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CHAPTER 3.1.3

Dimorphic Prosthecate Bacteria: The Genera *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron*

JEANNE S. POINDEXTER

Rationale for Clustering *Caulobacter*, *Asticcacaulis*, *Hyphomicrobium*, *Pedomicrobium*, *Hyphomonas* and *Thiodendron*

The genera treated together here comprise the *dimorphic prosthecate bacteria (DPB)*, in which reproduction regularly results in the separation of two cells that are morphologically and behaviorally different from each other (Fig. 1). One sibling is nonmotile and prosthecate, possessing at least one elongated, cylindrical appendage that is an outgrowth of the cell surface, including the outer membrane, the peptidoglycan layer, and the cell membrane, and that may also include cytoplasmic elements such as ribosomes; such an appendage is a *prostheca* (Staley, 1968). In natural populations, this prosthecate cell is usually also sessile by virtue of adhesive material associated with a cell pole or with the prostheca. The other sibling is flagellated, bearing (typically) one polar or subpolar flagellum, by means of which it is actively motile. This mode of reproduction is unique as a regular feature of a prokaryote reproductive cycle. It is regarded here as a reflection of an ecologic program common to these genera, viz., as a means of dispersing the population at each generation and thereby minimizing competition between siblings for resources.

In bacteria lacking motility, sibling separation in space depends on abiotic forces; if both are sessile, clones accumulate as cell clusters. In most motile unicellular bacteria, both siblings are motile; if they are also chemotactic, both cells will tend to follow the same gradients and so travel together. In the DPB, spatial separation is promoted by the motility of one cell and the immobility of its sibling.

Other prosthecate bacteria differ from those of the genera treated in this chapter either by lacking a motile stage (*Prosthecobacter*, *Filomicrobium*, *Ancalomicrobium*) or by producing motile cells only under certain environmental conditions (*Prosthecomicrobium*, *Rhodomicrobium*). *Thiodendron* is included in the DPB cluster even though its habit has so far been surmised only from polytypic populations in natural samples or enrichments; it bears one or two appendages that appear to be prosthecae, has been seen to form buds on those appendages, and is accompanied by motile cells that may be its own swarmers.*

Throughout the eukaryotic world, there are parallels for the asymmetric cell reproduction that is characteristic of the DPB, but very often with the consequence that only one of the two siblings continues to reproduce while the other differentiates and exhibits diminished or lost potential for subsequent reproduction. More relevant parallels are found at the organismic level, such as among fungi and invertebrate animals that are not freely mobile as adults but produce motile offspring or plants that produce propagules equipped for travel; the analogy to eukaryotes underlies the usage of the terms "mother" and "daughter" to refer to the nonmotile and motile siblings, respectively, of the DPB.

The parallel with dispersal units seems particularly apt as evidence accumulates that the motile siblings of DPB are in an analogously juvenile condition (Krasil'nikov and Belyaev, 1970; Matzen and Hirsch, 1982b; Moore, 1981; Moore and Brubaker, 1976; Moore and Hirsch, 1973; Morgan and Dow, 1985; Pate et al., 1973; Poindexter, 1964, 1981a; Swoboda and Dow, 1979; Wali et al., 1980). In each species so far studied, the motile cell is less actively growing than its prosthecate sibling; in *Asticcacaulis* and *Hyphomicrobium*, the swarmer is also typically considerably smaller than its prosthecate sibling.

This chapter was taken unchanged from the second edition.

* *Thiodendron* isolates have not been reported. The organism is known only from natural samples in which it has been interpreted as a sulfur-oxidizing prosthecate bacterium, probably with a motile stage. The absence of information regarding properties of monotypic populations does not justify a discussion beyond the excellent summary by Schmidt (1981).

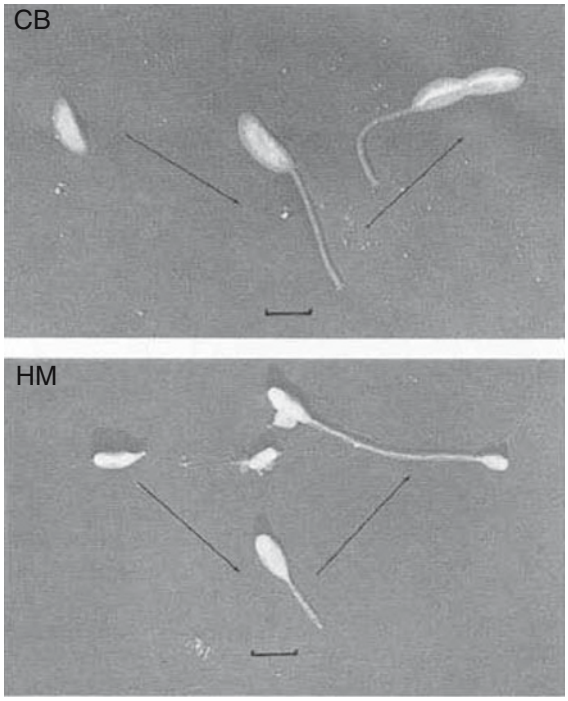

Fig. 1. Caulobacters (CB) and hyphomicrobia (HM). Left to right in each electron micrograph: swarmer, prosthecate cell, and reproducing cell. Transmission electron micrographs, Pt-shadowed cells. Bar = 1 μm.

Growth and progress toward reproduction are initiated in these swarmers only after a period in which their most evident activity is motility. The regulatory mechanisms by which growth and development are postponed have not been elucidated, although lipid, phosphorus, and nitrogen metabolism have been implicated as possibly influential metabolic processes (Chiaverotti et al., 1981; Emata and Weiner, 1983; O'Neill and Bender, 1989; Poindexter, 1984a; Mansour et al., 1980). Attachment is not necessary for the onset of development and growth, but presumably usually occurs prior to development in natural habitats. Although both swarmers and prosthecate cells are adhesive, attachment is probably initiated mainly in the swarmer stage (Hirsch, 1974; Leifson, 1962; Moore and Marshall, 1981; Zavarzin, 1961), since: 1) prosthecate cells are typically found attached, not afloat (although that may be because that is where they *can* be seen); 2) if pili are present in a strain, they occur predominantly or exclusively on swarmers (Schmidt, 1966; Umbreit and Pate, 1978); and 3) in laboratory-cultivated populations, the swarmer is demonstrably more active in initiating attachment (Newton, 1972). Moore and Marshall (1981) have suggested that, like pili, flagella may serve as a means of carrying adhesive material through the electrostatic barrier between cell and substratum, thereby aiding the establishment of stable contact (see Planktonic Versus Sessile Life of Prokaryotes in the second edition).

Thus, like the eukaryotes in which immotile organisms produce motile or transportable offspring, dispersal of DPB populations is the most reasonable interpretation of the major advantage of mobility in the juvenile stage. By fission or by budding, each normal reproductive event in these bacteria produces two siblings: one to grow and one to go. It is consistent with this developmental and reproductive habit that these bacteria exhibit the physiologic properties of oligotrophs (Poindexter, 1981b), most importantly: *tolerance of prolonged nutrient scarcity.* This is the principal physiologic property that can be exploited in their enrichment and isolation and is reflected in their distribution in nature. Once isolated, individual strains may tolerate much higher nutrient concentrations than are useful in their enrichment and isolation, but most isolates exhibit either growth inhibition or loss of control over cell form ("pleiomorphy") upon incubation in media designed for the cultivation of bacteria parasitic on plants or animals (see references in "Cultivation," this chapter). Such nutrient-rich media are generally suitable for the cultivation of copiotrophs, but morphology of those few DPB that are able to grow on such media may be so aberrant in such cultures that they may not be recognizable as DPB.

When cultivated under conditions that allow uniform dimorphic morphology and cell size from one generation to the next, cultivated DPB are morphologically identical to bacteria observed in natural materials. On the basis of the morphology of the reproductive stage, two fundamentally different types of DPB can be distinguished in natural samples, enrichment cultures, and pure cultures: caulobacters (*Caulobacter and Asticcacaulis*) and hyphomicrobia (*Hyphomicrobium*, *Pedomicrobium*, and *Hyphomonas*). Caulobacters reproduce by fission and hyphomicrobia by budding, almost always from the distal tips of prosthecae (hence the usage of the term "hyphae"). *Caulobacter* and *Asticcacaulis* can be distinguished from each other by the site of adhesion of sessile cells to substrata; *Caulobacter* cells adhere by the distal tip of the prostheca (hence "stalk"), while *Asticcacaulis* cells adhere by the cell pole and the prostheca is not adhesive. Among the hyphomicrobia, *Pedomicrobium* cells are distinguished morphologically from those of the other two genera by the production of buds whose long axes are perpendicular to the long axis of the hypha, rather than an extension of the hyphal axis; they also tend to produce more than one hypha per cell, but multiple prosthecae occur in stressed and aging populations of all DPB and are not a dependable morphological trait, particularly in

natural populations. Adhesiveness of a cell pole is typical of hyphomicrobia, but it is not a universal property of isolates under all conditions. The hyphae are not adhesive in any of the budding genera. The reverse orientation of sessile cells is helpful in distinguishing *Caulobacter* from hyphomicrobial cells in natural samples.

In addition to their developmental and reproductive habit and fundamentally oligotrophic physiology, these genera exhibit several other common properties that can be characterized only with pure populations. These properties are not unique to this group; on the contrary, they imply relationships to other eubacterial groups, particularly to pseudomonads, with which they often share their natural habitats. All are oxybiontic and grow in well aerated cultures; a tolerance of low pO_2 is exhibited by *Pedomicrobium* isolates, and a preference for such conditions is seen in *A. biprosthecum*. They seem universally cytochrome-positive and catalase-positive, although there are exceptional reports (e.g., Lapteva, 1977, 1987) of negative tests. Only one species (*C. crescentus*) has been examined for superoxide dismutase (SOD) activity; like certain *Pseudomonas* spp., *C. crescentus* possesses two SODs (Steinman, 1982). The Fe-SOD appears to be cytoplasmic, while the CuZn-SOD is periplasmic (Steinman and Ely, 1990). Unequivocal anaerobic growth is sustained only by *Hyphomicrobium* isolates, by dissimilatory respiration of nitrate to N_2.

All DPB are chemoheterotrophic, and variations in carbon-source preference are useful traits in generic and species differentiation. Ammonium ions are suitable as the sole source of nitrogen for isolates that can be cultivated in defined media; ammonia is assimilated by addition to glutamate or by reductive amination of pyruvate and glyoxylate (Doronina, 1985; Ely et al., 1978). Only *Hyphomicrobium* isolates have been reported able to use nitrate as the sole nitrogen source. In complex media, peptone is universally suitable as a nitrogen source. Poly-β-hydroxybutyrate (PHB) and polyphosphate are stored as reserves and may be detectable microscopically in cells in natural samples.

DPB possess outer membranes, and growing cells stain Gram-negatively. The outer membranes of the genera that have been characterized (*Asticcacaulis* [Jordan et al., 1974]; *Caulobacter* [Agabian and Unger, 1978]; *Hyphomonas* [Dagasan and Weiner, 1986; Shen et al., 1989]) exhibit a higher proportion of high-molecular-weight proteins than do those of other Gram-negative bacteria. Superficial layers of repeating protein subunits ("R-layers") are known in *Caulobacter* (Smit et al., 1981) and possibly in *Hyphomonas* (Dagasan and Weiner, 1986), in which two proteins account for more than half of the outer membrane proteins.

Most isolates are susceptible to antibiotics that inhibit prokaryotic protein synthesis (aminoglycosides, macrolides, tetracyclines), but may be resistant to synthetic antimicrobials such as sulfonamides; high concentrations of quinolones are required for inhibition of growth (J. S. Poindexter, unpublished observations). Any type of inhibitor, including mitomycin C, hydroxyurea, and cell wall synthesis inhibitors, may cause developmental aberrations (Haars and Schmidt, 1974; Koyasu et al., 1983; Moore and Brubaker, 1976; Moore and Duxbury, 1981; Weiner and Blackman, 1973).

Neutral to slightly alkaline reaction is optimal for growth, except for *Pedomicrobium* isolates, which grow well at pH 9.0. Moderate temperatures (20 to 30°C) are also generally optimal, but many isolates grow well at temperatures as low as 5°C, at least as primary isolates, and a few grow at temperatures above 35°C. Many isolates require vitamins, and growth is generally stimulated by B vitamins. When only one vitamin is required, it is biotin or B_{12}, while riboflavin and Ca-pantothenate are stimulatory for certain isolates.

The DNA base composition of bacteria of these genera is in the range of 59 to 67 mol% of GC, except in *A. excentricus* (55%) and *Hyphomonas hirschiana* (57%). RNA sequence analysis has revealed a closer relationship to pseudomonads than to other bacterial groups, although the relationship is "remote" (Stackebrandt et al., 1988); the presence of intracellular nucleases appears to interfere with the application of this analytic technique to phylogenetic interpretation of *Caulobacter* and *Hyphomonas*. Extensive studies of DNA-DNA hybridization have revealed little sequence similarity within the group, or even within a genus, except among isolates derived from the same initial sample, among variants of a single strain, or ~~in~~ only a few instances ~~among~~ isolates of clear phenotypic similarity (Gebers et al., 1984, 1985, 1986; Moore et al., 1978). Lack of DNA-DNA hybridization is not predictive of similarities among other molecules, such as outer-membrane proteins (Dagasan and Weiner, 1986). In the DPB, naturally-occurring plasmids have been detected only among caulobacters (Anast and Smit, 1988; Schoenlein and Ely, 1983). However, pseudomonad plasmids such as RP4 can be propagated and expressed in both *Caulobacter* and *Hyphomicrobium* (Anast and Smit, 1988; Chatterjee and Chatterjee, 1987; Dijkhuisen et al., 1984; Ely, 1979).

Possibly as an aspect of their oligotrophic nature, in the sense that they are not adapted to continual, well-supported growth, continuous

vegetative propagation of DPB results in diversification. This has been a persistent problem with all the genera and is perhaps best exemplified by Mevius' strain B; of the seven strains listed as *Hyphomicrobium aestuarii* in *Bergey's Manual* (Hirsch, 1989), six (MEV-533, MEV-533Gr, EA-617, EN-616, NQ-521 and NQ-528) are laboratory derivatives of one original isolate (Mevius, 1953). Most variations are detected as changes in colony morphology or texture and are presumably cell surface composition variants. This kind of change often occurs early in the purification of an isolate, especially of *Hyphomonas* (Moore et al., 1984). Pongratz (1957) reported one isolate of *H. polymorpha* as PR727 (rough) and PS728 (smooth); all five *Hyphomonas* isolates from the deep-sea vent community were all originally "rough," but two are now described as smooth (Moore and Weiner, 1989) and all have smooth variants (J. S. Poindexter, unpublished observations). Deposition of metal oxides is a trait that may be lost by initially positive isolates (Gregory and Staley, 1982; Tyler and Marshall, 1967a), reducing the usefulness of this characteristic in differentiating genera and species. Temperate bacteriophages also appear in long-cultivated strains (Driggers and Schmidt, 1970; Gliesche et al., 1988; Schmidt and Stanier, 1966), suggesting genetic perturbations during perpetual cultivation.

Despite this long list of similarities, there is no reason at present to regard this set of genera as a phylogenetically-related cluster. It is a group distinguished by its reproductive habit, which seems related to its ecology; its common ecologic program should be of sufficient advantage to have evolved more than once. The physiologic similarities arise largely from the oligotrophic nature of these organisms, which is consistent with a growth habit that promotes dispersal of successive generations.

Taxonomy by nucleic acid composition and sequence similarity has not resulted in a suggestion that these genera should be redistributed among bacterial groups differently from the placement inferred from traditional dependence on Gram-reaction, flagellation, cell morphology, nutrition, cytochrome content, and natural distribution. However, it has so far provided only scant evidence that they should be grouped together. Determination of rRNA cistron similarities revealed affinities among *Hyphomicrobium*, *Hyphomonas*, and *Caulobacter*, as well as between *Hyphomicrobium* and purple bacteria and nonmotile prosthecate bacteria (Moore, 1977). Similarities of rRNA sequences have also been detected among *Hyphomicrobium*, *Pedomicrobium*, and *Filomicrobium* (the last, a genus of nonmotile prosthecate bacteria), although this group appears to comprise a previously unrecognized fourth subgroup of the "alpha" subdivision of purple non-sulfur bacteria (Stackebrandt et al., 1988). Although presently inferred phylogenetic distances within this group seem great, they are smaller than the distance from any non-prosthecate bacteria.

Accordingly, aside from the possibility that the regular production of a motile stage is a late modification or a dispensable property among prosthecate bacteria, it seems appropriate at present to recognize the DPB as a cluster of genera that comprise a probably related subgroup of heterotrophic pseudomonads specialized for distribution in nature as uncrowded, oligotrophic populations.

Distribution of Dimorphic Prosthecate Bacteria

A single term can be used to describe the occurrence of DPB in waters: ubiquitous. This term applies without qualification to *Caulobacter* and is almost as appropriate for hyphomicrobia, as evidenced not just by the frequency of the detection of these two types within natural populations, but also by the relative ease of their enrichment and (with proper attention to their particular physiological traits) isolation. Lapteva (1987) has suggested that caulobacters, as typical aquatic bacteria, should be regarded as probably second only to pseudomonads in breadth of distribution and numbers. These two bacterial groups together may be responsible for the bulk of mineralization of dissolved organic material in aquatic environments, with caulobacters being especially important when nutrient concentrations and ambient temperatures are low (Allen, 1971; Staley et al., 1987).

When detected in unenriched samples, DPB are not found as crowds; even when total microbial density is high, DPB are usually sparse—with notable exceptions such as *Hyphomonas*-like organisms in some deep-sea hydrothermal vent samples (Jannasch and Wirsen, 1981) and *Pedomicrobium* in manganese concretions (Ghiorse and Hirsch, 1982; Marshall, 1980; Sly et al., 1988; Tyler and Marshall, 1967a, 1967b). Similarly, even when DPB are practically alone, as when total microbial counts are low or when they colonize submerged surfaces, they do not exploit the space by filling it with their progeny. Quantitative studies of their occurrence by direct plating, dilution for most-probable-number (MPN) calculation, or direct microscopic enumeration by scanning electron microscopy reveal population densities of 10^3 to 10^5 per ml of water or per gram of soil, detritus, or sediment; 10^6 DPB per such a unit, as reported by Lapteva (1987), is an exceptionally high density. By themselves, the

DPB have not been found responsible for turbidity of natural waters, nor are they detectable by sample odor as are, for example, sulfate-reducing bacteria and streptomycetes; microscopical examination of samples is required for their detection. The only macroscopic evidence of their possible presence is metal (Fe or Mn) oxide deposits on wet surfaces, but whether they cause or just adhere to such deposits remains to be established. Accordingly, "abundant" in referring to these organisms must be read as meaning 10^3 or more per ml or gram, "present" to mean detectable (usually microscopically and often only after enrichment), and "predominant" as relatively unaccompanied, but not necessarily "abundant."

Asticcacaulis is rarely sighted and even more rarely isolated. *Pedomicrobium* distribution is probably wider than evidence currently suggests, since a suitable method for enrichment and isolation of this type of hyphomicrobium has been developed only recently. *Hyphomonas* may be a type restricted to marine environments; its morphologic twin, *Hyphomicrobium*, is found in brackish waters (as well as soils and fresh water), but not marine sources. Distribution is discussed here principally with reference to *Caulobacter* and *Hyphomicrobium*.

Practically any type of seawater from harbors, estuaries, the open ocean, deep-sea hydrothermal vents, storage reservoirs contains *Caulobacter* and may also contain hyphomicrobia, probably *Hyphomonas*. *Caulobacter* may be the predominant form of aerobic chemoheterotrophic bacteria in oceanic samples (Jannasch and Jones, 1960), particularly in Antarctic waters (Takii et al., 1986; Waguri, 1976). DPB also occur in sediments, particularly if algal and/or plant material (including wood) has settled onto the bottom (Austin et al., 1979). DPB are typically found attached to submerged surfaces, whether or not they are detectable in bulk water, and so they settle with such materials.

They are also present in freshwater ponds, lakes, streams, rivers, and reservoirs (reviewed in Poindexter, 1981a), even temporary pools and puddles (Gebers, 1981; Masuda, 1957); in canals and lagoons of various trophic states (Lapteva, 1977, 1987; Staley et al., 1980, 1987; Stanley et al., 1979); in pipelines and water distribution systems, including domestic tap water; in wells (Masuda, 1957; Shah and Bhat, 1968); in home aquaria; in bottled spring water (Gonzalez et al., 1987); and in sewage. In short, if a site has been wet for any length of time, DPB will be present.

In this regard, it should be noted that the bulk of the carbon of some organic-rich sites in nature is insoluble, including plant litter and partly-treated sewage and activated sludge, as well as sediments, soils, and waters polluted with industrial wastes to the point of loss of translucency that is not due to microbial blooms. Such sites may support the growth of oligotrophic bacteria such as the DPB despite their "richness." Polymer-degrading bacteria such as cytophages are typical of such sites; presumably, the DPB subsist on soluble nutrients that are slowly released from insoluble organic materials by such bacteria.

Detection of DPB in sewage is sporadic, although they are known to occur in the highly aerobic type of sewage treatment known as activated sludge (Hartmans et al., 1986; Schmider and Ottow, 1986; Vedinina and Govorukhina, 1988), and phages lytic for DPB can be isolated from sewage effluents (Gliesche et al., 1988; Schmidt and Stanier, 1965). In anaerobic sewage treatment processes, only *Hyphomicrobium* appears to occur commonly, and then specifically in sewage carrying a high nitrate load. *Hyphomicrobium* is strongly favored in such sewage by the addition of methanol, which accelerates the removal of nitrate as N_2 as it selectively favors *Hyphomicrobium* multiplication (Claus and Kutzner, 1985).

Seasonal fluctuations have been characterized only for freshwater caulobacters; other DPB are not sufficiently abundant to allow meaningful quantitative determination of seasonal changes. Lapteva (1977, 1987) observed that caulobacters fluctuate with phytoplankton. Both types of aquatic organisms rise numerically in the springtime, may or may not decline (or descend in the water) during the summer, but definitely do so during the winter. They rise together again (in number and in location in the water, such as a lake) in the springtime, a pattern also noted by Staley et al. 1980. It seems likely that caulobacters respond readily to the release, in early spring, of nutrients immobilized during the winter and released abiotically during "spring turnover." They persist through the summer, but at that season are accompanied by a diversity of later-blooming bacteria.

The only clear limitation on the geographic distribution of DPB in fresh water is temperature; they have not been found in thermal springs.

DPB are found in soils, particularly from nutrient-poor soils such as "podzols."* They are also found in leaf-litter, in frequently-wetted agricultural and lawn soils (Belyaev, 1968; J. S. Poindexter, unpublished observations), and in metal ore deposits (Groudev et al., 1978).

* Podzol: a type of relatively infertile soil found typically in forests and consisting of a thin, ash-colored layer overlying a brown, acidic humus, the organic part of soil, resulting from the partial decay of leaves and other vegetable matter.

DPB are also found in the laboratory: *Hyphomicrobium* in enrichment cultures for nitrifying bacteria (Kingma Boltjes, 1936) and methane-oxidizers (Wilkinson and Hamer, 1972); *Caulobacter* in stock algal cultures (Bunt, 1961; Gromov, 1964; Klaveness, 1982; Li et al., 1984; Zavarzina, 1961); and both in enrichment cultures for N_2-fixing aerobes. Both *Caulobacter* and *Hyphomicrobium* can be isolated from laboratory water baths and distilled water (Callerio et al., 1983; reviewed in Poindexter, 1964). *Hyphomicrobium* is especially easy to detect and to isolate from any appended rubber tubing or plastic outlet of a distilled water reservoir, and *Caulobacter* is isolated from the outlets, the walls of the container, and the air-water interface.

With very few exceptions (*Hyphomonas polymorpha*, isolated from human nasal secretions [Pongratz, 1957]; *C. leidyi*, isolated from millipede hindguts [Poindexter, 1964]; and hyphomicrobia from turbot gills [Mudarris and Austin, 1988]), DPB are not detected in nor isolated from association with animals or decaying animal materials. They have not been reported as agents of food spoilage and are not pathogenic for laboratory animals~~ev~~en those that can persist long enough to stimulate an immune response (Famurewa et al., 1983).

In both soils and waters, DPB (and *Prosthecobacter*; see The Genera Prostheomicrobium, Ancalomicrobium and Prosthecobacter) are especially likely to be encountered as algal epibionts; examining the surfaces of algal thalli is often the most dependable way to detect DPB in a natural sample. Caulobacters are usually attached to algal structures, while hyphomicrobia are more often detected within algal jelly (Geitler, 1965; Hirsch, 1974). These associations are best observed by scanning electron microscopy (SEM) (Fig. 2). In living specimens examined by phase contrast microscopy, they are more difficult to discern; the phase halo of the relatively large algal cells interferes with the visibility of the stalks, while the *Caulobacter* cells may be overlooked waving on their stalks several micrometers away from the algal surface. Even in SEM images, very long stalks may not be traceable to their cells (Fig. 2; see also Tufail, 1987).

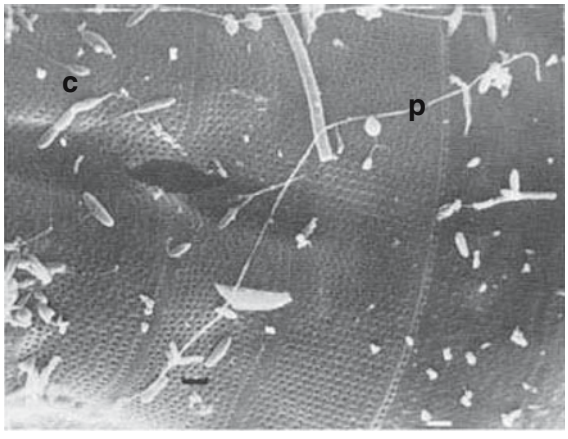

Fig. 2. Prosthecae (p) and prosthecate cells (c) attached to the filamentous diatom *Melosira*. Scanning electron micrograph. Bar = 1 µm.

As algal epibionts, DPB exhibit some preference for association with diatoms (Anderson and Poindexter, 1984; Nemec and Bystricky, 1962; Tufail, 1987). This may reflect any of: a preference of the caulobacters, at least, for siliceous surfaces (glass, sand grains, diatom frustules); strictly spatial coincidence, since diatoms are themselves common as epibionts of other algae and of plants; and environmental factors that favor, in common, the multiplication of these two types of organisms. In the laboratory, when mixed with an algal population, caulobacters attach readily to both phototrophic and heterotrophic diatoms and to algae with nonsiliceous walls (Fig. 3). In natural habitats, caulobacter numbers fluctuate in parallel with phytoplankton in general (Lapteva, 1987; Staley et al., 1987) and they are likely to be found on every kind of alga and on submerged plants as well (Kudryavtsev, 1978). Among the DPB, caulobacters in particular can be isolated from both natural and cultivated algal populations: from soil (Gromov, 1964), water (Gromov, 1964; Lapteva, 1987), and cultures (of *Nostoc* [Bunt, 1961], *Chlorella* [Zavarzina, 1961], *Phormidium, Tribonema, Chlorella,* and *Chlorococcum* [Gromov, 1964], *Anabaena* [Li et al., 1984], and *Cryptomonas* [Klaveness, 1982]).

The principal advantage of attachment to algae is presumed to be an immediate source of soluble organic substances in the algal exudate. During illumination, cyanobacteria and algae also release O_2, an environmental factor found by Lapteva (1987) to be a major positive influence on the occurrence of caulobacter populations. However, O_2 is also potentially toxic, and it has been suggested (Steinman and Eley, 1990) that algal association may have selected for the evolution in caulobacters of a periplasmic SOD in addition to their cytoplasmic SOD (see Steinman, 1982). Since O_2 can be especially damaging to phototrophs, epibiontic heterotrophs such as caulobacters may serve to consume sufficient O_2 to reduce the pO_2 in the immediate environment of photosynthetically active cells (Lupton and Marshall, 1981; Staley, 1971; Stanley et al., 1979), as well as to cycle carbon immediately (algal exudate to CO_2 to alga) through their oxidative metabolism (Allen, 1971). Such considerations suggest mutually beneficial association, not just coincidence.

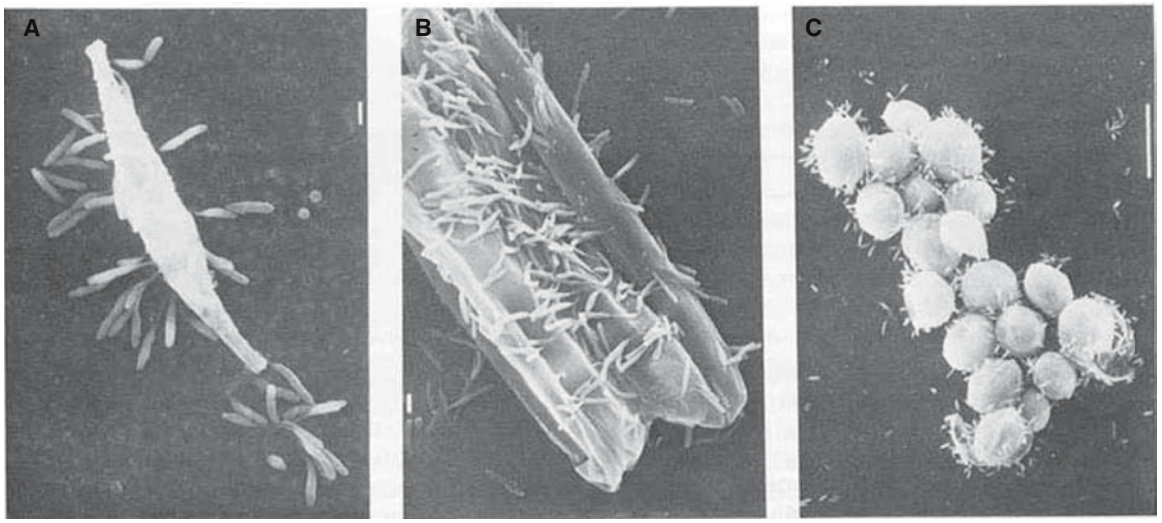

Fig. 3. Two-membered cultures of a marine caulobacter (VC13) attached to: (A) *Phaeodactylum tricornutum,* a phototrophic diatom; (B) *Nitschia* sp., a heterotrophic diatom; and (C) *Oöcystis* sp., a phototrophic green alga. Scanning electron micrographs by K. L. Anderson. Bar = (A and B) 1 μm; (C) 10 μm.

Enrichment and Isolation of Dimorphic Prosthecate Bacteria

Principles and General Procedure

The density of DPB in any natural sample will not be high and typically will be much lower than 10^6 per ml of water or per gram of solid material. Nevertheless, their tolerance of prolonged nutrient scarcity provides a dependable physiological basis for their enrichment. Successful enrichments from water, and from soil suspended in water, result when the sample is allowed to stand undisturbed for one to several weeks. Samples have often proved useful when stored for months and even years, and long-undisturbed samples can be regarded as pre-incubated enrichments.

Nevertheless, it is often more practical to promote microbial multiplication by supplementing the sample with organic nutrients; peptone added to 0.001 to 0.005% (w/v) is a suitable supplement, possibly indicating that microbial development in many natural samples is nitrogen-limited.

Periodic examination, by phase contrast microscopy, of the thin surface film (lifted with a loop, a slide, or a cover slip~~not~~ with a pipet), of growth scraped from the wall of the vessel, or of algal cells (if the culture has been illuminated) will reveal an accumulation of prosthecate cells. Other bacteria present may multiply, but they tend to die off or be consumed by protozoa; the prosthecate bacteria seem refractory to both of these processes.

At a point determined according to the proportion of accumulated prosthecate bacteria, a sample is streaked onto a suitable medium and incubated at room temperature (preferably) or at 30°C. A suitable plating medium for general purposes contains 0.05% peptone, plus possibly a vitamin mixture such as that formulated by Staley (1968), and 1.0 or 1.5% agar. As colonies become visible, they should be marked at two or three days and the plate reincubated. Colonies that become macroscopically visible at four to seven days will include *Caulobacter* and *Asticcacaulis* colonies, which are smooth, circular, and convex with an entire edge. Colonies that become visible only after seven days will include hyphomicrobia; development of *Hyphomicrobium* colonies can be accelerated by placing a flask or bottle of methanol in the incubator with the plates. Colonies of hyphomicrobia are initially hyaline, circular, cohesive, and often crateriform. Streaking on a mineral medium such as 337 (see "Cultivation," this chapter) and incubating in an atmosphere containing methanol vapors will increase selectivity of this step for *Hyphomicrobium*. Supplementation of the medium with 0.02% $MnSO_4$ or $FeSO_4$ (or iron powder or Fe-containing paper clips) may result in metal oxide deposition in the colonies, visible as brownish coloration. Many DPB deposit Fe and/or Mn oxides, at least upon primary isolation, and the presence of such deposits provides a macroscopic indication of possible DPB colonies.

At this point in the isolation of DPB, two major problems may be encountered. First, suitable samples often contain organisms that grow as a film on the surface of agar media; they may grow over and through colonies of DPB and be

carried onto subsequent streak plates. Their spreading can be retarded by the use of 2% agar, but this will also slow the development of DPB colonies. They can be avoided by transferring small, late-arising colonies as soon as possible (with the aid of a dissecting microscope, if necessary) to a secondary plate; the most suitable method is to transfer each colony with a sterile toothpick to a small area on a fresh plate. When growth accumulates in the inoculated patches, samples can be screened microscopically for DPB more conveniently than can the small colonies typical of the primary plates. Repeated restreaking from positive patches, then from isolated colonies, will eventually yield pure populations of DPB.

The second problem arises from the tendency of DPB to attach to other microbial cells, including other bacteria. As a consequence, initial colonies and patches containing DPB are often mixed with other bacteria (see, e.g., Harder and Attwood, 1978). This necessitates careful microscopic observation to avoid overlooking DPB, which may be only a minority in a mixed colony or patch, and repeated restreaking until all colonies on a plate contain only DPB, and only one type of DPB.

Microscopic screening is best accomplished in wet mounts examined with a phase contrast oil-immersion lens. This allows simultaneous evaluation of the presence of prosthecae and of motility. Prosthecae are less than 0.2 μm in diameter in caulobacters and only slightly wider in hyphomicrobia; they are not discernible in an ordinary light microscope. If phase contrast microscopy is not available, a droplet of crystal violet or methylene blue solution added to a wet mount will increase visibility of the prosthecae; the stain can be introduced from the edge of the cover slip after the mount has been examined for motility. Mordanted stains such as those used for visualizing flagella are also suitable for visualization of prosthecae.

Most DPB are adhesive, and in colonies and in pure cultures they tend to adhere to each other in rosettes united by a common mass of holdfast material. Only in *Caulobacter* is the holdfast located at the distal tip of the prostheca, resulting in rosettes in which the cells are at the periphery. In the other genera, adhesive isolates bear holdfast material directly on the cell surface, and cells in rosettes appear crowded while their prosthecae extend away from the cell cluster (Fig. 4). The presence of either type of rosette in colonies on primary isolation plates indicates the possible presence of DPB. Colonies and patches should be pursued to purity if rosettes of cells are present, even if prosthecae are not seen; such clones may eventually prove identifiable as *Asticcacaulis*.

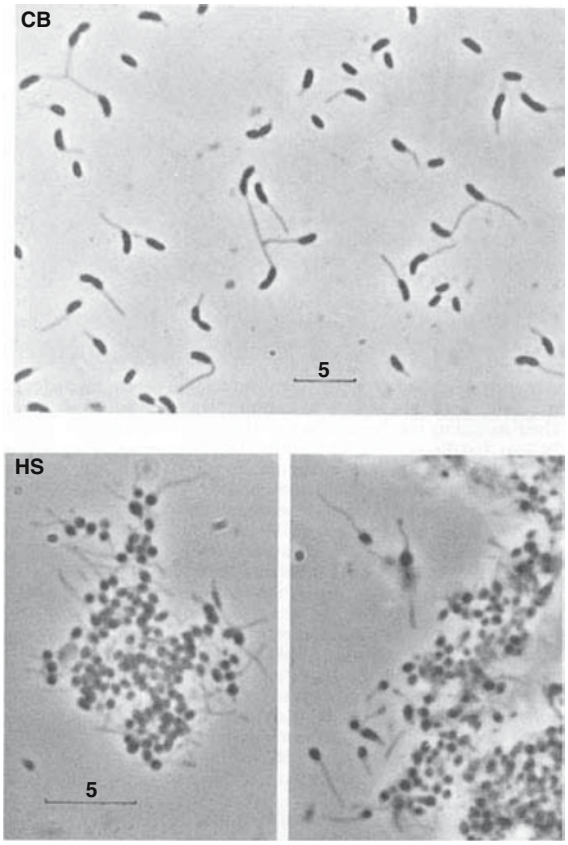

Fig. 4. (a) Caulobacterial (CB) and (b) hyphomicrobial (HS; lower panels) cell clusters formed in pure cultures. Phase contrast microscopy of cells in tannic acid + $HgCl_2$ (CB) or in seawater (HS). Bar = 5 μm.

Final identification of morphologic type should be made on the basis of electron microscopical examination of shadowed or negatively-stained specimens (see "Identification of DPB," this chapter).

The procedure just described is generally suitable for obtaining pure cultures of DPB. More specialized procedures for *Hyphomicrobium* and for *Pedomicrobium* are described below.

It is occasionally possible to isolate DPB without an enrichment step, and if the total microbial count of the sample is low because it was obtained from a strictly oligotrophic habitat, or if the sample is from a long-unused storage reservoir, direct streaking is worth attempting. A few useful guidelines are given below that can improve the development of a useful proportion of DPB and ensure their recognition once isolated.

1. Water samples should not be filtered to remove eukaryotic microbes and particulates. If filtration is for some reason desired, then the filter should also be used as inoculum for a separate enrichment culture. DPB are often more abundant on algal and fungal thalli and on

particles than in the ambient water, and the material on the filter may be a more suitable inoculum than the water.

2. Soil samples should be ground, not just shaken, to break up microbial clumps and to detach prosthecate cells from particles.

3. DPB can be collected on bait placed in the enrichment culture or in situ in the habitat. Glass surfaces such as microscope slides are the easiest to handle. (For simple observation of these organisms, this is the method used by Henrici and Johnson [1935]; somewhat more demanding, because of their physical delicacy, is the use of plastic-coated electron microscope grids [Hirsch and Pankratz, 1970].) To remove DPB, the bait should be rinsed well to remove casually-associated bacteria, then scraped (while wet) with a hard object to remove prosthecate cells. Breaking the prosthecae of caulobacters will not kill the cells, and hyphomicrobia do not attach by their prosthecae. Rubbing with a soft cotton swab will not detach these tightly-adhesive bacteria.

4. Dilution of the water sample or suspension of ground soil or sediment is often helpful. When DPB are present in sufficiently high proportion so that they will persist to higher dilutions than many of the other types present in a sample, dilution~~es~~pecially of samples to be supplemented with nutrients~~re~~duces the number of faster-multiplying bacteria and results in usefully high proportions of DPB after shorter incubation periods.

5. *Above all*, initial streaking must employ dilute media. Colony development will take several days, and colonies will be quite small, but the majority of DPB either do not grow on media containing 0.5% or more of soluble organic material or exhibit extreme pleiomorphy and absence of motility. Prosthecal development, in particular, is aberrant; in addition, cell shape is irregular, lysis is frequent, and viability in the colonies is low. Failure to accommodate this characteristic of DPB is probably the main reason they are so much more frequently sighted in natural samples than isolated from them; filtering them out before enrichment surely accounts for another major loss of DPB from natural samples prior to incubation. In addition, they are not reported in studies employing acridine orange (see, e.g., Kogure et al., 1979) because prosthecae fluoresce very weakly, or not at all, and most of the DPB strains tested either are not inhibited by nalidixic acid or develop aberrantly in its presence (see, e.g., Weiner and Blackman, 1973).

On the other hand, the purity of isolates can be checked by streaking on routine bacteriological "nutrient agar"(0.5% peptone plus 0.3% beef extract) for freshwater and soil isolates (Attwood and Harder, 1972; Gebers and Beese, 1988; Hirsch and Conti, 1964a) and on full-strength Zobell's 2216 (0.5% peptone, 0.1% yeast extract, 0.01% $FePO_4$ in 75% seawater) for marine isolates. Among marine DPB, only *Hyphomonas* will grow readily and with recognizable dimorphology on Zobell's medium, and even their growth is improved by diluting the medium three- to ten-fold (Havenner et al., 1979; Weiner et al., 1980).

Specific Enrichment Procedures for *Hyphomicrobium* and *Pedomicrobium*

Methanol-Nitrate Anaerobic Enrichment for *Hyphomicrobium* This method, developed independently by Sperl and Hoare (1971) and by Attwood and Harder (1972), is the only dependably rapid enrichment method for DPB. It yields a nearly pure population of *Hyphomicrobium* in three days to three weeks; the lower the organic content of the sample, the earlier the *Hyphomicrobium* population will develop (Attwood and Harder, 1972).

A sample of water or sewage (a few ml) or of mud or soil (not more than 1 gram) is added to a mineral medium (such as "337," see below) containing 0.5% KNO_3 and 0.5% methanol. The culture is incubated anaerobically, preferably in a tightly screw-capped vessel. When turbidity and bubbles (of N_2) appear, phase contrast microscopical examination usually reveals a high proportion (often more than 90%) of hyphomicrobia, which can then be purified by streaking on the same medium and incubating either aerobically or anaerobically. Methylotrophic denitrifying DPB isolated by this means, as well as strains isolated initially as aerobes but that are capable (sometimes only after adaptation [Sperl and Hoare, 1971]) of anaerobic growth with methanol and nitrate, have been assigned to four species of *Hyphomicrobium* (*H. vulgare*, *H. facilis*, *H. aestuarii* and *H. zavarzinii* [Hirsch, 1989]); thus, although highly selective for the genus *Hyphomicrobium*, the method yields several species among the isolates.

Humic Gel Selection for *Pedomicrobium* This method yields *Pedomicrobium* without a liquid enrichment step. Samples of soil or manganous deposits (e.g., from pipelines) suspended in water or saline, or of a water sample, are streaked on humic gel agar (for preparation, see Gebers and Hirsch, 1978) containing vitamins (to prevent pleiomorphy of *Pedomicrobium*) and cycloheximide (to inhibit fungi) at pH 5–6 (to inhibit other bacteria). After four to six weeks, yellow-brown cohesive colonies with dense cen-

ters and frayed edges appear. Isolation of *Pedomicrobium* requires vigorous disruption of these colonies (e.g., with the aid of a mechanical homogenizer) in a suspending medium and restreaking, since they are often overgrown by or contain other bacteria. After isolation, more rapid growth can be allowed by cultivation in acetate-yeast extract medium (PSM, below).

The principle underlying this direct isolation is the same as in the general procedure outlined above: during the first few weeks of incubation, other bacteria and fungi (and sometimes also algae and amebae) grow and multiply. *Pedomicrobium* colonies develop as the others decline (Gebers, 1981).

Identification of Dimorphic Prosthecate Bacteria

The genera of DPB are defined and therefore identified primarily on the basis of morphological traits. Accordingly, identification of an isolate as a representative of one of these genera requires microscopical examination, during the growth phase, of pure cultures that meet the following criteria.

1. Uniform dimorphic morphology.
2. Minimal to moderate accumulation of reserve polymers.
3. Not more than 50% of the population accounted for by swarmers.

These criteria are based on the following considerations.

1. Most isolates, particularly of hyphomicrobia, exhibit pleiomorphy even during the growth phase of a culture; all isolates do so in stationary phase. Pleiomorphy can often be reduced by vitamin supplementation of the medium (Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Matzen and Hirsch, 1982a; Vedenina and Govorukhina, 1988) and is invariably reduced by the use of media containing not more than 0.2% soluble organic nutrients. "Uniform dimorphic morphology" means all prosthecate cells look alike, all swarmers look alike, and all reproductive cells (the link between the two forms) look alike.

2. DPB accumulate large quantities of carbon or phosphorus reserves when these nutrients are available in excess. Reduction of the carbon or phosphorus source that minimizes such accumulation typically also yields cultures of more uniform morphology and reduces the proportion of "bizarre" forms (Tyler and Marshall, 1967b).

3. Because swarmers of these bacteria arise by cell reproduction and do not themselves reproduce, a swarmer proportion greater than 50% indicates a failure of cell maturation and implies that aberrant development will be observed among the prosthecate cells present (see, e.g., Dow et al., 1983). *A. excentricus*, whose nonprosthecate cells can divide in some media (Larson and Pate, 1975), is an exception to this criterion.

In any pure culture that meets these criteria, there will be three types of cells: prosthecate cells, swarmer cells, and reproducing cells. Their definitive characteristics are best determined by electron microscopy of shadowed or negatively-stained specimens (Fig. 5). The bands typical of (although not universal among; Poindexter,

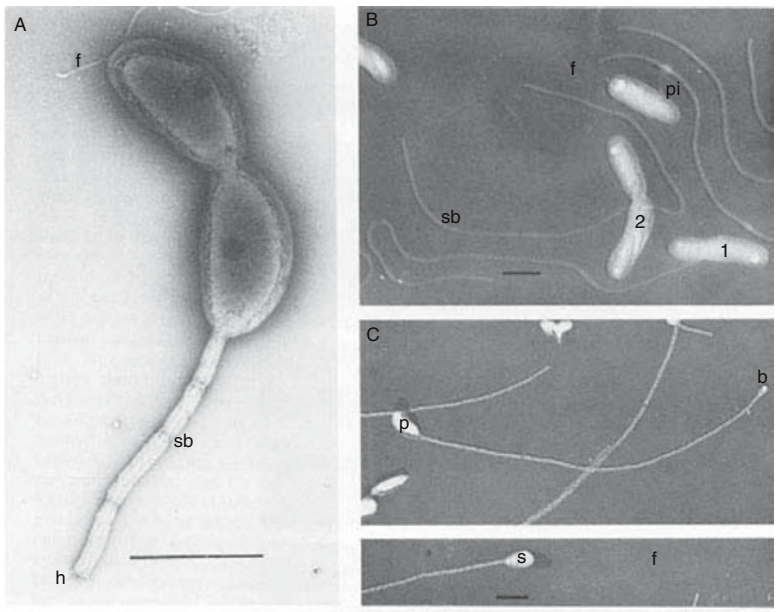

Fig. 5. (A) Negatively stained (K-PTA) *Caulobacter* cell. h, holdfast material; sb, stalk band; f, flagellum (with hook). (B) Pt-shadowed cells of *A. biprosthecum* with one (1) and two (2) prosthecae, and a swarmer with flagellum (f) and prosthecal initials (pi); sb, stalk band. The incipient swarmer of the dividing biprosthecate cell is flagellated. (C; two panels) Pt-shadowed *Hyphomicrobium* sp. cells; p, prosthecate ("mother") cell; b, very young bud; s, incipient swarmer with flagellum (f). Bar = 1 μm.

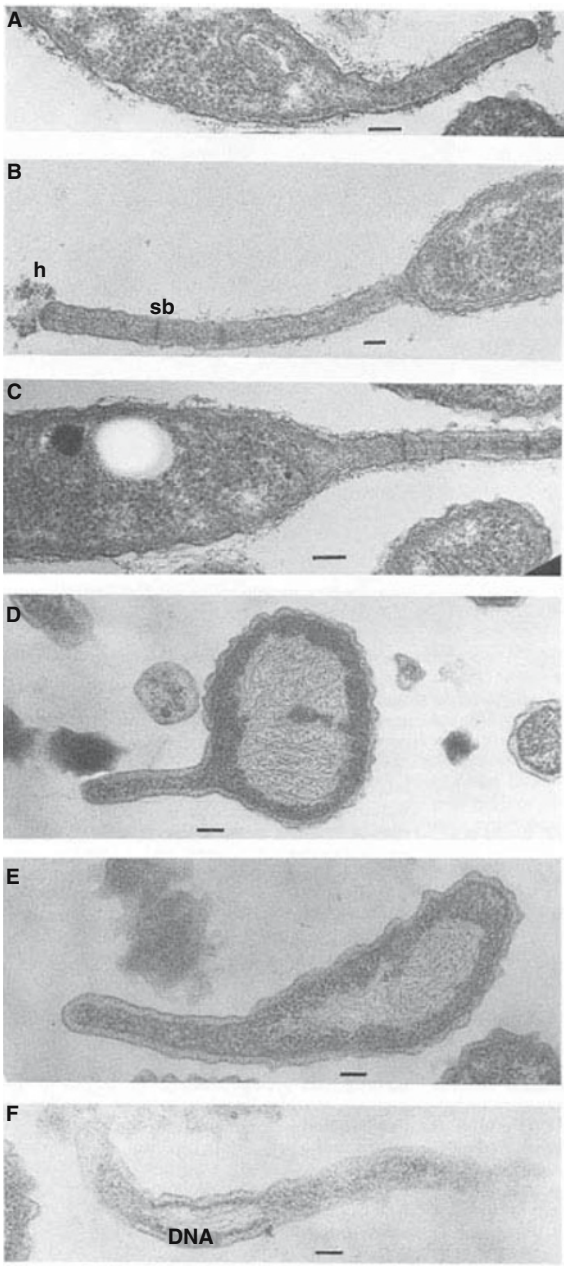

Fig. 6. Sections of prosthecae. (A–C) *Caulobacter* stalks; h, holdfast material; sb, stalk band. (D–F) *Hyphomonas* hyphae, with ribosome density as within the cell body cytoplasm; DNA, fibrils of DNA. Bar = 100 nm.

1989a, 1989b) caulobacterial prosthecae are discernible in such specimens. The identity of a cellular appendage as a prostheca can be established only by examination of thin sections (Fig. 6) that reveal the presence of all three components of the cell envelope: outer membrane, peptidoglycan layer, and cytoplasmic membrane. This technique also distinguishes the caulobacterial "stalk," which typically lacks cytoplasmic components through most or all of its length, from the (wider) hyphomicrobial "hypha," which includes cytoplasmic components throughout its length.

Cell shape is uniform in each developmental stage of each isolate, but variable within each genus. *Caulobacter* cells may be vibrioid, rod-shaped, or fusiform; *Asticcacaulis* cells are only rod-shaped. Known hyphomicrobia are rod-shaped or ovoid to nearly spherical, and incipient buds and swarmer cells may exhibit a shape different from that of mature, prosthecate cells. The morphology of the reproductive cell is distinctive for three of the genera; the fourth form is shared by two genera, as follows.

Caulobacter: approximately equatorial constriction; one pole prosthecate, one pole monoflagellate. There is one prostheca per cell.

Asticcacaulis: unevenly divided by septation; the prosthecate sibling is characteristically longer, with the prostheca subpolar or lateral, and the shorter sibling subpolarly monoflagellate. There may be one or two prosthecae per cell.

Pedomicrobium: the larger, prosthecate cell is the "mother" cell, and the smaller bud arises at the distal tip of the prostheca; as the bud grows, its long axis is perpendicular to the hyphal axis; the bud bears one subpolar flagellum. There may be one, two, or more prosthecae per cell.

Hyphomicrobium and *Hyphomonas*: morphology as in *Pedomicrobium* except that the long axis of the bud is a continuation of the hyphal axis. In *Hyphomicrobium*, the swarmer may possess more than one (polar or subpolar) flagellum. Typically one, rarely more than two prosthecae per cell.

Distinguishing among the genera of hyphomicrobia has long been difficult, particularly because of their tendency to pleiomorphy (Bauld and Marshall, 1971; Bauld and Tyler, 1971; Bauld et al., 1971). However, it is now apparent that this morphology occurs in at least three distinct physiotypes: 1) methylotrophs (now *Hyphomicrobium* [Hirsch, 1989]), among which requirements for organic growth factors are unknown, although vitamins may be stimulatory, and for which nitrate can serve as sole source of nitrogen; 2) non-methylotrophic organisms (now *Pedomicrobium* [Gebers, 1989]) that prefer organic acids as carbon sources, accept ammonia as sole source of nitrogen, and require organic growth factors; and 3) amino-acid-requiring isolates (now *Hyphomonas* [Moore and Weiner, 1989]), all of which are either marine isolates or can be maintained on marine media. Accordingly, distinction between the two genera that have similar morphology in the budding stage requires determination of dependence on amino

acids as macronutrients or of ability to grow with methanol or methylamines as the only organic substrates.

Cultivation of Dimorphic Prosthecate Bacteria

Although media designed to mimic the composition of animal tissues ("routine" bacteriological media) are unsuitable for the cultivation of these chemoheterotrophic bacteria (for example: Attwood and Harder, 1972; Bauld et al., 1971; Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Gromov, 1964; Havenner et al., 1979; Hirsch and Conti, 1964a; Kingma Boltjes, 1936; Kudryavtsev, 1978; Lapteva, 1977, 1987; Larson and Pate, 1975; Li et al., 1984; Loeffler, 1890; Poindexter, 1964, 1981a; Weiner et al., 1980), such media are *qualitatively* suitable; they need only be diluted at least three- to ten-fold. The importance of reducing total solute concentrations—principally of organic compounds, phosphate, and ammonium—cannot be over-emphasized. Growth of DPB in "rich" media or in severely unbalanced media is extremely poor if it occurs at all, and the cells are structurally fragile and morphologically aberrant. *Hyphomonas* isolates are the only members of this group that tolerate 0.5% or more of complex organic material, but in such media, viability even of this group is lower than on more dilute media, and the populations exhibit considerable genetic as well as structural and developmental instability.

The importance of employing dilute media was discovered during the first reported isolation of *Caulobacter* (Loeffler, 1890). In a study of methods for staining bacterial flagella, Loeffler encountered "eines höchst merkwürdigen Organismus" in a strongly diluted cabbage ("Kohlrabi") infusion. He purified the organism on Kohlrabi infusion solidified with gelatin, which the organism did not digest. On transfer to a richer, meat extract-peptone-gelatin agar medium, its growth was slower and development of prosthecae (described by Loeffler as thick thread-like appendages) was sparse. On return to the very dilute medium, the organism developed extremely long prosthecae that extended across the microscopic field. The organism is illustrated in Figs. 7 and 8 of Loeffler (1890) as it appeared on the two media. Uncertain of its nature, Loeffler named the organism *"Vibrio* (?) *spermatozoides"*; the cell form and pale yellow color of the colonies suggest that it was the organism known today as *C. vibrioides*. Because of his study, nutrient sensitivity in *Caulobacter* is the longest-known physiologic property of these DPB.

Many DPB are also sensitive to chelating agents such as nitrilotriacetate, ethylenediamine tetraacetate, and citrate and to phosphate, which binds many cations with affinities comparable to those of organic chelators (Hirsh, 1974; Hirsch and Conti, 1964a; Larson and Pate, 1975; Poindexter, 1984b). Thus, if chelators are used, their concentrations must be not more than 1 mM. Similarly, substances added as pH buffers (e.g., phosphate, HEPES, imidazole) are inhibitory or interfere with development at concentrations that provide dependable buffering capacity; imidazole is tolerated at the highest concentration (5 mM), but not by all isolates. Phosphate is provided in some media for some strains at 10 to 20 mM, but development is impaired (as evidenced by the accumulation of swarmers in late exponential and stationary phases), and the cells are relatively fragile. The majority of isolates exhibit requirements for vitamins (biotin, riboflavin, B_{12} or pantothenate) or for organic growth factors that are still unidentified. Pleiomorphy, in particular, is frequent among hyphomicrobia unless vitamins are provided (Gebers, 1981; Gebers and Beese, 1988; Gebers and Hirsch, 1978; Matzen and Hirsch, 1982a; Vedinina and Govorukhina, 1988).

Detailed recipes for isolation and cultivation of DPB are presented below. However, dilute (less than 0.2%) peptone media will support the growth of all DPB (even of methylotrophic hyphomicrobia, which will obtain their principal carbon source from laboratory air [Kingma Boltjes, 1936]). For nonfastidious isolates, a general basal medium should provide (in distilled water or in 50 to 75% seawater) a mixture of amino acids, vitamins, trace minerals (with relatively high proportions of Mg and Ca [Harder and Attwood, 1978; Hirsch and Conti, 1964a; Johnson and Ely, 1977; Poindexter, 1984a, 1984b]), not more than 1 mM phosphate, and ammonia at a molarity not more than twice the molarity of amino acids. A principal carbon source could be added as: a sugar (for instance, glucose, xylose, fructose, or maltose; filter sterilized) for *Caulobacter* and *Asticcacaulis*; methanol or methylamine for *Hyphomicrobium*; acetate or ribose for *Pedomicrobium*; and amino acids for *Hyphomonas*, with a total organic concentration not more than 0.1 to 0.2%. A neutral pH, an incubation temperature of 25–30°C, and aerobic conditions will allow growth of all types of DPB.

Cultivation of *Caulobacter* and *Asticcacaulis*

Isolates from fresh water and soil can be cultivated on dilute peptone-yeast extract medium (PYE). However, prostheca development is somewhat inhibited by the yeast extract, and

morphology is more regular in dilute peptone medium supplemented with $CaC1_2$ (PCa). Marine caulobacters have been cultivated only in complex medium (CPS). The majority of isolates must be cultivated and maintained on PYE, PCa, or CPS. For *C. vibrioides*, PYE must also be supplemented with riboflavin (0.1 μg/ml) to ensure perpetual subcultivation.

Growth in liquid medium occurs as a surface film on stationary cultures and as evenly-suspended turbidity in agitated cultures.

PYE Medium (Poindexter, 1964)

Peptone	0.2%
Yeast extract	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%

Use tap or distilled water.

PCa Medium (Poindexter, unpublished)

Peptone	0.2%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 2H_2O$	0.015%

Use distilled water.

CPS Medium (Poindexter, 1964)

Peptone	0.05%
Casamino acids	0.05%
Seawater	75–80%

Isolates of *C. crescentus* and *C. leidyi* grow readily in defined HiGg medium (see below), and *A. excentricus* can be cultivated in this medium supplemented with biotin (2 μg/L). Glutamate is not required as a growth factor or as a source of carbon or of nitrogen. Its role appears to be primarily as ammonia acceptor, since *C. crescentus*, at least, lacks glutamate dehydrogenase (Ely et al., 1978); growth rate and yield are maximal when the medium provides equimolar amounts of glutamate and ammonium chloride and glucose as principal carbon source (Poindexter, unpublished observations).

HiGg Medium (Poindexter, 1978)

Glucose (filter sterilized)	5–10 mM
Monosodium glutamate (filter sterilized)	5–10 mM
Phosphate (Na and K salts)	0.1–1 mM
NH_4Cl	5–10 mM
Imidazole	5 mM

Use Hutner's mineral base (Cohen-Bazire et al., 1957), prepared without vitamins.

Cultivation of *A. biprosthecum* in defined medium is dependent particularly on the presence of amino acids. Growth is optimal when five amino acids are provided as well as glucose, ammonia, and biotin (MS-B-AA medium).

MS-B-AA Medium (Larson and Pate, 1975)

Glucose	0.1%
Alanine, glutamate, serine, proline, aspartate	100 mg/ml of each
$(NH_4)_2HPO_4$ (optional)	0.075 mM
$NaH_2PO_4 \cdot H_2O$	0.5 mM
KH_2PO_4	0.5 mM
$MgSO_4 \cdot 7H_2O$	0.01%
Sodium citrate	0.01%
D-Biotin	4 μg/l

Trace salts (prepared at 1,000×; see Larson and Pate, 1975, for preparation); final concentration per liter:

$CaCl_2 \cdot 2H_2O$	10 mg
$CuSO_4$	1 mg
$CoCl_2 \cdot 6H_2O$	1 mg
$FeSO_4 \cdot 7H_2O$	10 mg
$K_2B_4O_7 \cdot 4H_2O$	1 mg
MoO_3	1 mg
$MnSO_4 \cdot H_2O$	10 mg
$ZnSO_4 \cdot 7H_2O$	10 mg

Cultivation of *Hyphomicrobium*

Hyphomicrobium isolates generally do not require organic growth factors and can be cultivated aerobically on defined media that provide a mixture of minerals, including nitrate or ammonia as nitrogen source and C_1 compounds as carbon sources (methanol, methylamine, trimethylamine). Many isolates can also be cultivated anaerobically in mineral media supplemented with methanol and KNO_3 as terminal electron acceptor or with methylamine and thioglycollate. Most isolates do not grow evenly dispersed in liquid media; in stationary cultures, cells accumulate as a pellicle and as a film on the vessel wall, and growth of many isolates is not evenly suspended even in continuously-agitated liquid cultures. All known isolates will grow on freshwater media, and some will also grow on seawater media or in media containing up to 5.5% NaCl. Temperature optima are wide, and growth may occur at 5 to 45°C.

Of many versions of the mineral medium 337," variations relate to quantities of trace salts; optimal quantities vary with the isolate. The following recipe is based on the recipes of Hirsch and Conti (1964a, 1964b), Moore and Hirsch (1972), Moore (1981), and Matzen and Hirsch (1982a) and would seem to be an average composition of general suitability.

Medium 337 General

The following are added per liter of distilled water:

KH_2PO_4	1.36 g
Na_2HPO_4	2.13 g
$(NH_4)_2SO_4$	0.5 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_{20}$	2–10 mg
$FeSO_4 \cdot 7H_2O$	2 mg
$MnSO_4 \cdot 7H_2O$ (20 mg, to detect Mn oxidation)	0.4–0.8 mg
$Na_2MoO_4 \cdot 2H_2O$	0.5–2.5 mg
Methanol (or methylamine, 67.5% solution)	5 ml
Vitamin B_{12} or vitamin mixture (Staley, 1968)	
KNO_3 (for anaerobic growth)	5 g

Cultivation of *Hyphomonas*

A defined medium (GAMS) has been developed for one isolate (*Hyphomicrobium neptunium*, now regarded as *Hyphomonas neptunium*), although growth is much slower than in Zobell's marine broth. All other isolates have been cultivated only on complex media, for which one-third strength Zobell's appears to be particularly suitable (Havenner et al., 1979). As with *Hyphomicrobium*, growth is typically clumpy, but smooth variants that grow more evenly dispersed regularly arise in stock cultures of *Hyphomonas* (Pongratz, 1957; Moore and Weiner, 1989; J. S. Poindexter, unpublished observations). All known isolates are marine (Weiner et al., 1985) and do not grow on freshwater media.

GAMS Medium (Havenner et al., 1979)

Glutamate, aspartate, serine, methionine	125 mM of each
Calcium pantothenate	0.26 µM
Seawater	30%

Cultivation of *Pedomicrobium*

Pedomicrobium isolates have not been cultivated on defined media; they may, like most *Caulobacter* isolates, require organic growth factors not yet identified. A generally suitable complex medium will provide an organic acid (acetate, malate, pyruvate, succinate, gluconate) at 10 mM as carbon source, a complex organic mixture (peptone, casamino acids, yeast extract) at 0.05% as nitrogen source, and a mixture of vitamins.

In isolates that deposit oxides of iron or manganese on their cells, this property can be observed macroscopically as yellow or brown coloration in colonies grown on media containing ferrous or manganous salts. Such colonies will develop a blue color when flooded with acidified 2% $K_4[Fe(CN)_6]$ or 0.4% leukoberbelin blue I (see Gebers, 1989). The property of metal oxide deposition may, however, be lost on laboratory cultivation and seems to be a "spasmodic" property in individual isolates of both *Pedomicrobium* and *Hyphomicrobium* (Tyler and Marshall, 1967a).

PSM (Gebers, 1981; Gebers and Beese, 1988)

Sodium acetate	10 mM
Yeast extract	0.05%
Starch (or 0.4% gelatin)	0.2%
Metals 44 (Cohen-Bazire et al., 1957)	1 ml/l
Vitamins (Staley, 1968)	10 ml/l

Adjust to pH 9.0.

PYVM (Gebers and Beese, 1988)

Peptone	0.025%
Yeast extract	0.025%
Vitamins (Staley, 1968)	10 ml/l
Hunter's base (Cohen-Bazire et al., 1957)	20 ml/l
DL-Malate	10 mM

Adjust to pH 7.5.

Maintenance of Dimorphic Prosthecate Bacteria

All DPB can be maintained as vegetative stock cultures on dilute peptone medium of appropriate ionic composition, refrigerated between transfers. They can also be preserved as frozen suspensions, with a cryoprotectant such as glycerol (*Hyphomicrobium*, *Hyphomonas*) or without a cryoprotectant (*Caulobacter*, *Asticcacaulis*). Hyphomicrobia and some *Asticcacaulis* isolates are also dependably preserved by lyophilization, with or without milk; however, this process is not dependable for *Caulobacter* isolates, particularly marine strains. *Hyphomicrobium* and *Pedomicrobium* are tolerant of desiccation and can be preserved as slant cultures in screw-capped tubes stored at room temperature; if dry, they can be revived by rehydration with liquid growth medium.

Ecological Roles and Potential Applications of Dimorphic Prosthecate Bacteria

The DPB exhibit two characteristics that could be exploited in the purification of waters: the property of adhesiveness and the ability to metabolize organic materials available in extremely low quantities. A wide variety of materials are suitable substrata for their attachment, including glass—a durable material available in increasingly burdensome quantities that could support (physically) equally durable populations of bacteria such as *Caulobacter* and *Hypomicrobium* in purification beds for groundwater and other waters bearing low levels of pollutants. Caulobacters have been demonstrated to be capable of rapidly mineralizing agricultural pollutants present in very low concentrations (Grimes and Morrison, 1975). They rise to prominence in enrichment cultures provided with hydrocarbons, including aromatic compounds, as sole carbon sources (Moaledj, 1978; Murakami et al., 1976) and retain the ability to oxidize aromatic compounds after years of cultivation on peptone media (Chatterjee and Bourquin, 1987). Efforts are in progress to expand their catabolic versatility by the introduction of *Pseudomonas* genes (Chatterjee and Chatterjee, 1987) and other plasmids (Anast and Smit, 1988).

Hyphomicrobia can mineralize a variety of pollutants, including aromatic hydrocarbons (Moaledj, 1978), dimethyl sulfoxide and dime-

thyl sulfide (DeBont et al., 1981; Suylen and Kuenen, 1986), methyl chloride (Hartmans et al., 1986), and various alcohols (Köhler and Schwartz, 1982). The unique capability of *Hyphomicrobium* to denitrify sewage supported (metabolically) by methanol, a relatively inexpensive carbon source, has already proved practicable (Claus and Kutzner, 1985). Since anaerobic stages of sewage treatment plants generate methane and at least one isolate of *Hyphomicrobium* is known to utilize this compound (see Hirsch, 1989), it would seem reasonable to expect that denitrification and methane utilization could be combined in *Hyphomicrobium* an isolate or by genetic manipulation (Dijkhuisen et al., 1984) thereby eliminating the need for methanol in the denitrification of sewage by *Hyphomicrobium*.

On the other hand, the same properties that would allow exploitation of these organisms probably enable them to foul submerged surfaces; they are particularly likely to become a nuisance in water distribution systems. Although often seen as members of biofouling communities on submerged surfaces, DPB have been regarded as relatively unimportant as pioneers, for two reasons, both based on microscopical studies. First, the microbial cells that appear earliest on experimental surfaces are not typically prosthecate (Corpe, 1978; Corpe et al., 1975; Marshall, 1976). Second, prosthecate bacteria do not predominate numerically at any stage of fouling. The first observation is consistent with the experimental evidence that attachment is initiated principally by the nonprosthecate, swarmer stage (Newton, 1972; Hirsch, 1974; Leifson, 1962; Moore and Marshall, 1981; Zavarzin, 1961), in which stage DPB are not distinguishable from other rods and vibrios by microscopy. The second is predictable, but does not preclude a significant role of DPB in initial conditioning or continued maintenance of the surface as suitable for attachment of other microorganisms and, later, of animals.

Beyond potential assistance from DPB in remedying problems of technological origin, their predictable presence in almost any sample of fresh or sea water and in many types of soils implies that their activities are compatible with and possibly of benefit to diverse microbial communities. At present, the only significant role of which they seem capable is as mineralizers. However, the frequent occurrence of DPB attached to plant and algal remains may reflect their participation in mobilization of detritus as polymer-digesting microbes slowly free organic carbon from insoluble forms. As Lapteva (1987) has pointed out, although DPB may be responsible for the bulk of mineralization only in waters of low organic content, their total contribution in the vastness of the biosphere's waters may be considerable.

Similarly, the high frequency of the occurrence of DPB as algal epibionts may reflect an influence on primary productivity that is not yet appreciated. In quantitative studies of *Pseudomonas* and *Caulobacter* in association with algae (*Gomphonema*, *Cyclotella*, and *Chlorella*), Allen (1971) observed that total productivity algal and bacterial was higher in two- or three-membered populations than when algae or bacteria were incubated as monotypic populations. Associations fared especially well when the source of carbon was organic material derived from a macrophyte *(Naxas)*. He inferred that within algal-bacterial associations, carbon could cycle between substratum and epibionts; such association would therefore be of mutual benefit to the associates. This intriguing possibility has not been pursued experimentally.

Nevertheless, the dimorphic habit of caulobacters and other DPB can be viewed as an adaptation particularly appropriate to attachment to living substrata. As new surface was generated by growth and reproduction of the substratum organism, each generation of epibiont would be prepared to relocate (by means of its swarmers) on virgin territory, while the substratum would not be overburdened by dense accumulations of epibionts. Clearly, the possibility that epibiontic prosthecate bacteria are advantageous to their algal associates and could have a direct and positive influence on primary productivity in aquatic (and possibly terrestrial) environments is worthy of exploration. Such a niche would be consistent with both the physiological properties and the unique developmental pattern of the dimorphic prosthecate bacteria.

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