The Phylogeny of Marine and Freshwater Caulobacters Reflects Their Habitat

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Caulobacter is a distinctive genus of prosthecate bacteria. Because caulobacters adhere to surfaces and are found in diverse locales, their role in oligotrophic environments and bacterial biofilm communities is of interest. The phylogenetic relationships of a group of marine and freshwater caulobacters were examined in part to address whether the taxonomic grouping of these bacteria (based primarily on morphological characters) was consistent with 16S rRNA sequence divergence. The caulobacters examined (9 marine and 11 freshwater species or strains) were affiliated with the alpha proteobacteria. They made up a diverse yet, with the possible exception of a strain of Caulobacter subvibrioides, coherent assemblage. The diversity was most apparent in a comparison of freshwater and marine isolates; an early divergence within the main caulobacter lineage generally corresponded to strains isolated from freshwater and marine habitats. The marine caulobacter assemblage was not exclusive; it also embraced strains of marine hyphomonads and Rhodobacter capsulatus. We hypothesize that these genera are derived from more ancestral caulobacters. Overall, the data are consistent with the interpretation that all of the caulobacters examined, with the possible exception of C. subvibrioides, are ancestrally related, albeit anciently, and that most often division by terrestrial and marine habitats corresponds to an early evolutionary divergence within the genus.

Given the simple shapes of most bacteria, morphologically conspicuous groups have long been a source of interest to general microbiologists. Among the better characterized of such bacteria are budding and/or prosthecate nonphototrophic bacteria. These include the genera *Hyphomicrobium*, *Hyphomonas*, *Pedomicrobium*, *Filomicrobium*, *Stella*, and *Caulobacter*. The taxonomy of these groups has relied primarily upon morphological criteria and required growth factors (27–29). In the case of caulobacters, beyond some consideration of the environment in which they are found, what visually appears to be a caulobacter is generally sufficient to be called one without challenge. This is due mainly to a lack of other defining physiological or metabolic traits, a recognized problem in the taxonomy of all of the prosthecate bacteria (27) and, indeed, other groups of environmentally relevant bacteria.

Such a paucity of factors upon which to base affiliations, aside from taxonomy issues, leads to a variety of questions concerning origins and coherence. Are the caulobacters derived from common ancestry (monophyletic), reflecting a single origin of their distinctive morphological and developmental characteristics? Or, do the structural components, notably the long stalk, represent the convergent discovery of a useful structural adaptation? For example, the stalk might be a means of increasing the surface area of the bacterium to enhance nutrient uptake that has been adopted by many species. Alternatively, the stalk with attendant adhesive at the tip (15) might reflect a common strategy to secure a position at the outer surface of complex bacterial biofilms formed on surfaces. A related question concerns evolutionary relationships between marine and freshwater species. Is specialization to life in marine and freshwater environments

an ancestral characteristic, or has their evolutionary history included transitions between freshwater and marine forms? For example, are some marine caulobacters derived from terrestrial forms and therefore secondarily modified to tolerate high salt concentration conditions?

A recent detailed morphological examination of a group of freshwater caulobacters isolated from wastewater treatment facilities indicated that most of the isolates were similar in many ways, including subtle shape characteristics and the presence of a uniformly similar paracrystalline surface (S) layer (16). Moreover, in that study hybridization probing with genes for the S-layer protein (33) and one of the flagellar filament proteins showed that most isolates could be identified with these gene probes, indicating similarity between the "typical" caulobacters found. On the other hand, restriction fragment length polymorphism analysis based on these gene probes did not successfully relate the isolates, indicating a greater genetic diversity than suggested by the phenotypic similarities.

Relatively little information is available concerning the genetic diversity and coherence of prosthecate bacteria, and there is even less that dissects individual genera. Early DNA hybridization (20) and more recent 5S and 16S rRNA sequence comparisons (14, 22, 35) suggested considerable diversity among representatives of certain of these genera and raised the question of their coherence. Only a modest amount of data applies specifically to caulobacters, and most is derived from 5S rRNA sequences. Given that the caulobacters are being actively examined for their range and contribution to biofilm communities and oligotrophic environments (3, 16, 17, 27) and receive considerable attention as model systems for prokaryotic development (21, 30), the intent of the present study was to evaluate more fully the genetic and phylogenetic diversity of freshwater and marine caulobacters by using comparative 16S rRNA sequencing.

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MATERIALS AND METHODS

Strains and growth conditions. The freshwater strains *Caulobacter crescentus* CB2A and CB15A, *C. subvibrioides* CB81, and *C. bacteroides* CB7 were from the laboratory culture collection of J. Smit and originally obtained from Nina Agabian. Their original isolation and characterization has been reported by Poindexter (25). The isolation of freshwater caulobacter strains FWC2, FWC14, FWC17, FWC18, FWC26, and FWC38 has also been reported; all but FWC2 were from wastewater treatment sources (16). The isolation and characterization of marine caulobacter strains MCS6, MCS10, MCS17, MCS18, and MCS24 has already been reported (3). Marine hyphomonas strain MHS3 was isolated in a fashion similar to that of the marine caulobacters as one of few hyphomonas strains able to grow on solid media (3).

All freshwater caulobacters were grown in peptone-yeast extract medium (20) supplemented with $MgCl_2$ and $CaCl_2$, each at 0.02%. Marine caulobacter strains were grown in salt-peptone-yeast extract medium, which was prepared by adding a 1/50 volume of 10% peptone–5% yeast extract to a 1.5% sea salt (Sigma Chemical, Co., St. Louis, Mo.) solution; both solutions were sterilized by autoclaving before mixing. For solid media, 15 g of agar was added per liter of medium. All cultures were grown at 30°C with shaking.

Cells were grown to mid-logarithmic density and centrifuged at 8,000 × *g* for 15 min. The cell pellets recovered were stored and transported at −70°C.

Sequencing of 16S rRNA. Total nucleic acid was extracted from 50 to 100 mg of cell pellets by using hot phenol and recovered as an ethanol precipitate (24). Nucleotide sequences were determined by the dideoxynucleotide method, by using reverse transcriptase and the 16S rRNA as the template (13). In addition to the standard deoxynucleotide reactions, a second set of reactions was run for most primers with inosine nucleotide substituted for guanosine (36). The inclusion of inosine resolved sequence ambiguities caused by premature termination or band compression in GC-rich regions of the transcript. Three universal primers for the small subunit rRNA (13) and four additional oligonucleotide primers were used to determine nearly complete 16S rRNA sequences (19). The sequences obtained have been deposited with GenBank and are also available from D. A. Stahl.

Sequences for 16S rRNA from *C. halobacteroides*, *C. maris*, and marine caulobacter strains VC1 and VC5 were obtained from Jed Fuhrman (14). The sequence of 16S rRNA from *C. crescentus* CB13 was obtained from Bert Ely.

Sequence analysis. Sequence similarities and evolutionary distances were determined as previously described (19), by considering only unambiguously homologous positions. All pairwise evolutionary distances were used to construct and evaluate phylogenetic trees. The optimal branching order was derived by using the computer algorithm of De Soete (5).

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences discussed in this report are M83796 to M83812, inclusive.

RESULTS AND DISCUSSION

Figures 1 and 2 display phylogenetic trees of caulobacters and related bacteria inferred by comparison of the 16S rRNA sequences determined in this study. Pairwise similarity values, from which these trees were inferred, are listed in Tables 1 and 2. These values were obtained from approxi-
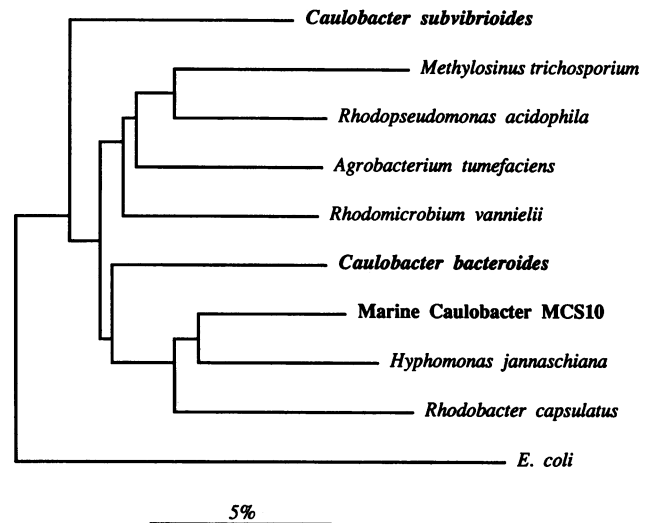

FIG. 1. Phylogenetic relationships among caulobacters and related bacteria listed in Table 1 as inferred from 16S rRNA sequence divergence (see Materials and Methods).

mately 1,200 and 1,000 homologous positions, respectively. Figure 1 displays general relationships of representatives of the major caulobacter lineages with other organisms affiliated with the alpha subdivision of proteobacteria.

All of the caulobacter sequences characterized (determined in this study and previous studies [12, 14, 22]) were affiliated with the alpha subdivision of proteobacteria (previously the purple bacteria). The alpha subdivision is one of four subdivisions (alpha, beta, gamma, and delta) now formally recognized within the proteobacteria (39). Together, the caulobacters analyzed in this study constituted a diverse collection that also included many noncaulobacter organisms of the alpha subdivision (e.g., *Rhodobacter cap-*
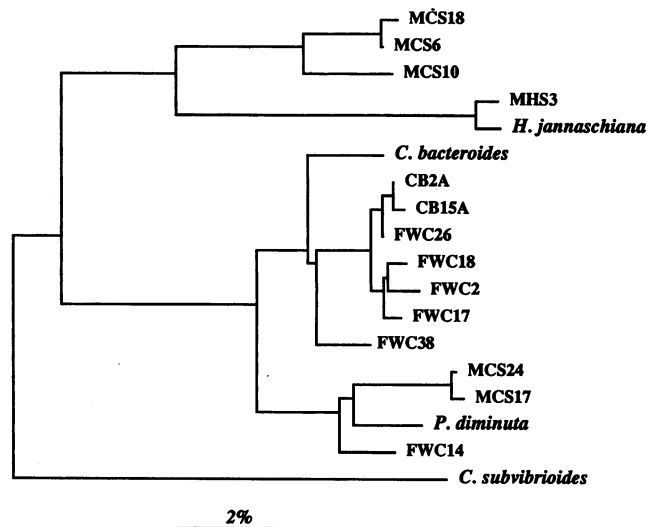

FIG. 2. Phylogenetic relationships among caulobacters, hyphomonads, and *P. diminuta*, listed in Table 2, as inferred from 16S rRNA sequence divergence. FWC refers to freshwater caulobacter isolates, while MCS and MHS designate marine caulobacter and hyphomonas strains, respectively.

TABLE 1. Pairwise 16S rRNA fractional similarity values for selected caulobacters and representatives
of the alpha subdivision of proteobacteria[a]

Organism	Fractional similarity of:									
	Rhodopseudomonas acidophila	*Methylosinus trichosporium*	*Agrobacterium tumefaciens*	*Rhodomicrobium vannielii*	*Escherichia coli*	*Caulobacter subvibrioides*	*Rhodopseudomonas capsulatus*	*Hyphomonas jannaschiana*	Marine caulobacter strain MCS10	*Caulobacter bacteroides*
Rhodopseudomonas acidophila										
Methylosinus trichosporium	0.901									
Agrobacterium tumefaciens	0.899	0.888								
Rhodomicrobium vannielii	0.900	0.882	0.895							
Escherichia coli	0.804	0.791	0.806	0.821						
Caulobacter subvibrioides	0.888	0.862	0.875	0.883	0.817					
Rhodopseudomonas capsulatus	0.871	0.845	0.881	0.867	0.788	0.851				
Hyphomonas jannaschiana	0.868	0.857	0.875	0.868	0.807	0.871	0.888			
Marine caulobacter strain MCS10	0.871	0.858	0.886	0.881	0.808	0.880	0.894	0.915		
Caulobacter bacteroides	0.895	0.866	0.883	0.886	0.809	0.876	0.867	0.877	0.893	

[a] Similarity values are based on approximately 1,200 nucleotide positions of continuous sequences. The 16S rRNA sequence of *Escherichia coli* (gamma subdivision of proteobacteria) is used as an outgroup. The GenBank accession numbers for those sequences not determined in this study are M29024 (*M. trichosporium*), M34128 (*R. acidophila*), M11223 (*A. tumefaciens*), M34127 (*R. vannielii*), and M34129 (*R. capsulatus*).

sulatus). The most distantly related of the caulobacters characterized were associated at approximately 88% 16S rRNA sequence similarity (Tables 1 and 2). For reference, studies of other phylogenetically coherent bacterial groups have shown that 50% DNA similarity corresponds to approximately 98 to 99% 16S rRNA sequence similarity (2, 6). The more general affiliation of budding and prosthecate bacteria with the alpha subdivision was reported earlier by Stackebrandt et al. (35).

A striking observation in the present study was the identification of an early divergence, not considering *C. subvibrioides* (see below), within the genus *Caulobacter*. This is defined in Fig. 1 by the divergence of *C. bacteroides* and marine strain MCS10. The specific affiliation of these two lines of descent was generally resistant to altered compositions of sequences used for phylogenetic inference. Notably, affiliation with either one of the two phylogenetically distinct lines of descent (ca. 88 to 90% similarity) generally corresponded to a marine or freshwater habitat.

One primary line of descent was composed exclusively of marine caulobacters (Fig. 2). This included MCS6, MCS10, and MCS18. It also included *C. maris*, *C. halobacteroides*, and caulobacter strains VC1 and VC5. These latter relationships were inferred from a partial sequence set (14) but are not included in Fig. 1 because of difficulties in representing comparisons of different-size data sets.

The other line of descent housed the freshwater caulobacters but was not exclusive, since it contained two marine forms. The freshwater or terrestrial caulobacters examined, in general, constituted a phylogenetically more restricted group. That is, none of the freshwater caulobacters characterized were affiliated with the marine line of descent. In a recent study of caulobacters isolated from wastewater treatment systems, we reported that most of the caulobacters noted in samples were similar in many morphological respects; all had short stalks, a crescent cell shape, a hexagonally packed paracrystalline S layer, and genomic DNA that hybridized with probes for the S-layer protein gene and a flagellar filament gene of *C. crescentus* CB15 (16). These typical caulobacters were in many ways indistinguishable

from *C. crescentus* strains that have been extensively studied and, on the basis of the current taxonomic scheme, are either *C. crescentus* or *C. vibrioides* (26). In the present study, these caulobacters (FWC2, FWC17, FWC18, and FWC26 and *C. crescentus* CB2A and CB15A) grouped closely (ca. 99% similarity), indicating that the morphological similarity was a reasonable predictor of relatedness. Although only a limited sequence was available for *C. crescentus* CB13, this strain also was clearly affiliated with the typical group (data not shown). Even though laboratory versions of this strain do not have an S layer, there are indications that it was lost by mutation during years of maintenance (33, 34) (a common finding with S layers of other bacteria [31]); in other respects, it is phenotypically similar to the typical caulobacters. Although the total number of terrestrial isolates examined was relatively small and emphasized recent isolates from wastewater treatment facilities, our working hypothesis is that this group represents a major lineage of terrestrial caulobacters.

The apparent exception to this pattern was the strain of *C. subvibrioides* examined in this study, which was seen to be only peripherally related to the greater caulobacter assemblage, although it had the morphological characteristics (i.e., the presence of a polar stalk contiguous with the cell membranes, a holdfast at the stalk distal tip, and a single polar flagellum on the swarmer cell [25, 32]) that presently define caulobacters. Thus, this again speaks to the inherent limitations of the use of morphology for inferring specific relationships and also implies an early origin of the caulobacter phenotype within the alpha subdivision. Although the marine hyphomonads and *R. capsulatus* appear to be derivative of caulobacter ancestry, we cannot establish with certainty that *C. subvibrioides* is ancestral to the alpha subdivision representatives displayed in Fig. 1. The branching order of the earliest divergences among the alpha subdivision organisms displayed in Fig. 1 demonstrates some instability, with an altered composition of organisms used for analysis.

The comparative study of freshwater caulobacters detected in wastewater treatment systems also revealed a

TABLE 2. Pairwise 16S rRNA fractional similarity values for the caulobacters and hyphomonads characterized in this study[a]

Organism	Caulobacter subvibrioides CB81	Hyphomonas jannaschiana	Marine hyphomonas strain MHS3	Marine caulobacter strain MCS10	Marine caulobacter strain MCS6	Marine caulobacter strain MCS18	Caulobacter bacteroides CB7	Marine caulobacter strain MCS17	Marine caulobacter strain MCS24	Pseudomonas diminuta	Freshwater caulobacter strain FWC14	Freshwater caulobacter strain FWC38	Freshwater caulobacter strain FWC17	Freshwater caulobacter strain FWC2	Freshwater caulobacter strain FWC18	Freshwater caulobacter strain FWC26	Caulobacter crescentus CB15A	Caulobacter crescentus CB2A
Caulobacter subvibrioides CB81																		
Hyphomonas jannaschiana	0.869																	
Marine hyphomonas strain MHS3	0.869	0.992																
Marine caulobacter strain MCS10	0.875	0.912	0.912															
Marine caulobacter strain MCS6	0.873	0.917	0.919	0.973														
Marine caulobacter strain MCS18	0.871	0.916	0.918	0.970	0.997													
Caulobacter bacteroides CB7	0.874	0.879	0.880	0.897	0.902	0.899												
Marine caulobacter strain MCS17	0.869	0.874	0.874	0.891	0.887	0.885	0.951											
Marine caulobacter strain MCS24	0.871	0.875	0.875	0.892	0.888	0.886	0.952	0.997										
Pseudomonas diminuta	0.876	0.883	0.883	0.898	0.896	0.894	0.954	0.971	0.972									
Freshwater caulobacter strain FWC14	0.879	0.882	0.882	0.891	0.891	0.889	0.964	0.972	0.973	0.977								
Freshwater caulobacter strain FWC38	0.882	0.879	0.879	0.904	0.907	0.906	0.974	0.949	0.950	0.953	0.962							
Freshwater caulobacter strain FWC17	0.874	0.888	0.880	0.897	0.899	0.896	0.973	0.944	0.945	0.947	0.956	0.978						
Freshwater caulobacter strain FWC2	0.874	0.883	0.883	0.897	0.899	0.896	0.969	0.940	0.941	0.943	0.952	0.970	0.992					
Freshwater caulobacter strain FWC18	0.876	0.881	0.881	0.898	0.900	0.897	0.971	0.942	0.943	0.945	0.954	0.976	0.992	0.992				
Freshwater caulobacter strain FWC26	0.877	0.880	0.880	0.901	0.903	0.900	0.978	0.945	0.946	0.951	0.961	0.983	0.995	0.987	0.993			
Caulobacter crescentus CB15A	0.873	0.881	0.881	0.898	0.900	0.897	0.974	0.941	0.942	0.948	0.957	0.979	0.991	0.983	0.989	0.996		
Caulobacter crescentus CB2A	0.875	0.882	0.882	0.899	0.901	0.898	0.976	0.943	0.944	0.949	0.959	0.981	0.993	0.985	0.991	0.998	0.998	

[a] Similarity values are based on approximately 1,000 nucleotide positions of continuous sequences.

number of caulobacter isolates that did not fit the pattern of typical caulobacters (16). Two of those strains (FWC14 and FWC38) were examined in the present study; they were significantly different from those of the typical caulobacter cluster (Fig. 2). Strain FWC38 was peripherally affiliated with the typical caulobacters (97 to 98% similarity). Strain FWC14 was even more distantly related to the typical caulobacters (ca. 95 to 96% similarity) and showed a relatively close relationship (97% similarity) to two marine isolates (MCS17 and MCS24). Interestingly, these strains have ties to freshwater environments, as described below. These organisms (MCS17, FWC14, and freshwater-tolerant strain MCS24) made up an assemblage that was distinct from the typical freshwater caulobacter group yet was clearly within the freshwater line of descent.

As noted above, since marine caulobacters were affiliated with both the marine and freshwater lines, as a group they were considerably more diverse than the freshwater forms. While some were relatively similar (e.g., MCS6 and MCS18), MCS17 and MCS24 were more closely related to freshwater caulobacters than to other marine strains. In the case of MCS24, this perhaps is not surprising. In a previous study of a group of marine caulobacters, it was noted that MCS24 was the only strain that could be cultivated in freshwater medium, although it grew with difficulty. The strain grows with no difficulty when small additions of magnesium and calcium (each at ca. 10 mM) are made to freshwater media (18). Strain MCS24 also has cross-walls in its stalk (3), a feature found only in some freshwater caulobacters. All of these features are consistent with the hypothesis that MCS24 represents a branch of marine caulobacters that has relatively recent terrestrial origins. Consistent with this scenario, the other marine form affiliated with freshwater caulobacters (MCS17) was isolated from a brackish environment (32).

Although all of the caulobacters examined appeared to constitute a coherent assemblage (with the possible exception of C. subvibrioides), it was not an exclusive assemblage.

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Representatives of other bacterial genera were associated with the greater caulobacter assemblage. These include *Hyphomonas jannaschiana*, marine hyphomonas strain MHS3, *R. capsulatus*, and *Pseudomonas diminuta* (23).

The close affiliation of *P. diminuta* with some of the caulobacter isolates (strains MCS17 and FWC14) was of interest. The molecular data suggested a relatively recent divergence of this organism from the caulobacter phenotype. Interestingly, when a caulobacter flagellar filament protein gene probe was used to survey a group of noncaulobacter (yet otherwise random) isolates from wastewater treatment facilities, a single positive score was obtained among 150 isolates (16). When this isolate was examined by a commercial species identification scheme (Biologue), a match to *P. vesicularis* was obtained (15). This *Pseudomonas* species is similar to *P. diminuta* on the basis of RNA homology (9); indeed, these two species form a highly distinctive branch of pseudomonads. Also, when several of the caulobacters were examined by the Biologue identification scheme, most did not closely match any species in the data base (caulobacters are not represented in the data base) but FWC38 scored an acceptable match to *P. diminuta* (15). Beyond the presence of a single polar flagellum, we have not found other morphological similarities between *P. diminuta* or the putative *P. vesicularis* isolate and the caulobacters, yet it is conceivable that these species might be comparable to caulobacters locked in the motile phase of the developmental cycle or are the result of deletion of the genes that control or specify the other developmentally regulated organelles expressed by caulobacters.

Recent 5S rRNA comparisons of representatives of the genera *Caulobacter*, *Hyphomonas*, and *Hyphomicrobium* demonstrated the coherence of each of these genera (22). The caulobacter isolates examined in that study are inferred to be affiliated with the assemblage we have here designated the freshwater line of descent (Fig. 2). Among the organisms compared in that study, hyphomonas isolates were distinct from caulobacters. However, identification of the marine line of descent in the present study has greatly expanded the recognized phylogenetic diversity among caulobacters. Members of the marine line of descent are more closely related to representatives of the genus *Hyphomonas* than to the freshwater line of descent. Thus, the hyphomonads and *R. capsulatus* (a photosynthetic organism) appear to be subsumed by the larger collection of caulobacters and thus presumably are derivative of the caulobacter phenotype (i.e., are of caulobacter ancestry). The existence of photosynthetic bacteria within the caulobacter assemblage suggests the possible existence of photosynthetic caulobacters, although, none have been reported.

Given that terrestrial versus marine habitats are reflected in the phylogeny, an outstanding question is habitat of origin; were the original caulobacters terrestrial or marine organisms? In this regard, it is interesting that as part of a project to develop plasmid electroporation capability in marine caulobacter strains, efforts to determine a minimum tolerated salt concentration led to the conclusion that several marine caulobacters grew faster in salt concentrations well below that found in most areas of the oceans (i.e., 1.5 to 2 versus 3 to 3.5% sea salts) (10, 11). This might argue for original freshwater ancestry. However, such questions can be more fully resolved only by additional comparative studies and direct inspection of the environmental distribution of the two now-recognized lines of descent, asking, for example, whether representatives of the marine line of descent are restricted to marine environments. Also, given

the great evolutionary diversity among caulobacters revealed by this study, their relationship to other prosthecate and budding bacteria (e.g., *Hyphomicrobium* sp.) must be more fully examined.

Direct studies of the environmental distribution of caulobacters will be facilitated by the information obtained in the present comparative study. The use of phylogenetically based probes (targeting the rRNA and specific for phylogenetically coherent microbial assemblages) is now well established and has been used for both determinative and environmental studies (1, 12, 37). Within the phylogenetic framework now established for caulobacters, several sequence regions that identify major groups within the genus have been defined (data not shown). Probes complementary to these regions could be used to evaluate directly their distribution (via hybridization to nucleic acid isolated from environmental samples) within marine and freshwater environments. In addition, the use of fluorescent probes for whole-cell hybridization (1, 4) should provide information on the morphology of those organisms identified by hybridization.

Analysis of environmental caulobacter isolates is continuing in several ways and will serve to compare the utility of various measures of relatedness. As detailed above, hybridization probing with genes for surface proteins demonstrated a link between many of the freshwater caulobacters, despite the prediction of the present study that for many the overall similarity of their genomic DNAs must be undetectably low. This was borne out with attempts to relate strains by restriction fragment length polymorphism analysis of the DNA fragments detected by hybridization with the surface gene probes, as detailed above. Yet a recent study of the antigenic cross-reactivity and physical properties of the S-layer proteins from typical wastewater treatment isolates demonstrated that nearly all produced a single protein that could be significantly purified with a single technique and was detectable by Western (immunoblot) analysis with antibody raised against the CB15 S-layer protein (38). Finally, *C. crescentus* CB15A and CB2A were shown to be highly similar (99.8% rRNA similarity), yet attempts to correlate regions of their genomes by pulsed-field gel electrophoresis of fragments derived from digests with restriction enzymes that cut infrequently (8) could not produce identifiable similarities between the two strains (7). Clearly, a variety of techniques will be necessary to refine the general relationships defined by 16S rRNA sequence comparisons.

The data in this study also indicate that reevaluation of the genus *Caulobacter* will eventually be necessary. Although they are a cohesive group, the similarity values noted for the caulobacters (Tables 1 and 2) are well below the values that separate other defined genera (37), frequently falling below 90%. This study included a relatively large number of strains of one genus, compared with comparable studies. Yet the discovery of significantly different species, such as *C. subvibrioides*, is one indication of the need for additional sequence comparisons before revision of the caulobacter classification is considered.

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