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Evolution

A phylogenetic analysis of the myxobacteria: Basis for their classification

(16S rRNA/δ purple bacteria/rRNA signature/rapid evolution)

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ABSTRACT **The primary sequence and secondary structural features of the 16S rRNA were compared for 12 different myxobacteria representing all the known cultivated genera. Analysis of these data show the myxobacteria to form a monophyletic grouping consisting of three distinct families, which lies within the δ subdivision of the purple bacterial phylum. The composition of the families is consistent with differences in cell and spore morphology, cell behavior, and pigment and secondary metabolite production but is not correlated with the morphological complexity of the fruiting bodies. The *Nannocystis exedens* lineage has evolved at an unusually rapid pace and its rRNA shows numerous primary and secondary structural idiosyncrasies.**

The myxobacteria have traditionally been perceived as a phylogenetically distinct and highly unusual group of evolutionarily "advanced" bacteria. This was true in the heyday of microbial taxonomy, when the bacterial world was seen as comprising three major classes, the Eubacteriae, Myxobacteriae, and Spirochaetae (1), and remained so when microbiologists later came to reject their earlier taxonomies and abandoned the search for a detailed natural microbial system as a futile exercise (2–4):

> ". . . we can also discern four principal subgroups [of prokaryotes], blue-green algae, myxobacteria, spirochetes, and eubacteria. . . . Beyond this point, however, any systematic attempt to construct a detailed scheme of natural relationships becomes the purest speculation . . ." (4).

The reason why myxobacteria commanded such special attention, and why microbiologists felt that they represented a phylogenetically distinct group, is obvious: In the morphologically mundane world of prokaryotes the myxobacteria exhibit impressive morphological complexity with their fruiting bodies. All myxobacteria have a developmental cycle in which tens of thousands of cells aggregate together to form these brightly colored bodies, often tree-like in form, usually visible to the naked eye (5). The rod-shaped cells within the fruiting body become metabolically dormant and environmentally resistant.

Fruiting-body morphology is still the primary basis of species classification even though it is clear that some genera form aberrant fruiting bodies or no fruiting bodies at all after continued cultivation (6, 7). During the process of isolating a pure culture, strains may change in such a way as to cease producing "typical" fruiting bodies. Type materials are, in some cases, represented by herbarium specimens of fruiting bodies, not by reference cultures (6). There is still some disagreement among taxonomists as to the precise placement of many genera and species, and the absence of well-defined and available type or reference strains further complicates taxonomic placement (6, 7). The present study was severely limited in that reference strains listed in *Bergey's Manual* (6) were not made available by the authors. The placement of reference cultures in accessible collections, such as the American Type Culture Collection, needs to be an established practice.

The use of molecular taxonomic approaches may help alleviate some of these problems, gradually replacing fruiting-body morphology as the ultimate taxonomic criterion and paving the way for modern myxobacterial systematics rooted in the genetic lineages of the organisms (8, 9). Nucleic acid sequencing is commonly used to establish phylogenetic relationships, and the 16S rRNA has come in for considerable use as a molecular chronometer (10). In the present study, 16S rRNA sequences were used to examine the phylogeny of representatives of the myxobacterial genera. The results suggest that all myxobacteria form a phylogenetically coherent order, one of at least three, within the δ subdivision of the purple bacterial phylum.

MATERIALS AND METHODS

Bacterial Strains. *Archangium gephra* (ATCC 25201), *Angiococcus disciformis* (ATCC 33172), *Cystobacter fuscus* (ATCC 25194), *Corallococcus (Myxococcus) coralloides* (ATCC 25202), *Melittangium lichenicola* (ATCC 25946), and *Stigmatella aurantiaca* (ATCC 25190) were obtained from the American Type Culture Collection and were cultivated in MD-1 (11). *Polyangium cellulosum* subsp. *ferrugineum* (ATCC 25531) was cultivated in CMA (0.5% Fleischmann's dry yeast/0.4% Difco casitone/0.15% $CaCl_2 \cdot 2H_2O$/0.15% $MgSO_4 \cdot 7H_2O$/0.001% vitamin B-12, pH 7.0). *Chondromyces crocatus* Cm c6, *Chondromyces apiculatus* Cm a2, and *Polyangium* sp. Pl 4943 were kindly furnished by H. Reichenbach (Gessellschaft für Biotechnologische Forschung, Braunschweig, Germany) and were cultivated in PMS [0.3% soluble starch/0.1% $MgSO_4 \cdot 7H_2O$/0.05% $CaCl_2 \cdot 2H_2O$/0.4% Difco casitone/0.4% Carnation nonfat dry milk/0.005% vitamin B-12 with trace-element solution (12) at 1 ml/liter]. *Nannocystis exedens* Na e1 (ATCC 15963) was also furnished by H. Reichenbach and cultivated in MD-1. Cells were grown at 32°C with shaking and harvested when the culture reached 2–5 × 10^8 cells per ml. Cells were removed from the growth medium by centrifugation at 10,000 × *g* and the pellet was frozen at −75°C until use.

Sequencing Methods. Total RNA was extracted by customary procedures (13, 14). The dideoxynucleotide chain-termination method (15, 16) adapted for direct rRNA sequencing using reverse transcriptase (17) was employed. Synthesized strands were labeled by the inclusion of [α-[^{35}S]thio]dATP (16). A standard set of primers (usually eight) specific for eubacterial 16S rRNAs (9, 18) was routinely used. All sequences are >90% complete.

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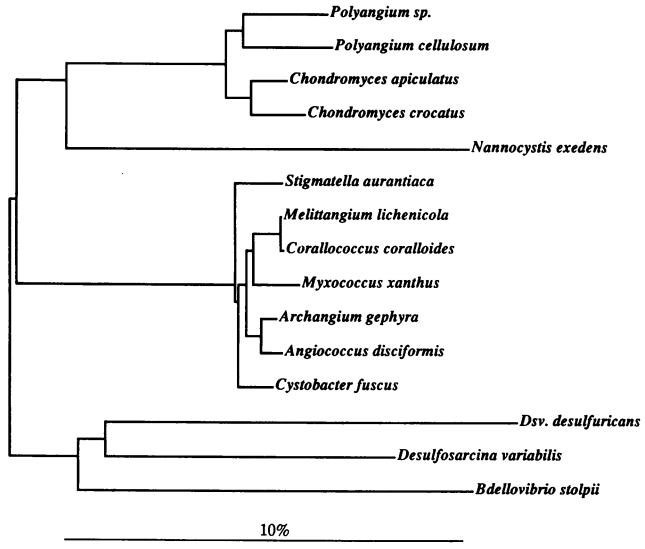

FIG. 1. Phylogenetic tree for the myxobacteria derived from the evolutionary distances of Table 1; see *Materials and Methods*.

Data Analysis. The sequences were aligned in our sequence editor, ae2, against a representative collection of bacterial 16S rRNAs (19). Corrected pairwise distances (expressed as estimated changes per 100 nucleotides) were computed from percent similarities by using the Jukes and Cantor correction (20) as modified by G. J. Olsen (described in ref. 18) to accommodate the actual nucleotide ratios. Dendrograms were constructed from evolutionary distance matrices by using the algorithm of De Soete (21).

RESULTS AND DISCUSSION

The myxobacteria are not, as previously believed, a phylogenetically highly distinct group of bacteria. Rather, they reside within the δ subdivision of the purple bacterial phylum together with the morphologically simpler sulfur- and sulfate-reducing bacteria and the bdellovibrios (9, 10). These relationships are shown in Fig. 1, a phylogenetic tree derived from the evolutionary distances of Table 1. The root of this tree has been determined using representatives from other purple bacterial subdivisions as outgroups and is not located within the myxobacterial group.

The myxobacteria comprise three distinct subgroups, Myxococcus, Chondromyces, and Nannocystis (which contains a single species). The last two appear to be specifically though distantly related. The phylogenetic distances separating the three subgroups are consistent with their classification as families. However, the definition of taxonomic rank based upon 16S rRNA sequences is still in a state of flux.

A substantial 16S rRNA sequence signature—listed in Table 2 and located in secondary structural representation in Fig. 2—distinguishes among the three myxobacterial subgroups. Fig. 2 also indicates the few shared derived characters (synapomorphies) that distinguish cleanly between the myxobacteria as a whole and the remaining δ purple bacteria, i.e., the tertiary pair 245·283 and the pair 1428·1472.

Higher-order structural features in the 16S rRNA also distinguish the three major myxobacteria families from one another. (*i*) The presumed ancestral form for the hairpin structure between positions 1128 and 1144 shows two unpaired bases, 1130 and 1131, which form a unilateral bulge in the stem, and the loop capping the structure comprises five or six nucleotides (see Fig. 2). However, all members of the Myxococcus subgroup eliminate these two bulged residues and shorten the capping loop to four nucleotides, whereas the Chondromyces subgroup retains the ancestral structure, with a loop of five nucleotides, which *Nannocystis* also does, except that in this case the loop comprises six nucleotides. (*ii*) In the adjacent hairpin structure, positions 1161–1175, the capping loop in almost all Bacteria comprises five nucleotides (Fig. 2). The Chondromyces subgroup, alone among the purple bacteria, shows six nucleotides in the loop, while *Nannocystis* reduces the number to four. (*iii*) The Myxococcus subgroup drastically alters the penultimate hairpin structure in the 16S rRNA (positions 1435–1466; see Fig. 2): Typically, this structure comprises 32–34 nucleotides, which form a 10-base-pair stalk interrupted by a single unilateral bulged residue (position 1441 in Fig. 2) and an asymmetric bilateral bulge (positions 1446 and 1447 vs. 1456), with a capping loop of four nucleotides. The Chondromyces subgroup and *N. exedens* exhibit this canonical structure; but the Myxococcus subgroup truncates it to an uninterrupted stalk

Table 1. Evolutionary distances among 16S rRNAs of various myxobacterial species

	Species	Evolutionary distance													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	*Dss. variabilis*	—													
2.	*Dsv. desulfuricans*	17.5	—												
3.	*Myx. xanthus*	16.8	20.2	—											
4.	*Crl. coralloides*	16.6	19.6	1.9	—										
5.	*Mel. lichenicola*	16.6	19.5	1.8	0.1	—									
6.	*Ang. disciformis*	17.0	19.5	2.0	1.9	1.8	—								
7.	*Arc. gephyra*	16.8	19.4	2.1	1.7	1.7	0.9	—							
8.	*Cys. fuscus*	16.3	19.5	2.7	2.1	2.0	1.8	1.7	—						
9.	*Sma. aurantiaca*	16.4	19.5	2.8	2.2	2.2	2.4	2.1	2.8	—					
10.	*Pol. cellulosum*	17.0	20.6	14.9	14.8	14.8	14.8	14.5	14.5	14.8	—				
11.	*Con. crocatus*	17.1	21.2	13.8	13.7	13.7	13.7	13.4	12.9	13.7	4.6	—			
12.	*Con. apiculatus*	17.1	21.3	13.8	12.9	12.9	13.2	12.8	12.8	13.0	3.5	2.2	—		
13.	*Pol.* sp.	17.3	21.4	14.7	14.4	14.4	14.1	13.9	13.7	14.4	4.3	5.1	4.3	—	
14.	*Nan. exedens*	20.6	21.4	19.2	19.0	18.9	18.0	18.3	18.5	18.7	16.6	16.0	16.0	16.4	—
15.	*Bde. stoplii*	18.2	20.5	18.6	18.1	18.0	18.9	18.5	18.5	18.1	20.4	19.8	19.1	20.0	21.7

The positions used in calculating the distances meet the condition that a known nucleotide is present in all sequences analyzed, and one base accounted for at least 50% of the composition at that position. The GenBank accession numbers for the myxobacterial 16S rRNA sequences used herein are as follows: *Myxococcus xanthus*, M34114 (9); *Myxococcus (Corallococcus) coralloides*, M94278; *Melittangium lichenicola*, M94277; *Angiococcus disciformis*, M94374; *Archangium gephyra*, M94273; *Cystobacter fuscus*, M94276; *Stigmatella aurantiaca*, M94281; *Polyangium cellulosum*, M94280; *Chondromyces crocatus* Cm c6, M94275; *Chondromyces apiculatus* Cm a2, M94274; *Polyangium* sp. Pl 4943, M94280; and *Nannocystis exedens* Na e1, M94279; with *Desulfosarcina variabilis*, M26632; *Desulfovibrio desulfuricans*, M34113, and *Bdellovibrio stolpii*, M34125, serving as outgroups.

Table 2. Small-subunit rRNA sequence signature defining and distinguishing the three main subgroups of myxobacteria from one another or from the remaining δ purple bacteria

Position(s) of base or pair*	Composition				
	Chondromyces group	Myxococcus group	*Nannocystis exedens*	Remaining δ purple bacteria	"Ancestral"†
36·548	C·G	U·A	U·A	C·G	C·G
38	G	A	U	G	G
45·396‡	U·A	C·G	G·C	U·R	—
116	C	A	C	A	A
146·176	G·C	U·A	G·C	G·C	G·C
245·283‡§	C·C	C·C	C·C	U·U	U·U
293·304	G·C	G·U	G·U	G·U	G·U
438·496‡	U·A	G·G	G·G	G·G	—
849	C	U	U	U	—
957	U	C	U	U	U
992	U	A	—	U	U
997·1044	U·A	A·U	C·G	U·A	U·A
1015	G	A	A	A	—
1045	C	U	A	C	C
1133·1141	C·G	G·C	A·U	A·U	—
1146	U	A	A	A	A
1253·1284	G·C	C·G	G·C	G·C	G·Y
1308·1329	U·A	A·U	U·A	U·A	U·A (Y·R)¶
1309·1328	G·C	A·U	G·C	G·C	G·C
1355·1367	R·Y	U·A	A·U	R·Y	R·Y
1428·1472§	U·A	U·A	U·A	A·U	R·Y
1437·1464	C·G	G·C	A·U	C·G	—

Positions were selected on the basis of compositional invariance in each of the myxobacterial subgroups and in the remaining δ purple bacteria (seven species) in the alignment.
*Position numbering defined by *Escherichia coli* sequence.
†Composition that is dominant in the vast majority of bacterial phyla.
‡Tertiary pairing (22).
§Distinguishes myxobacteria as a whole from the remaining δ purple bacteria.
¶Y·R (R·Y) pairs as used here do not include A·C-type pairings.

of eight pairs, capped by a loop of five nucleotides whose sequence, ACCAA, is unique.

The length of its branching in Fig. 1 suggests that the *N. exedens* lineage has evolved at an unusually rapid pace. Such lineages tend to show idiosyncrasies in their rRNA sequence and structure (10). *Nannocystis* is true to form. Out of a total of 45 positions in the δ purple bacterial 16S rRNA alignment in which all members, except for one or more members of the myxobacterial group, have a constant composition that appears ancestral for the entire purple bacterial phylum, *Nannocystis* provides an exception in 26 cases. The remaining myxobacteria show 11–18 exceptions.

The idiosyncrasies in the higher-order structure of *Nannocystis* 16S rRNA are even more telling, however. (*i*) The helix spanning the interval between positions 61 and 106 has a complex structure. Its middle section (shaded in Fig. 2) is highly variable phylogenetically in sequence, in overall length, and in structure; by contrast its base is constant in all respects, while the capping loop and underlying helix are constant in structure. Various phylogenetic groups present characteristic forms of this helix, by which they can be defined and distinguished. For the δ purple bacteria the portion of the helix above the base (positions 68–101) typically comprises 21–29 nucleotides: The terminal helix (three to seven pairs) and its capping loop are normal. However, the middle section, corresponding roughly to *E. coli* positions 70–77 and 92–99 (Fig. 2), is irregular, comprising four nucleotide "pairs," at least two of which are of the A·G type, which structure is separated from the overlying terminal helix/loop by a bulged nucleotide, at approximate *E. coli* position 93. *Nannocystis* truncates and simplifies the structure between positions 68 and 101; it comprises simply five (uninterrupted) normal pairs capped by a four nucleotide loop. (*ii*) Among the δ purple bacteria the helix between positions 197 and 220 comprises a stalk of eight base pairs interrupted by an asymmetric bilateral bulge loop, with a capping loop of four nucleotides. *Nannocystis* alone among the myxobacteria alters this structure, again by simplifying it, to an uninterrupted stalk of five normal pairs capped by a loop of four nucleotides. (*iii*) *Nannocystis* slightly alters the pattern of the helix whose cap crosses position 465, by deleting two nucleotides from the loop. (*iv*) As mentioned above, *Nannocystis* decreases the size of the loop crossing position 1170 from five to four nucleotides. (*v*) Finally, *Nannocystis* is one of only three (eu)bacteria to delete the nucleotide at position 993, and the only purple bacterium to do so (unpublished analysis).

The present results provide a framework for myxobacterial taxonomy rooted in 16S rRNA phylogeny. The myxobacteria would comprise three families, corresponding to the three subgroups defined above. Although this division is not consistent with taxonomies based upon fruiting-body morphology, it is consistent with the morphological and biochemical properties of the cells and spores. Members of the Myxococcus subgroup have long, slender cells that change their shape considerably during spore formation. The colonies absorb the diazo dye Congo red and form ridges of cells called ripples, which move processively within the colony to generate the appearance of waves (6, 7). Members of this subgroup also produce monocyclic carotenoid glucosides containing reddish keto pigments (23), 3-hydroxy and 2-hydroxy fatty acids, and antibiotics that are structurally distinct from those made by members of the other subgroups (24). Members of the Chondromyces and Nannocystis subgroups have much shorter vegetative cells that change their shape little upon myxospore induction; and their colonies neither absorb Congo red nor form ripples. Physiologically, the carotenoid glucosides of the Chondromyces subgroup lack the keto groups found in carotenoid glucosides of the Myxococcus subgroup, whereas *Nan-*

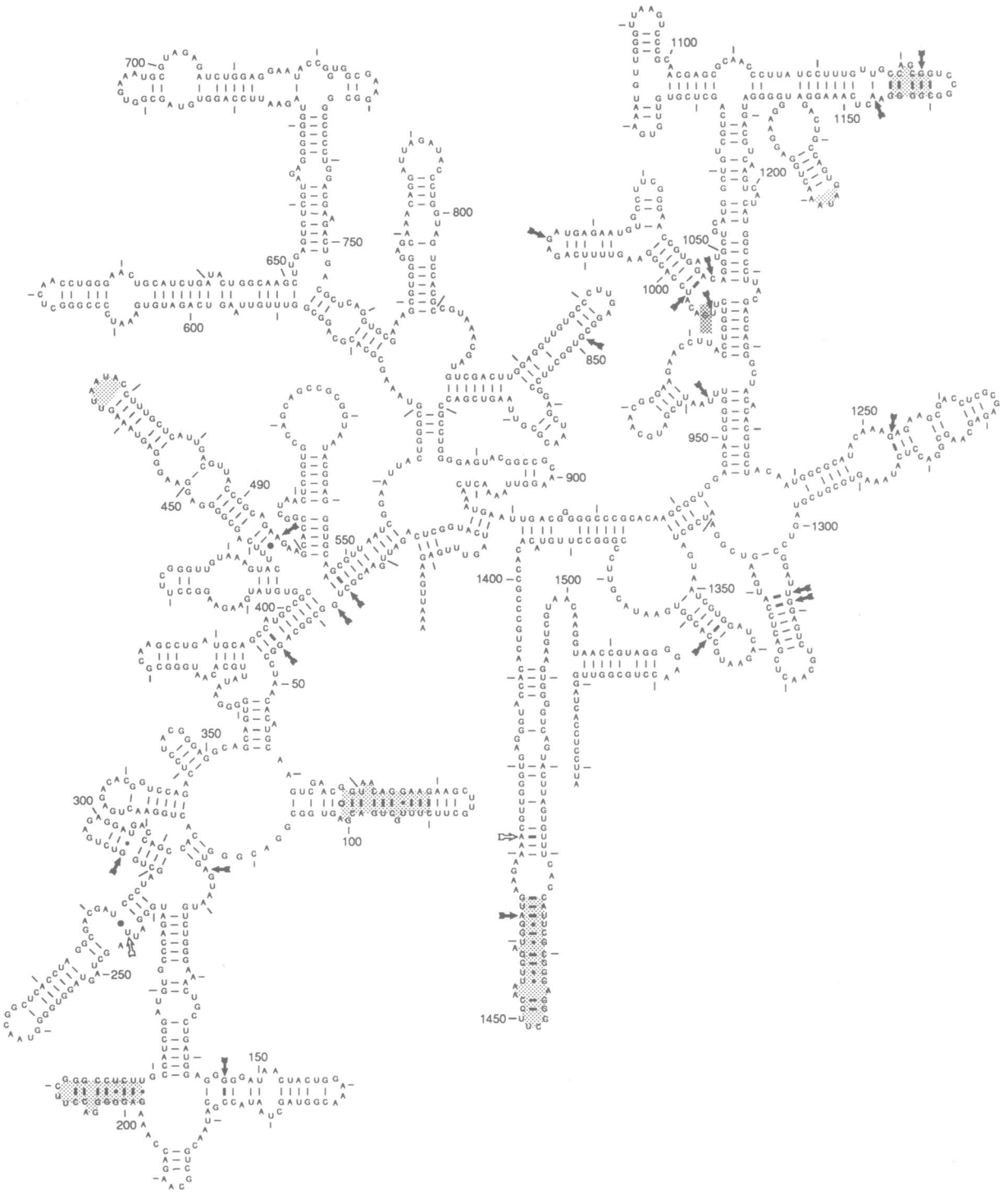

FIG. 2. Secondary structural representation of *E. coli* 16S rRNA (22) showing the signature positions (or base pairs) of Table 2, which define the subgroups of the myxobacteria (filled arrows). Also shown are the two positions that distinguish cleanly between the myxobacteria as a whole and the remaining δ purple bacteria (open arrows). The shaded regions indicate higher-order structural features discussed in the text.

nocystis forms monocyclic aromatic carotenoids, unique to that species (23). *Nannocystis* is also highly unusual in synthesizing sterols and squalene (25).

Previous classifications based largely upon characteristics of fruiting bodies have led to excessive taxonomic splitting; the families *Myxococcaceae, Archangiaceae*, and *Cystobacteraceae* (6, 7), separated by 16S rRNA distances of <3%, are more reasonably considered genera. The 16S rRNA phylogeny also offers a resolution to certain taxonomic conflicts. For example, *Crl. coralloides* has been placed in either the genus *Corallococcus* (7) or in *Myxococcus* (6). Clearly neither is the case. The 16S rRNAs of *Mel. lichenicola* and *Crl. coralloides* are so similar (see above) that they must be strains of the same species that form morphologically different fruiting bodies. In that the genera *Myxococcus, Corallococcus*, and *Melittangium* are separated by evolutionary distances of <2%, one should seriously consider placing all their species within a common genus. [The various species within many genera, *Leuconostoc* for example (26), are often separated by distances greater than this.]

Two other points of taxonomic dispute involve *Ang. disciformis* and the only cellulolytic myxobacterial species, *Pol.*

cellulosum. The former has been classified either as the lone species in the genus *Angiococcus* (7) or a member of the genus *Cystobacter* (6). Our results indicate that *Ang. disciformis* belongs in the same genus as *Arc. gephyra*, perhaps the same species (Fig. 1). In the case of *Pol. cellulosum*, the organism has been placed either in a separate genus, *Sorangium* (7), or lumped together with the noncellulolytic *Polyangium* species (6). The *Polyangium* strains used in this study are separated by an evolutionary distance of 4.3% and, in view of the physiological differences between them, should be placed in separate genera. Clearly a reassessment of myxobacterial taxonomy is in order.

A long-standing problem of myxobacterial ecology has been the enumeration of cells in environmental samples by viable counts. The lack of a selective medium, slow growth, and the propensity of large numbers of cells to exist in a polysaccharide matrix that is resistant to disruption have so far proven insurmountable difficulties. *Nannocystis* is regarded as the most abundant myxobacterium, but it has proven difficult to quantitate its abundance (7). *In situ* hybridization of environmental samples with group- or species-specific probes is gaining widespread popularity among microbial ecologists, and it offers the possibility of enumerating myxobacteria without cultivation (27). As discussed above, the helix spanning positions 62–106 (see Fig. 2) has distinct taxon-specific forms. The idiosyncratic truncated versions of this and the 198–219 helix seen in *Nannocystis* are both excellent candidates for developing species-specific probes.

The δ subdivision of the purple bacteria contains three groups of organisms whose phenotypes are so different that their phylogenetic relationship was unexpected: the sulfur- and sulfate-reducing bacteria, the myxobacteria, and the bdellovibrios (9). The dissimilatory sulfur- and sulfate-reducing bacteria are strict anaerobes that utilize a variety of oxidized sulfur compounds as electron acceptors. The bdellovibrios are aerobic bacterial predators that reproduce in the periplasm of enteric bacteria. The phylogeny of the δ subdivision brings into focus several important evolutionary transitions. The bdellovibrios and myxobacteria may have evolved their aerobic respiratory chain from that of a sulfur/sulfate-reducing ancestor (10). A second evolutionary milestone is reached with the appearance of the developmental and cell division cycles of the myxobacteria and the bdellovibrios, which are much more complex than those of the sulfur/sulfate-reducing bacteria. A final transition concerns the appearance of gliding motility, a property essential for fruiting-body formation as well as the other social and predatory behaviors of myxobacteria. Gliding, although taxonomically widely distributed among the Bacteria in general, is confined to a few δ subdivision members, i.e., *Desulfonema* and the myxobacteria. Was the ancestral purple bacterium a glider or was this trait introduced more recently into the lineage? A more careful analysis of the molecular mechanisms involved in these evolutionary transitions, coupled with a detailed phylogenetic analysis of the relevant genes, should help reveal the nature of the factors involved in the evolution of this interesting group of organisms.

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