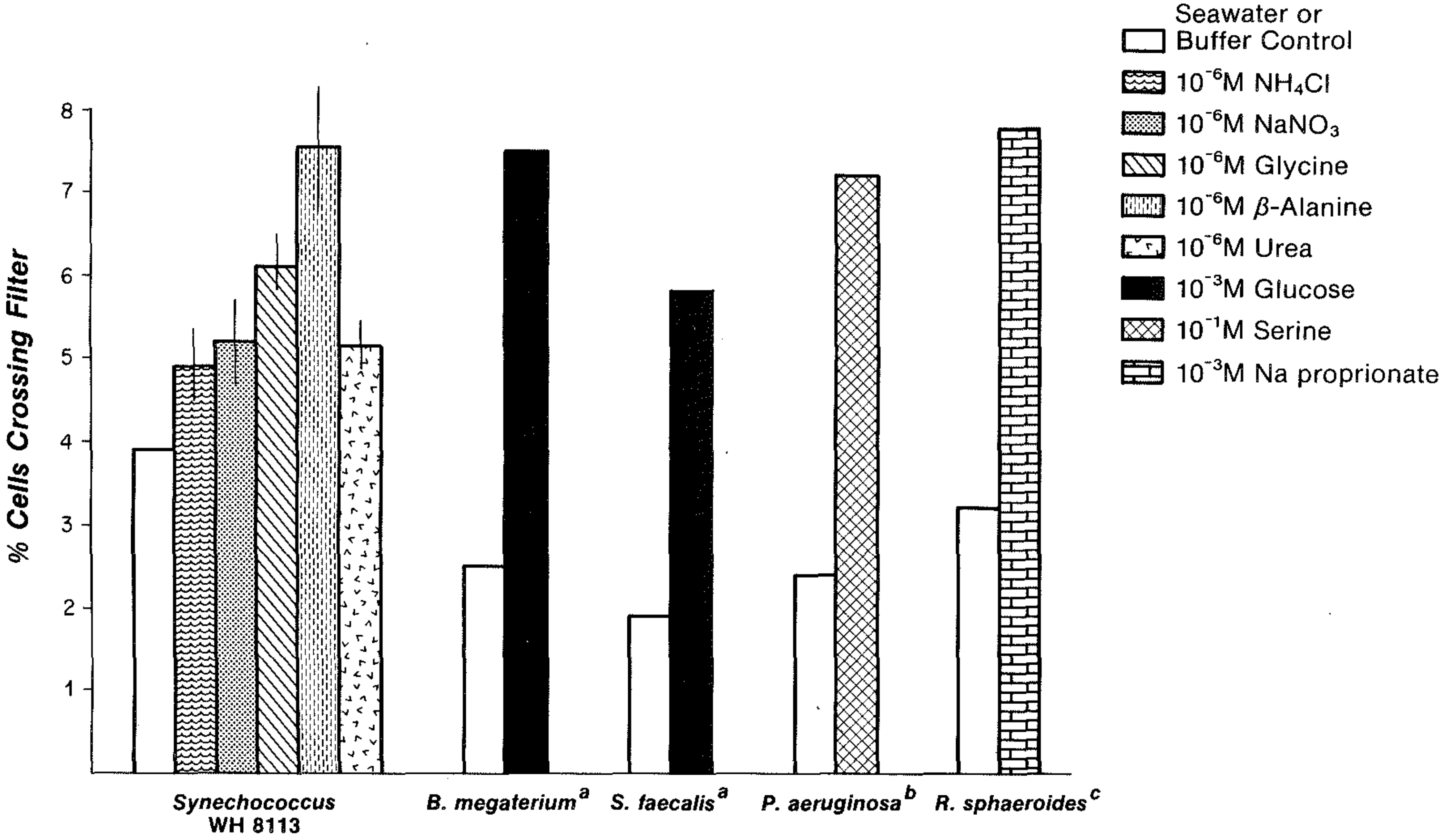
The cell responses observed in two types of experiments demonstrated that cells were capable of chemotaxis only when a gradient of chemoeffector was present. The control chambers employed in the control-control experiments included not only those in which only SSW was tested, but chambers with the same concentration of chemoeffector in both the upper and lower wells as well. Although there was no significant difference in the measured cell response between these controls, the cells were clearly capable of chemotaxis when placed in a spatial gradient of attractant (Figure 24). In addition, the lack of chemotaxis in experiments in which cells were tested in high concentration gradients of NH_4Cl and $NaNO_3$ (10^{-2} M) also illustrated that it was not the presence of the compound, per se to which the cells responded, but its gradient. Under these conditions, the cellular receptor capacity was presumably saturated, thus no gradient could be detected (Adler, 1973; Armitage et al., 1977).

Studies in other bacterial systems have shown that the potential for chemotaxis is not necessarily coupled to the ability to metabolize a specific chemoeffector, however such instances are generally based on steric similarities between a metabolizable substrate and its nonmetabolizable analog (Mesibov and Adler, 1972). Other studies have demonstrated that when the chemotactic response is mediated directly by a chemoeffector-inducded change in energy metabolism, chemotaxis and substrate uptake are coupled (Clancy et al., 1981; Taylor, 1983). Recently, it has been shown that chemotaxis in R. sphaeroides requires the transport but not the metabolism of several chemoeffectors (Ingham and Armitage, 1987). The ability of motile Syenchococcus WH 8113 to transport [^{14}C] glycine and [^{14}C] alanine indicated that this cyanobacterium has evolved a chemosensory system based upon metabolic substrates.

Several indications suggested that the ability to respond to a chemical gradient was expressed constitutively. Unlike many heterotrophic bacteria in which the potential for motility and chemotaxis must be actively selected by growth on swarm plates (Adler, 1973) or nutrient limiting chemostats (Pilgram and Williams, 1976; Terracciano and Canale-Parola, 1984), motile Synechococcus isolates have been maintained in rich batch culture for more that five years without noticable changes in levels of motility. Also attempts to induce a stronger chemotactic response in gradients of NH_4Cl (Figure 25), $NaNO_3$ (Figure 26) or glycine (data not shown) were unsuccessful.

When compared to the chemotactic response of other bacteria tested by this method, the percentage of cells that crossed the filter

Figure 30 Comparisons of the magnitude of chemotactic responses of Synechococcus WH 8113 to that of other bacteria using the some method. (a) Bacillus megaterium, Streptococcus faecalis (Armitage et al., 1977), (b) Pseudomonas aeruginosa (Armitage and Evans, 1983) and (c) Rhodobacter sphaeroides (Ingham and Armitage, 1987).


8
7
6
5
4
3
2
1
% Cells Crossing Filter
Synechococcus WH 8113
B. megaterium[a]
S. faecalis[a]
P. aeruginosa[b]
R. sphaeroides[c]
Seawater or Buffer Control
10^{-6}M NH_4Cl
10^{-6}M $NaNO_3$
10^{-6}M Glycine
10^{-6}M β-Alanine
10^{-6}M Urea
10^{-3}M Glucose
10^{-1}M Serine
10^{-3}M Na proprionate

and accumulated in the well with the chemoattractant was comparable (Figure 30). However in viewing this figure, one striking feature is the difference between the value of the cyanobacterial control chamber (4% of cells crossing the filter) and that of the other bacteria reported (2.5 - 3%). There are several possible explanations for this. First, this could reflect differences in interspecific swimming behavior. Both P. aeruginosa and R. sphaeroides are capable of rapid swimming speeds, up to 60 - 80 um s^{-1} (Macnab, 1978; Armitage and Macnab, 1987). The swimming patterns of these microbes also differ markedly from the random swimming paths displayed by motile Synechococcus. The differences in control values could also be due to variations in experimental procedure including incubation duration, cell to filter pore size ratio and the phase of growth the microbes were in when tested. Finally this difference could be attributed to the routine selection for chemotactic heterotrophic flagellated cells on swarm plates resulting in a much higher percentage of motile and chemotactic cells (Mesibov and Adler, 1972; Adler, 1973; Armitage et al., 1979; Armitage and Evans, 1983), while no such selection procedure is feasible for motile Synechococcus.

The many compounds that failed to elicit a chemotactic response in Synechococcus reflect the limited metabolic capabilies of this phototroph. This is further exemplified by the structural simplicity of those compounds that produced a positive response, particularly the 2 and 3 carbon amino acids. Although, this is in contrast to the metabolically diverse photosynthetic bacterium Rhodopseudomonas sphaeroides (now classed as Rhodobacter sphaeroides) which is capable of chemotaxis to a broad range of chemical stimuli (Armitage et al., 1979), this narrow range of Synechococcus chemoeffectors has ecological relevance. Calculations based on the assimilation rates of $[^{15}N]$ nitrate (Eppley et al., 1979a), ammonium (Eppley et al., 1979b) and $[^{14}C]$ urea (McCarthy, 1972) by open ocean and coastal clones of marine phytoplankton suggest that these three N-sources could account for all the nitrogen needed to maintain measured rates of carbon fixation and the observed C:N ratios. Furthermore, glycine and alanine have been reported to be among the amino acids present in highest relative abundance in Sargasso Sea water, as recently determined by shipboard HPLC measurement of dissolved free amino acids (Vaughan and Mopper, 1988).

Given that the utility of Synechococcus swimming motilty is confined to a scale of millimeters to centimenters, it is reasonable to assert that this motilty provides these strains with the ability to exploit local microenvironments of nitrogen enrichment, presumably conferring an ecological advantage to the motile strains. The relationship between motility, chemotaxis and an inferred ecological advantage has been studied in mixed cultures of motile chemotactic heterotrophic bacteria which have been demonstrated to have a survival advantange over nonmotile strains of the same bacteria when essential metabolites are limiting (Pilgram and Williams, 1976). This has also been considered from a theoretical viewpoint (Purcell, 1977; Lauffenburger et al., 1982; Jackson, 1987). Lauffenburger et al. (1982) have developed a mathematical model that evaluates the relationship between several parameters including cell motility and chemotaxis, nutrient diffusion and uptake and cell growth rates. When applied to nutrient limiting conditions, this model demonstrates, as was originally stated by Purcell (1977), that random motility cannot enhance growth by promoting the diffusion and cellular uptake rates of nutrients in solution. As indicated by the low Reynolds number inherrent to microorganisms, this is due to the viscous shear around the cell body, which essentially moves the local aqueous environment in tandom with the cell (Purcell, 1977). On the other hand, it is agreed that chemotaxis can augment nutrient uptake by increasing the number of encounters a cell has with sites of nutrient enrichment. According to Lauffenburger et al. (1982), this can result in an increased growth rate and population density, even when competing with bacteria possessing greater rates of nutrient uptake. Finally, Jackson (1987) has applied the established values describing peritrichous motility and algal "leakage rates" to model bacterial-algal interactions. Simulated runs and tumbles of a chemotactic marine bacterium in a seawater environment demonstrate that chemosensory behavior could allow such a cell to respond to the presence of an algal cell depending on the distance between the two organisms and the rate at which nutrients are released by the alga.

Experimental and theoretical considerations demonstrated that photoresponses would be of little, if any, value to motile Synechococcus cells inhabiting the euphotic zones of oligotrophic oceans. The simple nitrogenous compounds to which Synechococcus is attracted and the low threshold concentrations that elicited this attraction illustrate the degree of nutrient depletion in oceanic microbial ecosystems, which in turn highlights the utility of chemotaxis in such an environment. The threshold values measured in this study ranged from 0.1 nM to 1 nM, indicating that in agreement with theoretical considerations, random motility would be of no benefit to motile Synechococcus. However attraction to local regions of nutrient enrichment, as further demonstrated by the gradient-dependent nature of the observed chemotactic responses, could provide an ecological advantage for these strains.

The effects of nitrogen limitation and the role of zones of micronutrients either as marine snow or as phycospheres around larger phytoplankers has engendered lively discussion (Bell and Mitchell. 1972; Silver et al., 1978; Caron et al., 1982; Azam and Ammerman, 1984; Goldman, 1984; Alldredge and Cohen, 1987; Azam and Cho, 1987), although actual examination and applicability of these theories has been difficult. The presence of swimming motility in over half the open ocean Synechococcus isolates currently in culture coupled with the finding that at least one strain is capable of responding to gradients of simple nitrogenous compounds at concentrations of ecological significance helps support the notion that microscale nutrient dynamics may be important in maintaining the microbial communities in nutrient depleted oceans.

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APPENDIX I

ISOLATION AND GROWTH OF MARINE PLANKTONIC CYANOBACTERIA

ISOLATION AND GROWTH OF MARINE PLANKTONIC CYANOBACTERIA

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Proofs to J.B.W.

Shortened Title: Growth of Marine Planktonic Cyanobacteria

[ ] Isolation and Growth of Marine Planktonic Cyanobacteria

By John B. Waterbury and Joanne M. Willey

Cyanobacteria are widespread in marine habitats, especially in the warmer temperate and tropical regions, where they are important both as primary producers and nitrogen fixers. The overall taxonomic diversity of marine forms is comparable to that found in freshwater and terrestrial habitats. However, particular marine habitats (e.g., the intertidal and subtidal zones, coral reefs, salt marshes and the open ocean) often contain a characteristic and restricted diversity of forms which may differ markedly from season to season and from one geographical location to another[1,2].

This is particularly evident in the open ocean where only a few genera and species have been shown to occur abundantly and to be important components of the phytoplankton community. Principle among these are marine representatives of the genera *Synechococcus*, *Synechocystis*, *Trichodesmium* and *Richellia*. By contrast, the diversity of freshwater planktonic forms is much more extensive, encompassing over 100 species, 20 of which are capable of forming extensive water blooms[3]. This is probably due in part to the fact that freshwater planktonic habitats are diverse ranging from oligotrophic to highly eutrophic environments. The open oceans are relatively oligotrophic and the cyanobacteria that occur there reflect this in their growth requirements and sensitivities.

Marine representatives of the genus Synechococcus (sensu Rippka et al., 1979)[4] are small unicellular forms (0.6 x 1.4 um) that are abundant within the euphotic zone of the world's temperate and tropical oceans[5,6]. Marine representatives of the genus Synechocystis (sensu Rippka et al., 1979)[4] are novel unicellular forms capable of aerobic nitrogen fixation that have been isolated from the tropical Atlantic[7]. Members of the genera Trichodesmium and Richellia are filamentous forms capable of nitrogen fixation[2] that are found in the tropical oceans. Trichodesmium spp. are free living nonheterocystous forms, whereas Richellia spp. occur principally as intracellular symbionts in several species of diatoms[2].

The general principles of isolation and growth for cyanobacteria that are discussed elsewhere in this volume and in The Prokaryotes[8] are also applicable to marine planktonic forms.

Media

A variety of media has been used for the culture of marine cyanobacteria. Two in particular, one having a natural seawater base (medium MN[9] and the other having an artificial seawater base (medium ASN-III)[9] have been used successfully for the isolation and maintenance of pure cultures of a variety of marine cyanobacteria, mostly of coastal origin (e.g., intertidal and saltmarsh isolates). Neither of these media has been successful for the isolation and maintenance of the open ocean planktonic forms, necessitating the development of two new media designated SN[6], for the natural seawater medium and AN, for the artificial seawater medium. Their composition is shown in Table I. Medium SN is used for isolation and maintenance and medium AN is used experimentally when a defined medium is advantageous.

Preparation of Agar Plates

Solid media are prepared using Difco Bacto Agar that has been further purified using the following protocol[6]: 1/4 lb of agar is washed by stirring with 3 liters of double distilled water in a 4-liter beaker. After 30 min of stirring, the agar is allowed to settle, the wash water is siphoned off and the agar filtered onto Whatman F4 filter paper in a Buchner funnel. This procedure is repeated once more or until the filtrate is clear. The agar is then washed with 3 liters of 95% ethanol followed by a final 3-liter wash with analytical grade acetone. The agar is then dried at 50°C in glass baking dishes for 2-3 days and stored in a tightly covered container. Solid media prepared with the purified agar at a final concentration of 0.6% are sufficiently stable for streaking.

To prepare 40 agar plates from 1 liter of medium, the three following solutions are prepared and autoclaved separately: 1) 750 ml of filtered seawater in a teflon bottle, 2) 6.0 gm super-clean agar in 200 ml double distilled water in a 2 liter glass flask, and 3) the mineral salts for 1 liter of medium (Table I), in 50 ml double distilled water in a 125 ml glass flask. After autoclaving, the seawater and minerals are added to the agar flask. Vitamins[10] (Table I) and sterile sodium sulfite (2 mM final concentration) are added aseptically to the hot agar solution which is then cooled to 50° before the plates are poured. It is critical that the surface of agar plates be dry prior to streaking. Following inoculation the plates are stored upside down in clear plastic vegetable crispers to minimize evaporation and contamination by fungi. Colonies appear in 2-4 weeks and are then removed from the agar surface with drawn Pasteur pipettes, inoculated into liquid media and allowed to grow up between successive streakings.

Enrichment Cultures

Two media have been used for the enrichment of marine *Synechococcus*, medium SN in which the nutrients are diluted 10-fold and supplemented with 100 uM ammonium chloride (6) and medium F/40 supplemented with 10 uM ammonium chloride[11]. The sole strain of *Richellia* was enriched in the diluted SN medium used for *Synechococcus*. Enrichments for *Synechocystis* have been made in medium SN diluted 10-fold from which the combined nitrogen was omitted. The sole isolate of *Trichodesmium* was enriched in medium F/2[11,12].

Purification of Isolates

Isolates of *Synechococcus*, *Synechocystis* and *Richellia* have been purified by repeated streaking on solid medium (Table I). The isolate of *Trichodesmium* was purified by putting a clump of filaments in the center of an agar plate containing the solid medium in Table I, placing the plate in a light gradient to induce phototaxis and then cutting out agar blocks containing single filaments that had glided away from contaminating bacteria.

Stock Cultures

Axenic stock cultures are maintained in 50 ml of liquid medium in 125 ml glass flasks; *Synechococcus* and *Richellia* strains in full strength SN medium, *Synechocystis* in SN medium without combined nitrogen, and *Trichodesmium* in medium SN diluted four-fold. The conditions of growth used for the isolation, purification and maintenance of these cyanobacteria are shown in Table II. Stock cultures are transferred at one to three week intervals using heavy inocula.

Representative strains of these cyanobacteria are available from the culture collection of the Bigelow Laboratory for Marine Science, Boothbay Harbor, Maine 04575 U.S.A. The complete collection is housed at the Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543 U.S.A..

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Table I

COMPOSITION OF MEDIA

Ingredient	Amount $liter^{-1}$		
	Medium SN		Medium AN
	Liquid medium	solid medium	
Double distilled water			250 ml
250 ml	1000 ml		
Filtered seawater	750 ml	750 ml	--
NaCl	-	-	250 mM
$MgSO_4$ $7H_2O$	-	-	30 mM
$MgCl_2$ $6H_2O$	-	-	20 mM
$CaCl_2$ $2H_2O$	-	-	10 mM
KCl	-	-	10 mM
EDTA (disodium salt)	15 uM	15 uM	15 uM
$NaNO_3$	9 mM	2.5 mM	9 uM
NH_4Cl	-	100 uM	-
K_2HPO_4	90 uM	22.5 uM	90 uM
Na_2CO_3 H_2O	100 uM	100 uM	200 uM
$NaHCO_3$	-	-	3 mM*
Cyano-Trace Metals	1.0 ml	0.25 ml	1.0 ml
Va vitamin mix**	0.5 ml	0.5 ml	0.5 ml
Sodium sulfite	-	2 mM	-
Washed agar	-	6.0 gms	-
Final pH	8.0	8.0	8.3

Table I (Continued):

CYANO-TRACE METALS:***

Compound:	gm liter^{-1}
$ZnSO_4$ $7H_2O$	0.222
$MnCl_2$ $4H_2O$	1.4
$CO(NO_3)_2$ $6H_2O$	0.025
Na_2MoO_4 $2H_2O$	0.390
Citric Acid H_2O	6.250
Ferric Ammonium Citrate	6.0

Va VITAMIN MIX*** (10)

Vitamins:	gm liter^{-1}
Thiamine HCl	0.2
Biotin	0.001
B_{12}	0.001
Folic acid	0.002
PABA	0.01
Nicotinic acid	0.1
Inosotol	1.0
Calcium pantothenate	0.2
Pyridoxine HCL	0.1

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*Added after autoclaving.

**Va vitamin mix is added during enrichment and purification, in most instances purified stock cultures are maintained with only vitamin B_{12} (1 ug/L).

***Dissolve each compound separately, then add together and bring to 1 liter.

Table II

	CULTURE CONDITIONS		
	Temperature (°C)	Light regime	Light intensity* (uEin $m^{-2}sec^{-1}$)
Synechococcus	20-25	Constant or LD** (14/10)	10-30
Synechocystis	26-30	LD (14/10)	10-40
Trichodesmium	22-27	LD (14/10)	10-20
Richelia	20-25	Constant or LD (14/10)	10-30

* Light is supplied with Vitalux fluorescent lamps, Luxor Light Products, Lindhurst, NJ.

**LD - light/dark cycle in hours.

APPENDIX II

A CYANOBACTERIUM CAPABLE OF SWIMMING MOTILITY

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SCIENCE

A Cyanobacterium Capable of Swimming Motility

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A Cyanobacterium Capable of Swimming Motility

Abstract. *A novel cyanobacterium capable of swimming motility was isolated in pure culture from several locations in the Atlantic Ocean. It is a small unicellular form, assignable to the genus* Synechococcus, *that is capable of swimming through liquids at speeds of 25 micrometers per second. Light microscopy revealed that the motile cells display many features characteristic of bacterial flagellar motility. However, electron microscopy failed to reveal flagella and shearing did not arrest motility, indicating that the cyanobacterium may be propelled by a novel mechanism.*

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The cyanobacteria (blue-green algae) are morphologically and developmentally one of the most diverse groups of prokaryotes, ranging from simple unicellular forms to complex filamentous organisms, many of which display gliding motility during all or part of their developmental cycles. Gliding motility is expressed only when cells are in contact with a solid surface, and may reach speeds of several micrometers per second in some of the larger filamentous forms (*1*). The mechanism responsible for gliding motility remains one of the major unsolved problems in prokaryotic cell biology. Notably, visible external organelles have not been associated with this form of movement (*1*). In contrast, "swimming" (movement through a homogeneous liquid medium) generated by flagella is widespread among other groups of prokaryotes (*2*).

We report here the isolation and characterization of a cyanobacterium capable of swimming motility. To our knowledge, this is the first substantiated report of swimming motility in cyanobacteria (*3*). The swimming strains appear to lack bacterial flagella or other visible external organelles of motility, even though their swimming behavior has many of the features of flagellar motility when examined by light microscopy.

The swimming strains are marine unicellular forms belonging to the genus *Synechococcus* sensu Rippka (*4*). Members of this genus have been described that are capable of surface-associated irregular gliding at speeds of a few micrometers per minute (*1*, *5*). This property is rare, occurring in only 1 of 28 strains of *Synechococcus* in the Pasteur Institute culture collection (PCC 6910) (*6*). Representatives of the genus occur at high concentrations (*7*) and contribute significantly to primary biomass production (*8*) in the world's temperate and tropical oceans. As part of a study to determine the role of these cyanobacteria in the marine environment, we have isolated more than 40 closely related strains of marine *Synechococcus*.

Six strains of *Synechococcus* capable of swimming motility have been isolated (*9*), five from the Sargasso Sea and one from the temperate south Atlantic Ocean (Table 1). The swimming strains of *Synechococcus* are morphologically and physiologically similar to the many open-ocean nonmotile isolates in the Woods Hole culture collection. The swimming strains have coccoid to rod-shaped cells 0.7 to 0.9 μm in diameter and 1.25 to 2.5 μm in length (Fig. 1A) that divide by binary fission in a single plane. They have a typical synechococcoid ultrastructure (Fig. 1B) that is indistinguishable in thin sections from that of the nonmotile marine isolates of *Synechococcus*. The marine isolates of *Synechococcus*, like all other cyanobacteria, contain chlorophyll a as their primary photosynthetic pigment and phycobiliproteins as accessory light-harvesting pigments. The motile strains contain phycoerythrin as their predominant phycobiliprotein, a feature that characterizes all the open-ocean isolates of *Synechococcus* (*10*).

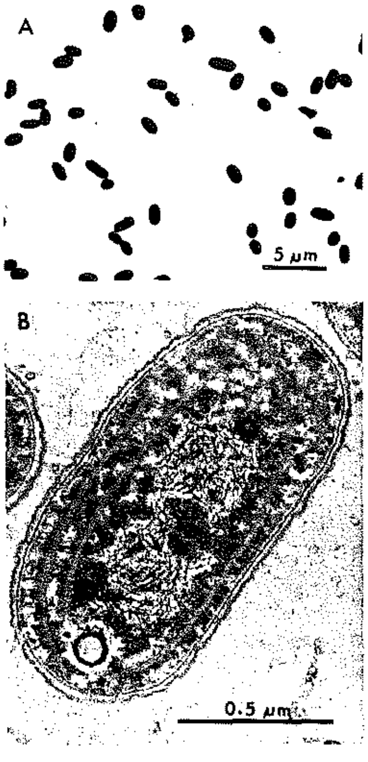

Fig. 1. (A) Phase-contrast photomicrograph of *Synechococcus* strain WH8011. (B) Transmission electron micrograph of a thin section of *Synechococcus* strain WH8112.

The swimming strains share three ecologically important physiological traits with all the open-ocean strains of *Synechococcus* so far examined. They are obligately marine, as evidenced by elevated requirements for Na^+, Cl^-, Mg^{2+}, and Ca^{2+} for growth (*11*). They are obligate photoautotrophs that cannot use sugars or organic acids as sole carbon sources either photoheterotrophically or chemoheterotrophically (*12*). They cannot fix nitrogen in the presence of air, nor can nitrogenase be induced under anaerobic conditions (*13*).

The DNA base ratios of the four axenic swimming strains (Table 1) form a tight cluster in the middle of the range for the entire open-ocean strain cluster, 54 to 62 moles percent guanine plus cytosine (*14*).

Swimming cells in liquid cultures examined during exponential growth by phase contrast or epifluorescence light microscopy had the following properties. More than half of the cells were actively motile, and moved through the medium without touching any surface. Long rod-shaped cells swam in relatively straight paths, whereas more coccoid cells looped and spiraled about, indicating that cell morphology affects directionality (Fig. 2). Individual cells sometimes rotate end over end at 3 to 5 revolutions per second. Occasionally cells attached themselves to the slide or cover slip and pivoted about one pole clockwise or counterclockwise at 0.5 to 1 revolution per second. Attached cells were never observed to glide along a surface.

Swimming speed, measured from video tracings of individual cells at 1/6-second intervals, ranged from 5 to 25 μm/sec. The variation in speed was associated with the age and condition of individual cultures and not with variations in light intensity. Translocation was accompanied by cell rotation, as demonstrated by swimming cells in the process of division in which the two daughter cells were attached at an angle; thus both thrust and torque contributed to their movement. Swimming cells of *Synechococcus* could often be observed for more than 1 minute without showing interruptions in translocation analogous to the characteristic "tumbles" of flagellated bacteria (*2*). As in other forms of movement in prokaryotes, experiments with a variety of inhibitors indicate that swimming in *Synechococcus* is driven by proton motive force (*15*).

The swimming strains of *Synechococcus* did not show photokinesis or photophobic responses, nor did they demonstrate positive or negative phototaxis in liquid or on semisolid preparations (*16*). Rates of motility were similar for cells incubated with light of different intensities or kept in the dark for up to 12 hours. This suggests that, in the dark, motility may be driven by oxidative metabolism of glycogen reserves in a manner similar to that shown for gliding in *Oscillatoria princeps* (*17*). Lack of a photophobic response by swimming *Synechococcus* is indicated because they did not accumulate in a light trap experiment and they did not reverse direction or alter their swimming pattern in response to a rapid reduction in light intensity (*1*).

The most striking feature of these strains is the apparent absence of flagella or any other organelle that might be associated with motility. Transmission electron microscopy of negatively stained preparations did not reveal flagella, whereas flagella were routinely observed in control bacteria examined by this technique (*18*). Examination of thin sections by TEM did not reveal flagella or unusual features in the outer cell envelope or the periplasmic space (Fig. 1B) (*19*). Preliminary examination by high-intensity dark-field light microscopy, a technique that allows visualization of individual bacterial flagella (*20*), also did not reveal flagella.

Shearing experiments provided further evidence that the swimming strains of *Synechococcus* lack conventional bacterial flagella. Brief periods of blending are sufficient to shear flagella from bacterial cells without affecting cell viability (*21*). We conducted shearing experiments with the motile strains of *Synechococcus* grown in basal medium (*9*) with 8.75 m*M* sodium nitrate. Control bacteria, *Escherichia coli* H102 and *Vibrio parahaemolyticus*, which have unsheathed peritrichous and sheathed polar flagella, respectively, lost motility after 10 to 15 seconds of shearing; whereas swimming in *Synechococcus* was not impaired even after 15 minutes of continuous blending (*22*).

Several properties of *Synechococcus* indicate that the ecological advantage of motility is not associated with responses to light. First, the motile cells lack classical photokinetic and photophobic behavior. Second, a cell swimming constantly in a straight line at 25 μm/sec could cover only 2 m in 24 hours, a distance of almost no consequence with respect to light quantity or quality in the open ocean. Finally, because of their size, *Synechococcus* cells behave like colloidal particles in seawater, which results in their movement being dominated by the physical mixing processes in the water column. It seems more likely that motility enables these cyanobacteria to respond chemotactically to nutrient-enriched micropatches or microaggregates, which are currently believed to be important to nutrient cycling in the euphotic zone (*23*).

Table 1. Isolation data and DNA base composition of swimming strains of *Synechococcus*.

Strain	Isolation data			DNA base ratio (mol % GC)*
	Location	Date	Depth	
		Axenic		
WH8011	34°N, 65°W	June 1980	25 m	59.3
WH8103	28°N, 67°W	March 1981	Surface	58.9
WH8112	36°N, 66°W	October 1981	20 m	59.8
WH8113	36°N, 66°W	October 1981	60 m	60.5
		Nonaxenic		
WH8401	39°S, 49°W	March 1984	10 m	ND†
WH8406	30°N, 77°W	December 1984	50 m	ND

*Moles of quanine plus cytosine per 100 mol of DNA. †ND, not determined.

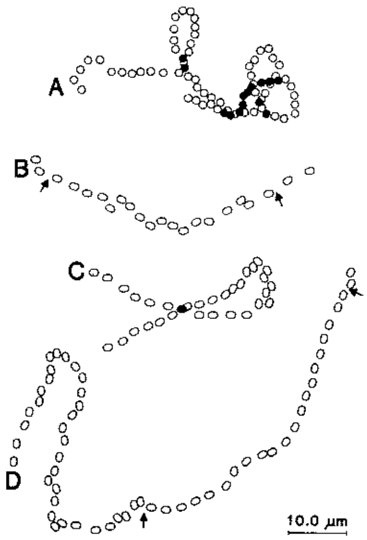

Fig. 2. Tracings of swimming *Synechococcus* at 1/6-second intervals, illustrating patterns and speed of movement. Sealed wet mounts of cell suspensions were recorded by phase-contrast video microscopy. (A and B) Strain WH8103. (C and D) Strain WH8113. Filled tracings show the path of a cell where it crosses previous tracings. Average speed between arrows: (B) 13.3 μm/sec and (D) 15.5 μm/sec.

The existence of swimming cyanobacteria will be particularly fascinating if future studies confirm that the swimming strains lack flagella or other external organelles. It is difficult to imagine how a cell the size and shape of *Synechococcus* could be driven through a liquid medium at 25 μm/sec by any other known mechanism.

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22. Shearing experiments were conducted with a Waring two-speed drive unit and an Eberbach MicroJar modified with an ice chamber surrounding the vessel and operated at 17,000 rev/min (Thomas Scientific). *Escherichia coli* H102 was grown at 37°C in Difco Bacto-Penassay broth made in both distilled water and 3/4 strength seawater. *Vibrio parahaemolyticus* was grown at 25°C in medium (*9*) with 5 m*M* ammonium chloride, 1 percent Difco Bactopeptone, and 0.2 percent Difco yeast extract.
23. J. C. Goldman, *Bull. Mar. Sci.* **35**, 462 (1984).

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