Role of Chemotaxis in Establishing a Specific Nitrogen-Fixing Cyanobacterial-Bacterial Association

Abstract. *A specific association with the cyanobacterium* Anabaena oscillarioides *was established by positive bacterial (pseudomonad) chemotaxis to* Anabaena oscillarioides *heterocysts. This association enhanced nitrogen fixation in* A. oscillarioides, *and positive chemotaxis was particularly strong during periods of active nitrogen fixation. Addition of compounds known to elicit positive chemotaxis in pseudomonads interfered with the establishment of the association, while removal of these compounds led to reestablishment of the association.* Anabaena oscillarioides *excretion products, some of which are exuded from heterocyst–vegetative cell junctions, are likely to be responsible for positive chemotactic responses. Chemotaxis-controlled associations such as this one explain in part why aquatic bacterial-algal and bacterial-particle associations occur sporadically and are heterogeneously distributed in time and space.*

Chemotaxis, the ability of microorganisms to be attracted or repelled along chemical concentration gradients, is a characteristic among diverse, motile aquatic taxa (*1*). As early as 1888 Pfeffer (*2*) demonstrated the ability of the filamentous green alga *Spirogyra* to aerotactically draw bacteria to extracellular regions bordering the alga's ribbon-shaped chloroplast during photosynthetic periods. Filamentous cyanobacteria (blue-green algae) similarly exhibit close and specific associations with bacteria during active growth. Associations range from the general presence of a variety of heterotrophic bacteria in mucilaginous sheaths to highly specific associations between physiologically distinct cyanobacterial cells and bacterial epiphytes (*3*).

The N_2-fixing genera *Anabaena* and *Aphanizomenon* form biochemically and morphologically distinct heterocysts, thick-walled cells considered to be the sites of N_2 fixation (*4*). Heterocysts are often covered by bacteria, while neighboring non–N_2-fixing vegetative cells remain relatively free of bacterial epiphytes (*3*). Heterocyst-bacterial associations are particularly prevalent during N_2-fixing blooms in nature, but they can also be maintained in laboratory populations (*3*). Independent studies have shown that cellular N_2 fixation and growth of host cyanobacteria are enhanced in these types of associations (*5*). We implicated localized removal of O_2 in regions supporting actively respiring bacterial epiphytes as being one mechanism maintaining optimal N_2 fixation rates in such associations (*6*). The extent to which these N_2-fixing genera form nuisance blooms in natural waters appears to be positively correlated with the frequency and magnitude of heterocyst-specific bacterial associations (*3*).

Despite the ecological roles such microbial consortia play in promoting and sustaining cyanobacterial bloom activities, virtually nothing is known of the physiological mechanisms responsible for establishing and maintaining such associations. Microscopic observations of the colonization of axenic *Anabaena oscillarioides* populations by natural microflora indicate that motile bacteria can readily discriminate heterocysts from vegetative cells. The ability to discriminate is particularly acute during N_2-fixing periods. Bacteria interacting with *A. oscillarioides* filaments can be observed to "bump" into both vegetative cells and heterocysts. As a rule, they do not immediately adhere to either cell type on initial encounter, but rather exhibit complex flagellar rotation patterns as described for other systems (*1*). When bacteria encounter heterocysts flagellar rotation is often enhanced; typically a 5- to 10-minute period of rapid flagellar motion takes place. This motion precedes attachment at the heterocyst–vegetative cell junction. When bacteria are situated in this region flagellar motion stops. Once attachment has taken place, active growth of both host and epiphyte can be observed. However, bacteria rarely penetrate cyanobacterial cell walls.

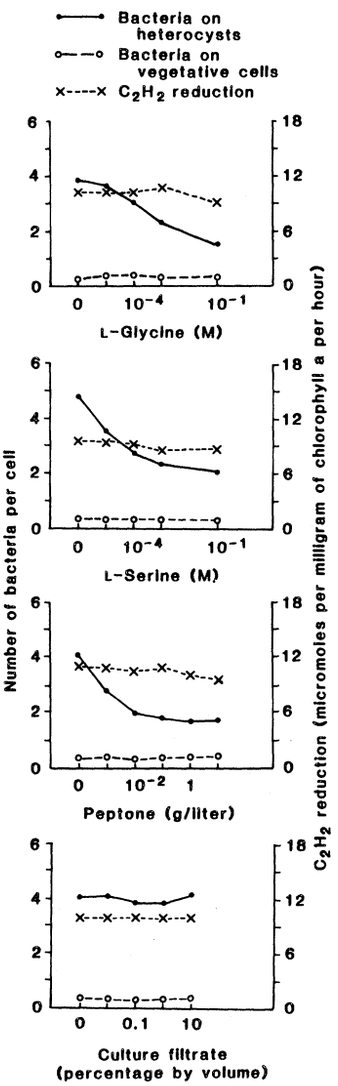


Fig. 1. Effects of various concentrations of L-glycine, L-serine, peptone, and pseudomonad culture filtrate (as a control) on the frequencies of pseudomonads attached to heterocysts and vegetative cells of *A. oscillarioides*. Concurrently, none of the above substrate additions significantly altered C_2H_2 reduction in *A. oscillarioides* in a 1-hour incubation period.

Bacterial density on heterocysts is closely related to rates of N_2 fixation. When N_2 fixation is inhibited by the addition of NH_4^+ there is a significant ($P < 0.001$, Student's t-test) decrease in the number of bacterial epiphytes or heterocysts (Table 1). In contrast, active N_2-fixing populations of *A. oscillarioides* readily form and maintain heterocyst-bacterial associations (Table 1). Pseudomonads commonly associated with *A. oscillarioides* show the following characteristics: (i) a stimulatory effect of isolates on N_2 fixation when combined with axenic host populations (N_2 fixation occurs exclusively in the host and not in associated bacteria); (ii) strong affinity for *A. oscillarioides* heterocysts, even after having been separated from their cyanobacterial hosts for 10 to 20 generations; and (iii) chemotactic behavior, with a variety of L–amino acids eliciting strong positive responses (*5*).

We postulated that chemotaxis might be involved in these associations and performed a series of experiments to evaluate this possibility. Serine, glycine, and peptone (a complex bacterial medium containing peptide residues and inorganic salts), which previously elicited chemotactic responses in pure cultures of pseudomonads, were individually added over a range of concentrations, each in triplicate, to N_2-fixing cultures of

Table 1. Effects of 1 mM NH_4^+ (as NH_4Cl) on C_2H_2 reduction and bacterial attachment to heterocysts and vegetative cells of *A. oscillarioides*. T_0 indicates conditions before addition of NH_4^+, while T_1 indicates conditions after a 2-hour incubation with or without NH_4^+.

Treatment	Number of bacteria per heterocyst	Number of bacteria per vegetative cell	C_2H_2 reduction (micromoles per milligram of chlorophyll a per hour)
T_0	3.71 ± 0.08	0.18 ± 0.02	9.23 ± 0.13
T_1 (with NH_4^+)	1.92 ± 0.07	0.23 ± 0.04	0.47 ± 0.04
T_1 (without NH_4^+)	3.85 ± 0.11	0.16 ± 0.02	8.39 ± 0.17

A. oscillarioides and pseudomonads in stoppered 50-ml Erlenmeyer flasks. The flasks were placed on an oscillating shaker (200 rev/min at 25°C) under photosynthetically active radiation (200 μE/m^2-sec). An additional set of flasks receiving the same treatments was examined for nitrogenase activity responses by the acetylene reduction assay (*7*). After 1 hour of incubation the flasks were removed and the contents were preserved at 4°C with 0.01M sodium cacodylate–buffered (pH 7.8) glutaraldehyde (2.5 percent by volume). A 250-μl sample was then observed at ×1000 with a Zeiss model K phase-contrast microscope. At least 150 heterocysts and 1000 vegetative cells were randomly examined from each preparation to determine mean numbers of bacteria attached per cell and to observe patterns of bacterial distributions.

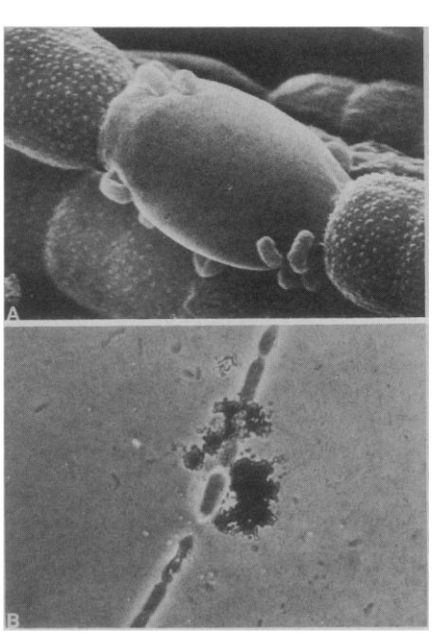
In a second set of experiments a pseudomonad previously isolated from *A. oscillarioides* and maintained on 0.25 percent peptone agar was transferred to 0.1 percent liquid peptone containing 10 nM tritiated methyl thymidine having a specific activity of >60 mCi/mmol (final activity, 10.5 μCi per milliliter of culture medium). At least eight generations (10 to 12 hours) were grown under these conditions in logarithmic phase. Pseudomonad yields were then centrifuged at 1600 rev/min, washed twice with unlabeled medium, and released into axenic cultures of *A. oscillarioides*. *Anabaena oscillarioides* and pseudomonads were allowed to interact for 30 minutes and 1 hour under illuminated conditions in 50-ml Erlenmeyer flasks placed on an oscillating shaker. Specific associations of labeled pseudomonads and *A. oscillarioides* formed during these periods were detected by microautoradiography. A gentle filtration and freeze fixation technique was used to prepare autoradiographs (*8*). Processed autoradiographs were viewed and photographed with phase-contrast optics at ×1000.

The addition of all three soluble chemotactic substrates led to significant ($P < 0.01$) reductions in the number of bacteria epiphytic on heterocysts, the negative effect being concentration-dependent (Fig. 1). While dramatically reducing bacterial attachment to heterocysts, amino acids and peptone had little effect on nitrogenase activity within 2 to 3 hours (Fig. 1). Positive chemotactic agents caused fewer bacteria to be located around heterocysts. Bacteria were observed to swim more randomly, albeit actively, in cultures receiving chemotactic agents. We interpret these alterations in bacterial swimming and attachment characteristics to the presence of chemoattractants overriding the attraction provided by chemical gradients surrounding heterocysts. On removal of chemoattractants (by filtration and rinsing of *A. oscillarioides* and associated bacteria), heterocyst-specific attachment resumed.

Pseudomonads labeled with ^3H readily and specifically preferred heterocysts and heterocyst–vegetative cell junctions to vegetative cells when released into axenic N_2-fixing *A. oscillarioides* cultures (Fig. 2). Microautoradiographic examinations revealed eight times as many bacteria attaching to heterocysts as to vegetative cells after release and a 2-hour incubation. Viability (as judged by motility) was maintained among 98.5 percent of pseudomonad cells released during such incubations. After a 4-hour incubation period, the presence of pseudomonads enhanced *A. oscillarioides* chlorophyll a–specific acetylene reduction rates 23 percent over those in axenic cultures (Fig. 3).

Chemotaxis played a key role in the establishment and maintenance of this cyanobacterial-bacterial association. Products of N_2 fixation, which appear to be excreted at heterocyst–vegetative cell junctions (*5*), are implicated in eliciting positive chemotactic responses among diverse naturally occurring heterotrophic bacteria as well as in specifically isolated populations. Amino acids are probable chemotactic agents, since they are known to be excreted by N_2-fixing *Anabaena* (*9*) and elicit very strong positive chemotactic responses in pseudomonads (*10*). Our study does not preclude other, yet to be identified, associations between bacteria and nonnitrogenous attractants released from heterocysts, vegetative cells, and akinetes. Certainly, observations of bacteria oriented in mucilaginous excretions bordering vegetative cells and akinete-associated bacteria abound (*3*), but the attractants responsible for such bacterial recruitment and growth remain unknown.

We have shown that biotic attachment surfaces represented by cyanobacteria are far from homogeneous with regard to



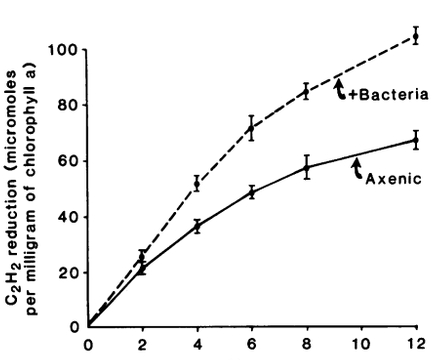


Fig. 2 (left). (A) Scanning electron micrograph of pseudomonads specifically attached to a heterocyst of *A. oscillarioides*. Note the tendency to favor the polar regions of heterocysts as attachment sites. (B) Microautoradiograph illustrating the specific accumulation of ^3H-labeled pseudomonads around an *A. oscillarioides* heterocyst. Groups of exposed (darkened) silver grains cover ^3H-labeled bacteria in this preparation. Fig. 3 (above). Chlorophyll a–specific C_2H_2 reduction in axenic and bacterially colonized (by pseudomonads) populations of *A. oscillarioides*. Triplicate determinations were made over the indicated time intervals after the release of pseudomonads in *A. oscillarioides* cultures. Purified pseudomonad isolates consistently failed to reduce C_2H_2.

surface chemical conditions conducive to specific associations with bacteria. Chemical gradient formation and patchiness near surfaces promote the establishment of physiologically diverse epiphytes, including pseudomonads, whose individual metabolic characteristics lead to enhancement of a localized metabolic process (N_2 fixation) in host cyanobacteria. Parallel associations between vegetative cells and bacterial populations may specifically serve to enhance photosynthetic production by recycling CO_2 and inorganic nutrients (*11*).

Although chemotaxis has been shown to play a role in promoting diverse aquatic algal-bacterial associations, this process may also be instrumental in establishing specific abiotic particle-bacterial interactions in marine and freshwater habitats. Both habitats represent very dilute pools of dissolved energetic and nutritional growth substrates, but such substrates are known to be concentrated on particle surfaces (*12*). The ability to chemotactically sense and seek nutrient-enriched surfaces would offer a distinct advantage under nutrient-limited conditions. Chemotaxis also complements other means of initiating bacterial attachment, including electrostatic forces, random encounters, and mutual accumulation of particles and bacteria along vertical density gradients in the water column. However, in contrast to these mechanisms, chemotaxis offers selectivity based on biologically active molecules released (by diffusion or excretion) from particle surfaces. A great deal of variability in types and magnitudes of microbial colonization has been reported among diverse suspended particles (*12, 13*). Such variability may in part be due to diverse chemotactic agents characterizing the spectrum of biotic and abiotic surfaces present in natural waters.

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A Hydrophobic Transmembrane Segment at the Carboxyl Terminus of Thy-1

Abstract. *The mode of integration of the glycoprotein thy-1 within the membrane has been controversial due to an apparent lack of a transmembr hydrophobic segment. Rat and mouse complementary DNA and genomic clo encoding the thy-1 molecule have been isolated and sequenced. These studies h enabled us to determine the intron-exon organization of the thy-1 gene. Furtherm they have revealed the existence of a sequence which would encode an extra segn (31 amino acids) at the carboxyl terminus of the thy-1 molecule. These extra an acids include a 20–amino acid hydrophobic segment which may be responsible integration of thy-1 within the plasma membrane.*

Thy-1 is a membrane glycoprotein found predominantly on the cell surface of thymocytes and brain cells (*1, 2*). Originally identified in mice, proteins similar to thy-1 are present in many species (*3–6*) although the distribution of thy-1 among hematopoietic cells seems to vary (*7, 8*). Thy-1 proteins isolated from rat and mouse brains have been sequenced and consist of a protein moiety of 111 and 112 amino acids, res tively (*9, 10*). These sequences v lacking a hydrophobic segment, whi necessary for integration of thy-1 wi the lipid bilayer of the membrane. has prompted speculation that thy- covalently linked to some hydroph component such as glycolipid which chors the thy-1 molecule to the m brane. The DNA sequence analyse

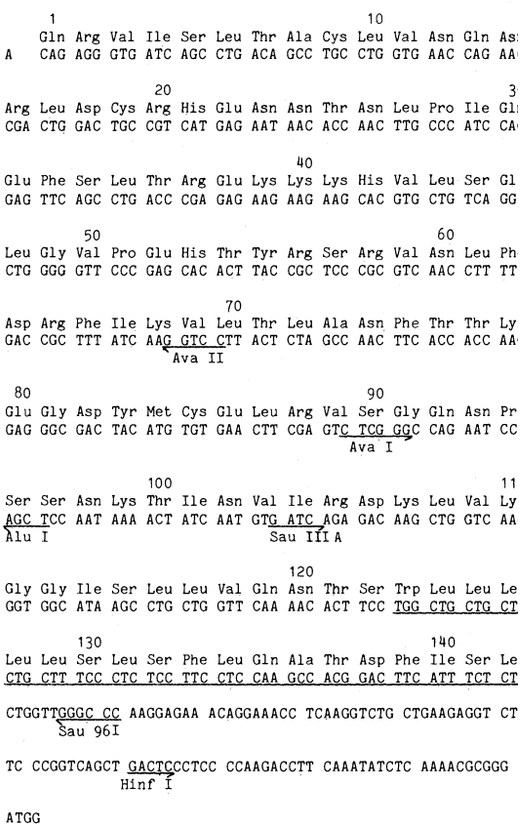

Fig. 1. The nucleotide sequence of the cDNA insert of the thy-1 cDNA clone pT86 and the predicted amino acid sequence of the complete rat thy-1 antigen. The DNA of pT86 was cleaved with restriction endonucleases (BRL) and fragments corresponding to the insert were purified by polyacrylamide gel electrophoresis and electroelution. Purified fragments were treated with calf intestinal alkaline phosphatase (Boehringer), labeled at both 5′ ends with T4 polynucleotide kinase plus [γ-^{32}P]ATP (*11*), and cleaved secondarily to generate subfragments with only one labeled end. The restriction enzyme sites shown and both Pst I sites are those that were labeled at the 5′ end. The subfragments were separated by acrylamide gel electrophoresis, electroeluted, and subjected to partial chemical degradation sequence analysis (*15*). Both strands of the insert cDNA were sequenced. The hydrophobic 20–amino acid segment is underlined and the termination codon is indicated by an asterisk.