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# Evolutionary Relationships among Sulfur- and Iron-Oxidizing Eubacteria

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**Some 37 reverse transcriptase, partial 16S rRNA sequences from sulfur- and/or iron-oxidizing eubacteria, including sequences from species of the genera *Thiobacillus*, *Thiothrix*, *Thiomicrospira*, *Acidophilium*, "*Leptospirillum*," *Thiovulum*, and *Chlorobium*, have been determined. In addition, 16S sequences from a number of unnamed sulfur- and/or iron-oxidizing bacteria from hydrothermal vent sites, from invertebrate-bacterial endosymbioses, and from various mineral recovery operations also have been determined. The majority of sequences place their bacterial donors in one or another of the subdivisions of the *Proteobacteria*. However, three unnamed facultatively thermophilic iron-oxidizing isolates, Alv, BC, and TH3, are affiliated with the gram-positive division. One $H_2S$-oxidizer, from the genus *Thiovulum*, is affiliated with *Campylobacter*, *Wolinella*, and other genera in what appears to be a new subdivision of the *Proteobacteria*. Three "*Leptospirillum*"-helical vibrioid isolates, BU-1, LfLa, and Z-2, exhibit no clear phylum level affiliation at all, other than their strong relationship to each other. A picture is emerging of an evolutionary widespread capacity for sulfur and/or iron oxidation among the eubacteria.**

Sulfur- and iron-oxidizing bacteria have been subjects of some interest for more than 100 years (35). Interest in their evolution stems, at least in part, from a realization of the enormous influence of chemolithotrophic metabolisms in shaping our planet. The notion that such chemolithotrophic metabolisms might be primitive traits has since gone in and out of vogue a number of times (3, 16, 27, 28). The existence of sulfur-metabolizing archaebacteria, evolutionarily so far removed from the more familiar thiobacilli, and more recent discoveries of (arguably) more primitive variations of sulfur oxidation-reduction metabolisms (15, 16) have added new dimensions to evolutionary thinking about lithotrophic metabolism. The phylogenetic diversity of the iron- and sulfur-metabolizing phenotypes clearly suggests that a closer molecular inspection of the genes encoding the enzymes of these pathways could provide some important insights into their evolution and the evolution of the organisms harboring them. A 16S rRNA phylogeny of the sulfur- and iron-oxidizing bacteria should provide a useful framework for focusing such analyses.

The 16S rRNA analyses presented here focus on the phylogenetic affiliations of eubacterial iron and sulfur oxidizers. Partial or complete 16S rRNA nucleotide sequences from 37 iron- and/or sulfur-oxidizing strains have been obtained for these analyses. Genera represented are *Thiobacillus*, *Thiomicrospira*, *Thiothrix*, *Acidophilium*, "*Leptospirillum*," *Thiovulum*, *Chromatium*, and *Chlorobium*. In addition, fully half of the sequences in the collection derive from unculturable or unclassified isolates. Thus, a fairly broad (though certainly not exhaustive) representation of genotypes is included.

Sequences were derived by the 16S rRNA reverse transcription method (18). 16S data on a large number of relevant or related nonlithotrophic bacteria also were necessarily generated as part of this study and were considered in analyzing the phylogenetic relationships of the iron and sulfur oxidizers. The data are, for the most part, treated by distance-averaging methods (24, 25, 33) and are presented as rooted or unrooted trees of the various eubacterial divisions or phyla into which the iron and sulfur oxidizers fall. 16S trees for the alpha, beta, and gamma subdivisions of the "purple bacteria and relatives" are considered in some detail, since the vast majority of the iron and sulfur oxidizers inspected fall into these groups. This division or phylum now is referred to as the *Proteobacteria* (32). The relationship of *Thiovulum* to *Campylobacter* and related genera, and of that grouping to the other subdivisions of the *Proteobacteria*, also is described in the context of certain diagnostic structural features of the 16S rRNA. Placement of the iron-oxidizing strains ALV, BC, and TH3 makes use of available gram-positive signature positions previously assigned by Woese (37). Finally, there is one small group of iron oxidizers, the "*Leptospirillum*" sp. strains Z-2, BU-1, and LfLa, which simply is not placeable with the currently available data and methods. These strains may represent a new phylogenetic division of the eubacteria.

Since the rRNA sequences define subgroups of sulfur- and iron-oxidizing bacteria in a manner which (presumably) is independent of their chemolithotrophic potential, they should serve as a useful framework for comparing the biochemistry and molecular genetics of the sulfur and iron metabolic pathways of these bacteria and for unambiguously establishing the affiliations of new isolates. Further development of rRNA-targeted "group"-specific probes also might permit some interesting ecological applications of the growing data base.

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TABLE 1. Bacterial strains

| Genus and species | Strain | Accession no. | Habitat or physiology | Source | Reference(s) |
|---|---|---|---|---|---|
| *Acidiphilium angustum* | KLB | ATCC 35903 | Coal mine drainage, Pennsylvania | P. L. Wichlacz | 36 |
| *Acidiphilium cryptum* | Lhet2 | ATCC 33463 | Acidophilic heterotroph | A. P. Harrison | 8, 10, 19 |
| *Acidiphilium facilis* | PW2 | ATCC 35904 | Coal mine drainage, Pennsylvania | P. L. Wichlacz | 36 |
| *Acidiphilium rubrum* | OP | ATCC 35905 | Coal mine drainage, Pennsylvania | P. L. Wichlacz | 36 |
| Unnamed "acidiphilium" | QBP | | Coal mine drainage, Pennsylvania | P. L. Wichlacz | 36 |
| *Leptospirillum ferrooxidans* | Z-2 | ATCC 29047 | Copper deposit, Armenia | G. A. Zavarzin | 1, 12 |
| Unnamed "leptospirillum" | BU-1 | DSM 2391 | Copper mine, Bulgaria | S. Groudev | 8 |
| Unnamed "leptospirillum" | LfLa | | Uranium mine water, Mexico | P. R. Norris | 13 |
| *Thiobacillus acidophilus* | | ATCC 27807 | Mixotroph | P. A. Pienta | 7, 10, 19 |
| *Thiobacillus ferrooxidans* | m1 | DSM 2392 | Coal strip mine refuse | A. P. Harrison | 9, 11 |
| *Thiobacillus ferrooxidans* | LM2 | | Hot spring sediment, Iceland | P. R. Norris | 22 |
| *Thiobacillus ferrooxidans* | F221 | | Uranium mine | K. Bosecker | 9 |
| *Thiobacillus ferrooxidans* | | ATCC 23270 | Type strain | ATCC[a] | 12 |
| *Thiobacillus ferrooxidans* | | ATCC 19859 | Copper mine leachate | ATCC | 9, 12 |
| *Thiobacillus ferrooxidans* | Lp | IFO 14245 | Coal mine, western Pennsylvania | P. R. Dugan | 9 |
| *Thiobacillus ferrooxidans* | PH | IFO 14262 | Coal mine, Missouri | A. P. Harrison | 9 |
| *Thiobacillus neapolitanus* | X | | Obligate chemolithoautotroph | J. Shively | 19 |
| *Thiobacillus perometabolis* | | ATCC 23370 | Chemolithoheterotroph | J. Brierley | 19 |
| *Thiobacillus tepidarius* | | DSM 3134 | 43°C Roman bath, United Kingdom | D. P. Kelly | 41 |
| *Thiobacillus thiooxidans* | | DSM 612 | Acidic sulfate soil | H. Hippe | 9 |
| *Thiobacillus thiooxidans* | | ATCC 19377 | Type strain | ATCC | 9 |
| *Thiobacillus thioparus* | | ATCC 8158 | Type species | J. Brierley | 19 |
| *Thiobacillus versutus* | A2 | ATCC 25364 | Facultative chemolithotroph | ATCC | 10, 19 |
| Unnamed "thiobacillus" | NF13 | | Marine hydrothermal vent | H. Jannasch | 31 |
| Unnamed "thiobacillus" | AG33 | | Marine hydrothermal vent | H. Jannasch | 31 |
| Unnamed "thiobacillus" | NF18 | | Marine hydrothermal vent | H. Jannasch | 31 |
| *Thiomicrospira* sp. | L12 | | Marine hydrothermal vent | H. Jannasch | 19, 30 |
| *Thiothrix nivea* | JP2 | ATCC 35100 | Neotype strain | W. R. Strohl | 20, 33 |
| *Thiovulum* sp. | | | Enrichment culture | D. A. Stahl | 33 |
| Unnamed facultative thermophile | TH3 | | Copper mine dump, New Mexico | P. R. Norris | 2 |
| Unnamed facultative thermophile | BC | | Coal mine drainage | P. R. Norris | 22 |
| Unnamed facultative thermophile | ALV | | Coal mine drainage | P. R. Norris | 22 |
| Unnamed symbiont | | | *Calyptogena magnifica* | D. Distel | 6 |
| Unnamed symbiont | | | *Bathymodiolus thermophilus* | D. Distel | 6 |
| Unnamed symbiont | | | *Lucinoma annulata* | D. Distel | 6 |
| Unnamed symbiont | | | *Codakia orbicularis* | D. Distel | 6 |
| Unnamed symbiont | | | *Riftia pachyptila* | D. Distel | 6 |

[a] ATCC, American Type Culture Collection.

## MATERIALS AND METHODS

**Bacterial strains.** Strains used in this study are listed in Table 1; growth conditions for cultivable bacteria are available in the references provided there. Others (e.g., *Thiovulum* strains and the symbionts) were collected and purified prior to lysis and nucleic acid extraction (Table 1). *Chlorobium limicola* forma thiosulfatophilum was obtained from M. Madigan as freeze-dried cell mass.

**RNA preparation.** RNA was prepared essentially as described previously (18). Cell cultures were preferentially harvested in mid- to late-log growth, collected by centrifugation, washed in a low-salt buffer, and again collected by low-speed centrifugation. For the chemolithotrophs, particularly the iron oxidizers, it generally was important to remove as much excess spent medium from the cell pellets as possible prior to cell lysis. Cell pellets either were used immediately or were stored frozen at −80°C until processing. Lysis usually was accomplished by simple sodium dodecyl sulfate (SDS) addition (in a buffer containing 0.1 M Na EDTA, 0.1 M NaCl, 0.05 M Tris-HCl [pH 7 to 7.5], and 1% [wt/vol] SDS), but passage through a French pressure cell, enzyme pretreatment (e.g., lysozyme, proteinase K), etc., also were used in some instances.

In most cases, total nucleic acid was prepared by standard phenol methods (e.g., two phenol-isoamyl alcohol [24:1, wt/vol] extractions, followed by three [or more] phenol-chloroform-isoamyl alcohol [24:24:1, wt/wt/vol] extractions until little or no interface material remained, and then two chloroform extractions to remove residual phenol). Nucleic acids were recovered and concentrated from this final aqueous phase by precipitation from ethanol and then resuspended in a small volume of 10 mM Tris-HCl (pH 7)–0.1 mM EDTA (TE buffer). The absorbance at 260 nm was measured, and the concentration of nucleic acid was adjusted (by further dilution or reconcentration) to 3 to 6 mg/ml. RNA then was selectively precipitated by adding NaCl (2.0 M final concentration) to the concentrated nucleic acid solution and holding it at 4°C overnight. This salt precipitation step is most efficient if the nucleic acid concentration is kept above 2.0 mg/ml. The precipitated RNA was collected by centrifugation, resuspended in TE buffer, and further desalted by one additional ethanol precipitation step (0.2 M sodium acetate and 2 volumes of ethanol), after which the recovered RNA was resuspended in TE buffer at approximately 2 mg/ml and stored frozen at −80°C.

**Sequence analysis.** Sequencing was by the reverse transcriptase method, using the three universal 16S rRNA primers described previously (18). Approximately 1,000 nucleotides of each rRNA sequence were determined. For some sequences, terminal deoxynucleotidyl transferase chase re-

actions were performed in parallel with the normal reverse transcriptase chase reactions (17). For these reactions, standard reverse transcription reactions were followed by a brief heating step (90°C). The reactions were quick cooled, and chase nucleotides (1 mM [each] C, A, T, and G) were added as usual except that 5 to 10 U of deoxynucleotidyl transferase (Supertechs, Inc., Bethesda, Md.) rather than reverse transcriptase was added with the chase nucleotides. The reaction mixtures were then incubated for 15 min at 37°C and the reactions were stopped as usual.

Sequences were aligned by using conserved primary and secondary structural features (17, 24). Regions of ambiguous sequence alignment were excluded from the analyses, as were positions which consistently yielded ambiguous nucleotide assignments or band compression anomalies in reverse transcription sequencing. The distance method and treeing algorithms used for phylogenetic analysis of the derived sequences were as described elsewhere (24, 25, 33).

**Nucleotide sequence accession numbers.** The 16S rRNA sequences of the iron- and sulfur-oxidizing bacteria used in the present analysis have been deposited with GenBank and assigned accession numbers M79363 to M79443 and M80290.

## RESULTS AND DISCUSSION

**Phylogeny of iron and sulfur oxidizers: an overview.** The 16S sequences of the bacteria listed in Table 1 were collected over a period of approximately 3 years. Analyses on some of these sequences (e.g., those of the symbionts and of *Thiovulum* strains) have been presented elsewhere (6, 29) but are included here for the overall perspective they provide.

For the most part, the sulfur and iron oxidizers are dealt with on a phylum-by-phylum basis in the context of their nonlithotrophic relatives. Limiting the analyses in this way generally permitted maximum utilization of the nucleotide positions available for each analysis. (The data set is limited to those alignable positions represented in all sequences in the set [17, 24]). Trees of this (phylum level) evolutionary scale generally exhibited a high level of concordance with trees based on complete 16S or 5S rRNA sequence information insofar as the data overlap. In contrast, kingdom level eubacterial trees based on partial (three-primer) reverse transcription data exhibit substantial topological instability with respect to the specific branching order of some phyla. Complete 16S gene or reverse transcription sequences clearly are preferable for this application. For this overall perspective, we defer to previously published trees which make use of available complete 16S rRNA sequences from representatives of these phyla (37). Similarly, certain very close relationships were found to be unstable with respect to precise branching orders (e.g., strains F221, ATCC 23270, etc.; see Fig. 5) and are indicated only as a cluster of highly related strains. The reader should refer to appropriate DNA-DNA homology studies (9) for fine-structure detail on the relationships among these bacteria.

Many more 16S sequences from nonlithotrophs than are shown in the presented trees are available in various published and unpublished sequence collections (23). Because of the sheer mass of the present collection, only a relatively few, phylogenetically representative 16S sequences from nonlithotrophs are shown in Fig. 2 to 7. However, many additional sequences were used to construct many more trees (of slightly or significantly different organism-sequence composition) than those presented. Such changes in the organism composition of the trees or in differences in the sequence positions included in the analyses did not result in any topologies significantly different from those presented. Thus, the topologies presented in Fig. 2 through 7 are generally representative of the 16S rRNA relationships existing among the represented organisms and are not peculiar to specific sequences or domains of sequence. Nevertheless, certain interesting topological ambiguities remain and are discussed in the context of each phylum description as they arise.

Figure 1 shows a tree of the molecularly defined eubacterial divisions. While the divisions themselves are readily defined by patterns and extents of 16S rRNA sequence variation, the exact branching orders have not yet been convincingly demonstrated in every case, even when complete 16S rRNA sequences were used (37). Certain tentative, higher-order affiliations have been pointed out by Woese (37), such as that between the cyanobacteria and the gram-positive bacteria and that of those groups with the *Proteobacteria*. These tendencies also are observable but not clearly demonstrable by using representative three-primer, partial 16S rRNA sequences. Similarly, the tentative association of the green sulfur bacteria with the bacteroides-flavobacteria group is only marginally favored by the partial 16S data. With the exception of *Chlorobium* sequences, the sequences in the present study place their respective sulfur- and/or iron-oxidizing donors in one or another of the divisions of the gram-positive bacteria-*Proteobacteria*-cyanobacteria "subdomain" of the eubacterial tree evident in Fig. 1. These relationships are depicted in a series of progressively more detailed trees of the various divisions, described as follows.

**The *Proteobacteria*.** According to previous 5S and 16S studies, four main "subdivisions" of the *Proteobacteria* are discernible. These have been termed the alpha, beta, gamma, and delta subdivisions. Iron- and/or sulfur-oxidizing chemolithotrophs are found in at least the alpha, beta, and gamma subdivisions (19). The delta subdivision contains, along with other nonchemolithotrophic relatives (e.g., *Bdellovibrio* spp., myxobacteria, etc.), the majority of sulfur- and sulfate-reducing eubacteria (e.g., *Desulfovibrio* spp., *Desulfococcus* spp., etc.). Figure 2 provides an overview of the subdivisions of the *Proteobacteria*. The topology is based on distance analysis of (nearly) complete 16S rRNA sequences of *Escherichia coli* (gamma), *Pseudomonas testosteroni* (beta), *Agrobacterium tumefaciens* (alpha), *Desulfovibrio desulfuricans* (delta), *Campylobacter jejuni*, and *Thiovulum* spp. These analyses strongly support the topology shown in Fig. 2, but they do not yield a "robust" placement of the root (which falls somewhere within the shaded area). Note that, depending upon the exact placement of the root, the *Thiovulum-Campylobacter* group can be viewed either as a deep offshoot of the delta subdivision or as an independent subdivision. In any case, the root of the *Thiovulum-Campylobacter* group falls very close to the root of the *Proteobacteria*. For consistency of usage, we shall refer to the *Thiovulum-Campylobacter* group as a subdivision of the *Proteobacteria*.

***Thiovulum*.** The genus *Thiovulum* belongs to a group of eubacteria which includes members of the genera *Campylobacter*, *Wolinella*, *Bacteroides* (21, 26, 29, 34), and *Flexispira* (reference 26a and unpublished observations). The whole grouping appears to be generally affiliated with the *Proteobacteria*, but not specifically with any of its subdivisions (21). Two natural subgroups of the *Thiovulum-Campylobacter* subdivision are evident. One contains *C. jejuni* and relatives along with a number of *Bacteroides* and *Wolinella* species, arbitrarily referred to as the *Campylobacter*

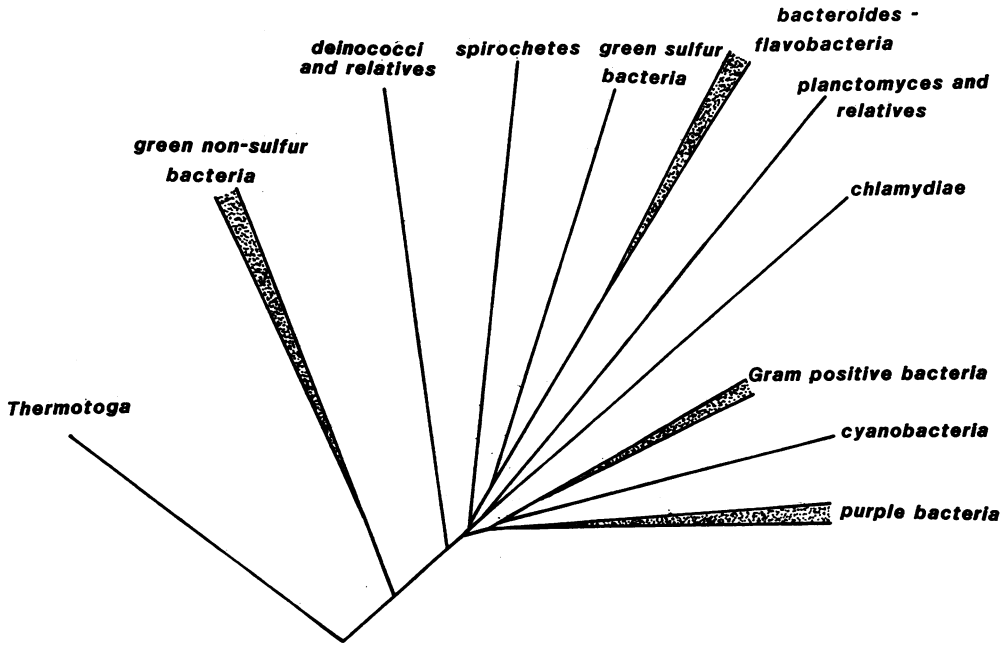


FIG. 1. The eubacterial kingdom. The major lineages (divisions or phyla) of the eubacterial kingdom derived from 16S rRNA sequences are shown. (Redrawn from Woese [37] with permission). Branch lengths are proportional to calculated evolutionary distances (4, 14).

subgroup. *Campylobacter cryaerophila* and *Campylobacter nitrofigilis* clearly are affiliated with the *Campylobacter* subgroup (discussed further below). The other (*Thiovulum*) subgroup contains *Thiovulum* spp., *Campylobacter fennelliae*, *Campylobacter cinaedi*, *Flexispira rappini*, and *Wolinella succinogenes*.

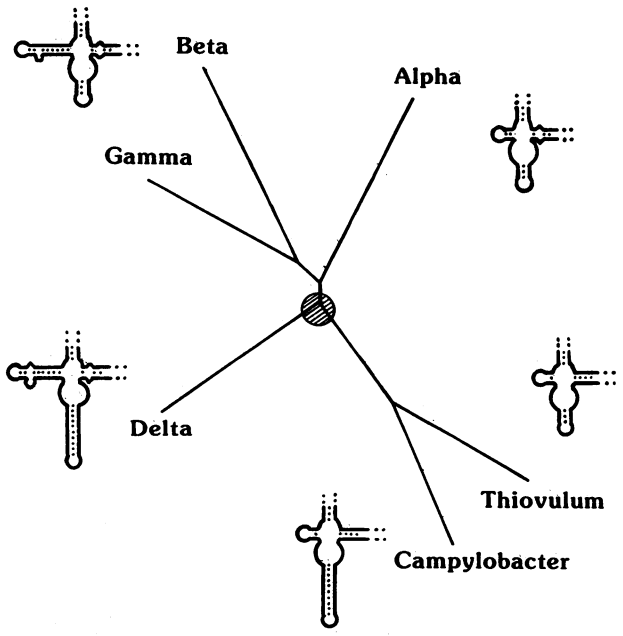


FIG. 2. The *Proteobacteria*. The 16S rRNA relationships among the *Proteobacteria A. tumefaciens* (alpha), *P. testosteroni* (beta), *E. coli* (gamma), and *D. desulfuricans* (delta); *C. jejuni* (17); and the genus *Thiovulum* (see text) are shown. Branch lengths are proportional to calculated evolutionary distances (24). The structures represent the 184-to-219 region of 16S rRNA (see text for details).

Besides the average distance type analysis shown in Fig. 3, a number of interesting 16S rRNA structural features exist which support (i) the cohesiveness of the whole subdivision, (ii) its "close but distinct" relationship with the *Proteobacteria*, and (iii) the natural bifurcation of the subdivision into two subgroups. Among these features are (i) a conserved (unknown) modification at ca. position 978 which terminates reverse transcription at this point in all members of the subdivision, (ii) a conserved deletion of the helix corresponding to positions ca. 455 to 477, and (iii) a unique compensating base pair change involving positions 921 (A) and 1396 (U) which phylogenetically strengthens the case for a proposed extension of the helix which circumscribes the 3′ domain of the 16S molecule.

Another interesting structural signature involves two stems in the 184-to-219 region (one from ca. positions 184 to 193 and the other from ca. positions 198 to 219, using the *E. coli* numbering convention). The patterns of length variation in these two stems serve to divide the *Campylobacter* subgroup along the lines discussed above and as shown Fig. 3, and they further suggest a close but separate relationship with the *Proteobacteria* as emphasized in Fig. 2. Bacteria in the alpha subdivision have short versions of both helices (37); the beta and gamma bacteria all exhibit the combination short stem (positions 184 to 193)-long stem (positions 198 to 219); and the delta bacteria all exhibit the long-long pattern in these two helices. Members of the *Campylobacter* subgroup (e.g., *C. jejuni*, *Campylobacter sputorum*, *Bacteroides gracilis*, *C. cryaerophila*, etc.) exhibit a fourth pattern, long (184 to 193)-short (198 to 219), which would argue for placing them as a distinct division or phylum of the eubacteria; however, other members of the *Thiovulum-Campylobacter* subdivision (including *Thiovulum* spp., *Campylobacter pylori*, *Flexispira* spp., *W. succinogenes*, etc.) contain

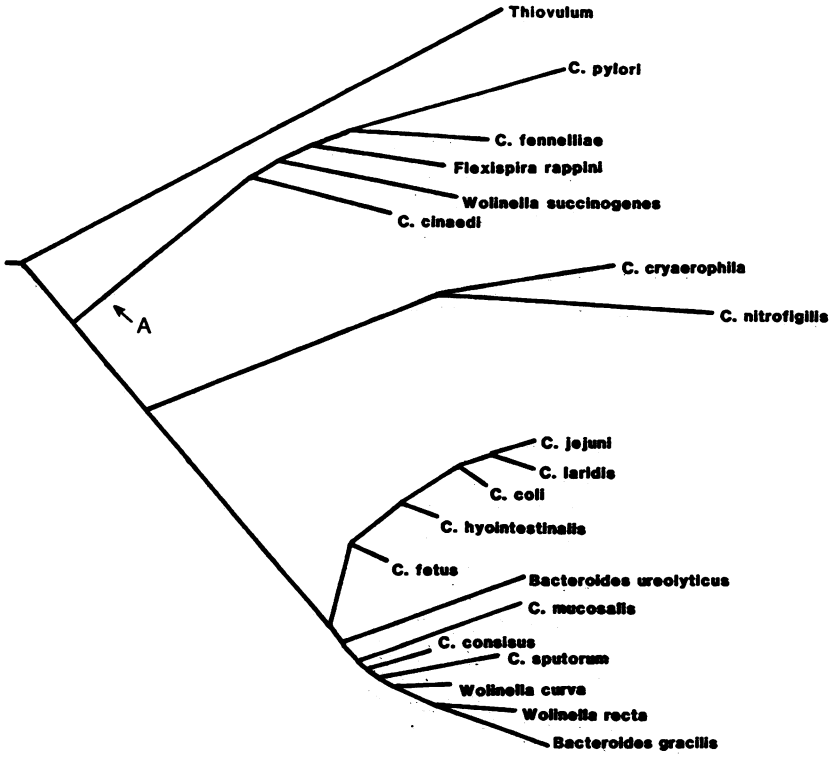


FIG. 3. The *Thiovulum-Campylobacter* subdivision. The 16S rRNA relationships among the members of the *Campylobacter-Thiovulum* subdivision are shown. Sequences on which the relationships are based are amalgams derived from Lau et al. (21), Paster and Dewhirst (26), Romaniuk et al. (29), and Thompson et al. (34); the sequence of *F. rappini* is from our unpublished observations. The horizontal components of the branch lengths are proportional to evolutionary distances (24). For significance of arrow A, see text.

the short-short pattern seen in the alpha bacteria (Fig. 2; see also reference 37 for additional discussion of these "signature" structures).

The structural analysis discussed above clearly aligns the genus *Thiovulum* with *C. pylori* and related species, all of which share the short-short helical pattern. However, the exact nature of this relationship is not entirely clear from the partial data available. Although the genus *Thiovulum* is depicted in Fig. 3 as the deepest member of the division, depending upon the organisms and exact nucleotide positions included in the analyses, a secondary placement of the root at the position marked A occasionally is obtained. In any case, the genus *Thiovulum* is quite distinct from all other sulfur oxidizers that have been characterized by 5S or 16S rRNA analysis, as well as by morphology and behavior.

**The alpha *Proteobacteria*.** The detailed topology of the alpha group or subdivision of the *Proteobacteria* has been reviewed recently by Woese et al. (37, 39). Three distinct subgroups have been defined by catalog similarity and signature analyses of 16S rRNA sequences and have been given the temporary designations alpha-1, alpha-2, and alpha-3. The tree shown in Fig. 4 is based on reverse transcription data from the included sulfur oxidizers, and the corresponding portions of complete 16S sequences either are available in the literature (e.g., *A. tumefaciens*) or are unpublished (37a). Two quite distinct clusters of sulfur oxidizers are apparent.

The "heterotrophically inclined" *Thiobacillus acidophilus* (ATCC 27807), *Acidiphilium cryptum* (ATCC 33463), *Acidiphilium facilis* (ATCC 35904), *Acidiphilium rubrum* (ATCC 35905), *Acidiphilium angustum* (ATCC 35903), and the unnamed *Acidiphilium*-like strain QBP cluster specifically with *Rhodopila* (formerly *Rhodopseudomonas*) *globiformis* (ATCC 7950) and therefore belong to the so-called alpha-1 subgroup. As is evident in Fig. 4, *A. angustum*, *A. rubrum*, and strain QBP form a tight cluster and are highly

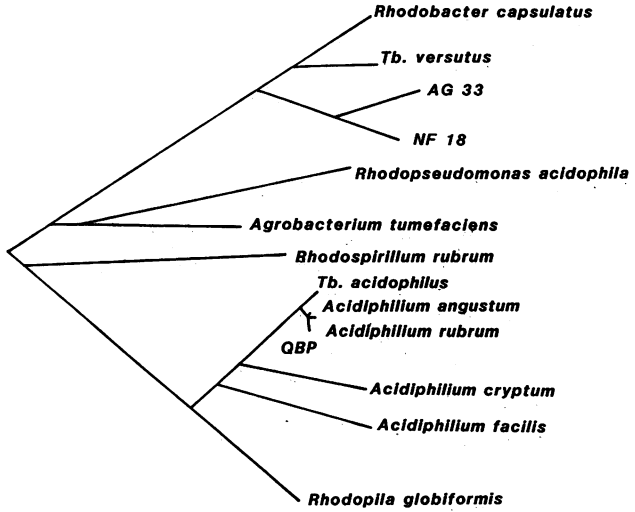


FIG. 4. The alpha subdivision. The 16S rRNA relationships among the members of the alpha subdivision of the *Proteobacteria* are shown. The horizontal components of the branch lengths are proportional to evolutionary distances (24).

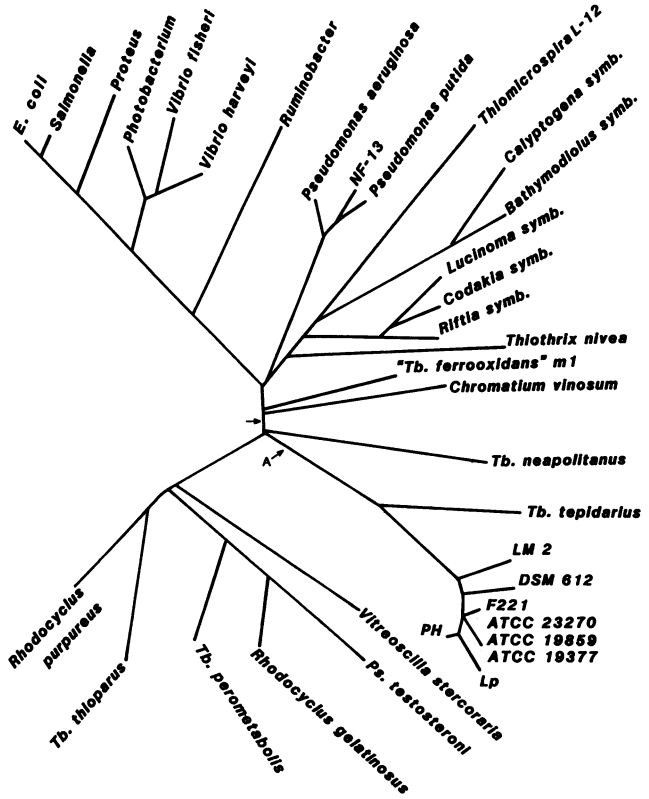


FIG. 5. The beta and gamma subdivisions. The 16S rRNA relationships among the members of the beta and gamma subdivisions of the *Proteobacteria* are shown. The branch lengths are proportional to evolutionary distances (24). Arrows are explained in the text.

related to one another at the 16S rRNA level. This cluster, in turn, is more closely related to *T. acidophilus* than it is to the other represented *Acidiphilium* species, *A. cryptum* and *A. facilis*. As determined by 5S rRNA analysis, this grouping likely also contains the facultative chemolithotroph *Thiobacillus novellus* (19), albeit as a deeper branching. Thus, the genus level discrimination between these *Acidiphilium* and *Thiobacillus* isolates, as based solely on relative "preferences" of the latter for lithotrophic rather than heterotrophic energy metabolism, are equivocal.

*Thiobacillus versutus* (ATCC 25364, formerly *Thiobacillus* strain A2), along with the sulfur-oxidizing hydrothermal vent isolates AG33 and NF18 (31), groups with *Rhodobacter capsulatus* (formerly *Rhodopseudomonas capsulatum*) and therefore is placed in the alpha-3 subgroup (39).

**The beta and gamma *Proteobacteria*.** The network shown in Fig. 5 is based on reverse transcription data from the included iron and sulfur oxidizers and the corresponding portions of other 16S sequences that either are available in the literature (*E. coli*, *Proteus vulgaris*, *Ruminobacter* [formerly *Bacteroides*] *amylophilus*, *P. testosteroni*) or are unpublished (*Chromatium vinosum*, *Rhodocyclus purpureus*, *Rhodocyclus gelatinosus* [37a]).

The position of the root separating the two subdivisions corresponds, at least roughly, to the point marked with an arrow in Fig. 5. However, the dense clustering of sulfur and iron oxidizers very near the probable position of the root makes its exact location difficult to ascertain. One interesting possibility, with which the present data also are consistent, is that the root may actually lie along the branch leading to *Thiobacillus tepidarius* (marked A for alternate), although such a placement was favored in only a small minority of the trees inspected. By analogy with analyses of complete 5S and 16S rRNA sequences and 16S cataloging, the root placement shown is at least reasonably accurate. Complete 16S or 23S rRNA sequences on a few of the root-proximal iron or sulfur oxidizers should shed further light on this.

The gamma subdivision includes most of the commonly encountered gram-negative bacteria, including the enteric bacteria, vibrios, fluorescent pseudomonads, photosynthetic purple sulfur bacteria (e.g., *Chromatium* spp.), *Legionella* spp., and others (37, 40, 41). Sulfur- and iron-oxidizing chemolithotrophs are found in a number of these groups.

NF13, a sulfur-oxidizing hydrothermal vent isolate (31), is part of the fluorescent pseudomonad subgroup of the gamma subdivision, represented in Fig. 5 by *Pseudomonas aeruginosa* and *Pseudomonas putida*. *Thiomicrospira* strain L-12, a free-living hydrothermal vent isolate (30); the bacterial endosymbionts of the hydrothermal vent-associated invertebrates *Calyptogena magnifica*, *Bathymodiolus thermophilus*, and *Riftia pachyptila*; the endosymbionts of the marine marsh mollusks *Codakia orbicularis* and *Lucinoma annulata* (and *Lucinoma aequizonata*); and *Thiothrix nivea* JP2 form a loose cluster branching near the base of the fluorescent pseudomonad cluster. Because of the short segment lengths involved, this grouping may in fact represent two or three closely related clusters, one being *Thiomicrospira* strain L-12 and the symbionts, the second being the fluorescent pseudomonads, and the third being represented by the genus *Thiothrix*. All are "stable" and are consistently placed in the gamma subdivision, however. The *Thiothrix* 16S rRNA sequence has one unique structural feature of note: the stem corresponding to positions 455 to 477 (using the *E. coli* position numbering) is absent. This is not uncommon among the eubacteria as a whole, but it is the only example of its occurrence within the gamma subdivision of which we are aware (except for the genera *Thiovulum* and *Campylobacter*, etc., as discussed above).

Assuming the correct placement of the root in Fig. 5, the gamma subdivision also includes the iron oxidizer "*Thiobacillus ferrooxidans*" m1 (9) and *Chromatium vinosum*. By analysis of 5S rRNA sequences and 16S catalogs, a number of (but not all) *Beggiatoa* and *Vitreoscilla* species (33) also tree near the root of the gamma subdivision.

The detailed topology of the beta subdivision is difficult to infer with any great confidence. Its members are still fairly poorly represented in both the 5S rRNA and 16S rRNA sequence collections. The 16S rRNA catalogs cover the group most comprehensively, and they define two main subdivisions and a number of less-well-resolved, deep branchings, e.g., *Neisseria* species and *Spirillum volutans* (5, 41). The so-called beta-1 subgroup is represented in Fig. 5 by *P. testosteroni* and *R. gelatinosus*. It also contains, as a deep branching, *Thiobacillus perometabolis* (ATCC 23370). This placement is consistent with earlier 5S sequence analysis (19) and 16S catalog placement of its very close relative, *Thiobacillus intermedius* (41).

The beta-2 subgroup is represented by *R. purpureus*. It also contains, as a deep branching, *Thiobacillus thioparus* (ATCC 8159). This placement is similar to that inferred for *Thiobacillus denitrificans* by 16S rRNA cataloging (41). The present placements of both *T. thioparus* and *T. perometabolis* are somewhat in disagreement with their previous placement by 5S rRNA analysis, which placed them both as deeper branchings of the beta subdivision. The present placements are quite stable with respect to organism com-

position and the utilization of different portions and extents of sequence in the analysis. However, a more detailed analysis of complete 16S sequences for the beta bacteria clearly is warranted. *Vitreoscilla stercoraria* (strain VT1) is placed just outside the beta-1–beta-2 bifurcation, similar to its placement by 5S analysis and consistent with the 16S rRNA-based placement of its close relative, *Neisseria gonorrhoeae* (5, 16a).

Branching very near the beta-gamma root is a large group of iron and sulfur oxidizers. It contains (proceeding from the root shown in Fig. 5) the following: *T. tepidarius* DSM 3134, a moderately thermophilic isolate from a Roman bath (42); LM 2, an elemental sulfur-preferring, facultative thermophile isolated from a hot spring sediment from Lake Myvam, Iceland (22); *Thiobacillus thiooxidans* DSM 612, isolated from acidic sulfate soil in The Netherlands by H. Hippe; and a mixed group of iron and sulfur oxidizers whose 16S rRNA sequences all are sufficiently similar that their distinct branching order cannot be ascertained precisely, including *T. ferrooxidans* F221 (isolated from a uranium mine in Forstav, Austria, by Klaus Bosecker), *T. ferrooxidans* ATCC 23270 (type strain), *T. ferrooxidans* ATCC 19859 (from acid copper-leaching water), and *T. thiooxidans* ATCC 19377 (from a Libyan sulfur-producing lake).

By previous DNA-DNA hybridization measurements (9, 12), *T. ferrooxidans* F221, ATCC 23270, and ATCC 19859 all are related (group 3a) at the 85% homology level (F221-ATCC 19859). *T. thiooxidans* ATCC 19337 and *T. ferrooxidans* ATCC 19859 are related only at the 9 to 12% DNA-DNA homology level (9). Also included in this grouping are the iron-oxidizing strains (*T. ferrooxidans*) Lp and PH. Lp (IFO 14245), isolated from a coal mine in western Pennsylvania (9), and PH, isolated from a coal strip mine in Missouri (9), also are distinguishable from one another and from *T. ferrooxidans* ATCC 19859 by DNA-DNA hybridization, all being around the 20 to 30% homology level. By previous 5S rRNA analysis (33), the beta subdivision also contains a number of other *Vitreoscilla* strains (e.g., *Vitreoscilla filiformis* ATCC 15551 and L1401-7) and *Leptothrix discophora* (Stokes).

**"*Leptospirillum ferrooxidans*" Z-2, BU-1, and LfLa.** Isolates Z2, BU-1, and LfLa are closely related to one another (ca. 94% similar) but are not specifically related (i.e., above the ca. 80% similarity level) to any other bacterium whose 16S rRNA sequence presently is available. Some 350 partial or complete 16S rRNA sequences, representing all the eubacterial phylogenetic divisions or phyla so far described, were available for these comparisons. These leptospirilla are novel not only among the sulfur and iron oxidizers but also among the eubacteria. As such, they definitely warrant closer inspection. Z-2 was isolated from a copper deposit in Armenia, USSR (1, 12); BU-1 was isolated from the Gramatikovo copper mine in southeastern Bulgaria (9); and LfLa was isolated from a uranium mine at Los Amoles, Sonora, Mexico (13). Figure 6 simply depicts the relationships among Z-2, BU-1, and LfLa. The long segment emphasizes the close relationship among the three, relative to their common divergence from the eubacteria.

Because of the novelty of these strains, a nearly complete 16S rRNA sequence was obtained from one member of the group, BU-1, by using additional primers developed subsequent to the original sequencing efforts (17). As with the original comparisons, no convincing specific affiliation with any of the defined eubacterial divisions could be established (this work and reference 37a). Interestingly, the BU-1 sequence exhibits the long stem (positions 184 to 193)-long stem (positions 198 to 219) pattern characteristic of the delta subdivision of the *Proteobacteria*.

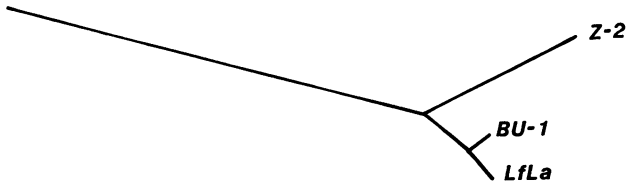


FIG. 6. The iron-oxidizing leptospirilla. The 16S rRNA relationships among "*Leptospirillum ferrooxidans*" Z-2, BU-1, and LfLa are shown. The horizontal components of the branch lengths are proportional to evolutionary distances (24).

**TH3, BC, and ALV.** Strains TH3, BC, and ALV all are facultatively thermophilic iron oxidizers. All three are characterized as gram negative (or gram indeterminate), yet they clearly group, by 16S rRNA analysis, with the gram-positive eubacterial division. TH3 was isolated from the Chino mine dump of the Kennecott Copper Corp., Hurley, N.Mex. (2). ALV was isolated from a coal spoil heap near Alvecote, Warwickshire, United Kingdom (22). BC was isolated from a drainage channel of a washed coal pile from the Birch Coppice Collier, Warwickshire, United Kingdom (22). The specific relationship of ALV, BC, and TH3 to the gram-positive bacteria is based on larger eubacterial trees in which all of the known divisions or phyla are represented. They always branch similarly to the positions shown in Fig. 7, that is, very near the root of the gram-positive division.

On the basis of an analysis of 16S rRNA catalogs, Woese and collaborators (37) have defined two major subdivisions of the gram-positive division. One includes bacteria whose DNAs all contain more than 55% G+C and which are characteristically actinomycetelike in phenotype (represented in Fig. 7 by *Streptomyces lividans*, *Arthrobacter globiformis*, and *Mycobacterium bovis*). The second major division includes bacteria whose DNAs contain less than 50% G+C, e.g., the genera *Bacillus*, *Clostridium*, etc. Two more gram-positive groups, as yet only sparsely represented in the 16S rRNA sequence collections (or catalogs), have recently been identified. One of these contains the phototrophs *Heliobacte-*

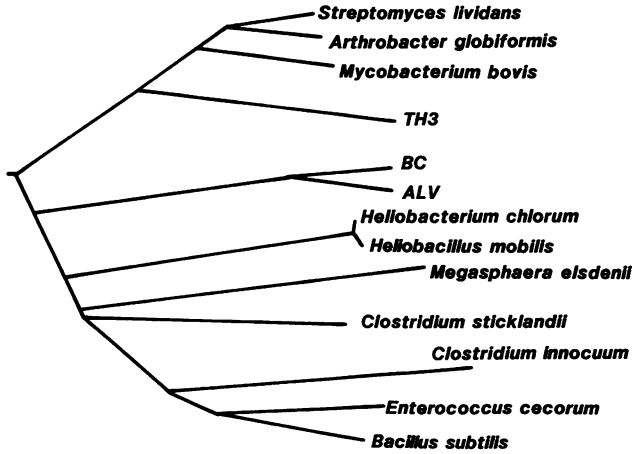


FIG. 7. The gram-positive division. The 16S rRNA relationships among the major gram-positive eubacterial lineages are shown. The horizontal components of the branch lengths are proportional to evolutionary distances (24).

TABLE 2. Groupings of iron- and sulfur-oxidizing eubacteria

Class (division) *Proteobacteria*
- Subdivision (alpha)
  - Subgroup (alpha-1)
    - *Rhodospirillum rubrum*
    - *Rhodopila globiformis*
    - *Acidiphilium cryptum* Lhet2 (ATCC 33463)
    - *Acidiphilium facilis* PW2 (ATCC 35904)
    - *Thiobacillus acidophilus* (ATCC 27807)
      - *Acidiphilium angustum* KLB (ATCC 35903)
      - *Acidiphilium rubrum* OP (ATCC 35905)
      - Unnamed "acidiphilium" QBP
  - Subgroup (alpha-2)
    - *Rhodopseudomonas acidophila*
    - *Agrobacterium tumefaciens*
  - Subgroup (alpha-3)
    - *Rhodobacter capsulatus*
    - *Thiobacillus versutus* A2 (ATCC 25364)
    - Unnamed "thiobacillus" AG33
      - Unnamed "thiobacillus" NF18
- Subdivision (beta)
  - Subgroup (beta-1)
    - *Rhodocyclus gelatinosus*
    - *Pseudomonas testosteroni*
    - *Thiobacillus perometabolis* ATCC 23370
    - *Thiobacillus intermedius* (5S rRNA)
  - Subgroup (beta-2)
    - *Rhodocyclus purpureus*
    - *Thiobacillus thioparus* ATCC 8158
    - *Thiobacillus denitrificans* (16S catalog)
  - Subgroup (beta-?)
    - *Vitreoscilla stercoraria*
  - Subgroup (beta-?)
    - *Thiobacillus neapolitanus* X
    - *Thiobacillus tepidarius* DSM 3134
    - *Thiobacillus ferrooxidans* LM 2
    - *Thiobacillus thiooxidans* DSM 612
    - *Thiobacillus thiooxidans* ATCC 19377
      - *Thiobacillus ferrooxidans* ATCC 23270
      - *Thiobacillus ferrooxidans* ATCC 19859
      - *Thiobacillus ferrooxidans* F221
    - *Thiobacillus ferrooxidans* Lp (IFO 14245)
    - *Thiobacillus ferrooxidans* PH (IFO 14262)
- Subdivision (gamma)
  - Subgroup (gamma-1)
    - *Chromatium vinosum*
  - Subgroup (gamma-3)
    - *Thiobacillus ferrooxidans* m1 (DSM 2392)
    - *Beggiatoa leptomitiformis* (16S catalog)
    - *Leucothrix mucor* (16S catalog)
    - *Pseudomonas aeruginosa*
      - *Pseudomonas putida*
      - Unnamed "thiobacillus" NF13
    - *Thiothrix nivea* JP2 (ATCC 35100)
      - *Thiomicrospira* sp. L12
      - Unnamed symbiont of *Bathymodiolus thermophilus*
        - Unnamed symbiont of *Calyptogena magnifica*
      - Unnamed symbiont of *Riftia pachyptila*
        - Unnamed symbiont of *Codakia orbicularis*
        - Unnamed symbiont of *Lucinoma annulata*
    - *Ruminobacter amylophilus*
    - *Photobacterium* and *Vibrio* species
    - *Escherichia*, *Salmonella*, and *Proteus* species
- Subdivision (*Thiovulum-Campylobacter*)
  - Subgroup (*Campylobacter*)
    - *Campylobacter jejuni*
      - *Campylobacter laridis*
      - *Campylobacter coli*
      - *Campylobacter hyointestinalis*
      - *Campylobacter fetus*
    - *Bacteroides ureolyticus*
    - *Campylobacter mucosalis*
    - *Campylobacter consisus*
    - *Campylobacter sputorum*
    - *Wolinella curva*
    - *Wolinella recta*
    - *Bacteroides gracilis*
    - *Campylobacter cryaerophila*
    - *Campylobacter nitrofigilis*
  - Subgroup (*Thiovulum*)
    - *Thiovulum* sp.
    - *Campylobacter pylori*
      - *Campylobacter fennelliae*
      - *Flexispira rappini*
      - *Wolinella succinogenes*
      - *Campylobacter cinaedi*

Class (division) gram-positive bacteria
- Subdivision (high G+C)
  - *Streptomyces lividans*
  - *Arthrobacter globiformis*
  - *Mycobacterium bovis*
  - Unnamed facultative thermophile TH3
- Subdivision (low G+C)
  - Unnamed facultative thermophile BC
    - Unnamed facultative thermophile ALV
  - *Heliobacterium chlorum*
    - *Heliobacillus mobilis*
  - *Megasphaera elsdenii*
  - *Clostridium sticklandii*
  - *Clostridium innocuum*
  - *Bacillus subtilis*
    - *Enterococcus cecorum*

Class (division) "*Leptospirillum*"
- "*Leptospirillum ferrooxidans*" Z-2 (ATCC 29047)
- Unnamed "leptospirillum" BU-1 (DSM 2391)
- Unnamed "leptospirillum" LfLa

*Continued*

TABLE 2—*Continued*

*rium chlorum* and *Heliobacillus mobilis*. The other contains members of the genera *Megasphaera*, *Selenomonas*, and *Sporomusa*. TH3 appears to be firmly affiliated by overall sequence homology with the high-G+C subdivision as a deep branching. It contains gram-positive signature nucleotides at positions 1207 (C) and 513 (A) and a strong "high-G+C signature" at position 1167 (U).

In the tree shown in Fig. 7, BC and ALV are placed too close to the root of the gram-positive division to convincingly predict (or preclude) a specific affiliation with any particular group of the low-G+C subdivision. Using a nearly complete 16S rRNA sequence for ALV yields a placement identical to that shown in Fig. 7, which is based on partial sequences derived from three primers. Signature sequences at positions 513 (A) and 1198 (A) support their assignment as gram-positive bacteria. Positions 168 (G), 906 (G), 955 (U), 1167 (A), and 1401 (A) support a specific affiliation with the low-G+C branch of the gram-positive bacteria. Positions 1202 (G) and 1207 (G) support their placement as a unique branch, not specifically affiliated with any other group of gram-positive bacteria.

**Summary.** A great deal about the phylogeny of the iron- and sulfur-oxidizing chemolithothrophs has been learned during the course of these and previous rRNA analyses. A picture emerging is of very widespread dissemination of these metabolisms, among both the archaebacteria and eubacteria. However, the nature of the evolutionary relationships among the various phenotypic homologs of the iron and sulfur oxidation-reduction pathways is still unclear. The traditional physiological groupings based on iron, sulfur, and

heterotrophic metabolisms create some interesting taxonomic inconsistencies; the groupings overlap for many bacteria. The phylogenetic groupings presented here are internally consistent, at least, and may serve as a useful framework for synthesizing a natural and useful taxonomic system for the iron- and sulfur-oxidizing bacteria.

The bacteria and groupings displayed in Fig. 2 to 7 are assembled in Table 2 for simplified reference. Conventions regarding the naming of phylogenetically defined groupings are still in a state of considerable flux. For example, it recently has been proposed (38) that a new taxon above the level of kingdom, called domain, be established. The Archaea, Bacteria, and Eucarya domains are proposed to include the current members of the archaebacterial, eubacterial, and eukaryotic kingdoms (39), respectively. However, no kingdom level structure for the Bacteria has been proposed, so the domain Bacteria and kingdom eubacteria do not yet have any distinguishing features. Similarly, the proposal (32) to rename the "purple bacteria and relatives" the *Proteobacteria* was accompanied by a proposal to establish the taxon "class" for that group but to retain the vernacular terms alpha, beta, gamma, etc., as group names for the "subclasses." In order to acknowledge these proposals and yet avoid confusion, we use here the following terms according to their current usage (32, 37, 38): domain, kingdom, class (= division = phylum), subdivision (= group), and subgroup. The term "subdomain" is used to name the grouping of eubacteria that include the gram-positive, *Proteobacteria*, and cyanobacterial divisions to avoid confusion with the current definition of kingdom.

On the basis of numbers alone, the genus *Thiobacillus* belongs to the beta subdivision of the *Proteobacteria*. The few exceptions include the "heterotrophically inclined" *Thiobacillus acidophilus* and *T. versutus*, which belong to the alpha subdivision, and "*T. ferrooxidans*" m1, which belongs to the gamma subdivision as a deep branch. *T. acidophilus* probably is best considered as a species of the genus *Acidiphilium*. *T. versutus*, along with the hydrothermal vent isolates AG33 and NF18, probably deserves consideration as a new genus. No clear demarcation between sulfur- and iron-oxidizing thiobacilli is found in their 16S rRNAs.

Among the gamma subdivision, the placements of *T. ferrooxidans* m1 and *T. nivea* should be viewed as provisional. They are single representatives of unusual 16S rRNAs. NF18 is strongly and specifically affiliated with the fluorescent pseudomonads. The sulfur-oxidizing endosymbionts definitely cluster together, but their relationship to *Thiomicrospira* strain L-12, while stable and specific, is not close. The 86 to 90% 16S rRNA sequence similarity between *Thiomicrospira* strain L-12 and the symbionts would not, for example, be expected to correspond to any measurable DNA-DNA homology.

The relationship of the genus *Thiovulum* to the campylobacters is interesting but of unknown significance. It clearly would be useful to inspect 16S rRNAs from other species of *Thiovulum*. The data appear to strongly suggest that the entire *Thiovulum-Campylobacter* group is a subdivision of the *Proteobacteria*. The affiliation of the iron oxidizers TH3, BC, and ALV with the gram-positive division is novel and unambiguous. The iron-oxidizing leptospirilla BU-1, LfLa, and Z-2 may represent a new division of the eubacteria, perhaps affiliated with the gram-positive bacteria-*Proteobacteria*-cyanobacteria subdomain. They certainly are not affiliated with the spirochete division of the eubacteria.

A great deal about the reverse transcriptase methodology and its application to bacterial systematics also has been learned. The use of only three "universal" primers, for instance, probably is not to be recommended any longer. Virtually complete sequences now can be obtained with very little additional effort by the use of additional primers complementary to five additional highly conserved priming sites (17). The additional data permit more precise higher-level (i.e., phylum and kingdom level) branchings to be inferred. Likewise, the added refinement of terminal deoxynucleotidyl transferase chase reactions reduces significantly the number of ambiguous positions resulting from some bacterial rRNA templates. Empirically, these ambiguities most often arise in the less conserved regions of the molecule; therefore, the information gained is useful for sharpening the resolution of close relationships and for designing nucleic acid hybridization probes of high specificity. Both additions permit a more useful integration of distance (or parsimony) type analyses and the signature analysis described by Woese (37).

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