Evolutionary Relationships among Ammonia- and Nitrite-Oxidizing Bacteria

A. TESKE,[1]† E. ALM,[1] J. M. REGAN,[2]‡ S. TOZE,[1]§ B. E. RITTMANN,2‖ AND D. A. STAHL[1,2,3]*

Department of Veterinary Pathobiology,[1] Department of Civil Engineering,[2] and Department of Microbiology,[3] University of Illinois, Urbana, Illinois 61801

Comparative 16S rRNA sequencing was used to evaluate phylogenetic relationships among selected strains of ammonia- and nitrite-oxidizing bacteria. All characterized strains were shown to be affiliated with the proteobacteria. The study extended recent 16S rRNA-based studies of phylogenetic diversity among nitrifiers by the comparison of eight strains of the genus *Nitrobacter* and representatives of the genera *Nitrospira* and *Nitrospina*. The later genera were shown to be affiliated with the delta subdivision of the proteobacteria but did not share a specific relationship to each other or to other members of the delta subdivision. All characterized *Nitrobacter* strains constituted a closely related assemblage within the alpha subdivision of the proteobacteria. As previously observed, all ammonia-oxidizing genera except *Nitrosococcus oceanus* constitute a monophyletic assemblage within the beta subdivision of the proteobacteria. Errors in the 16S rRNA sequences for two strains previously deposited in the databases by other investigators (*Nitrosolobus multiformis* C-71 and *Nitrospira briensis* C-128) were corrected. Consideration of physiology and phylogenetic distribution suggested that nitrite-oxidizing bacteria of the alpha and gamma subdivisions are derived from immediate photosynthetic ancestry. Each nitrifier retains the general structural features of the specific ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, apparently are not derived from an ancestral nitrifying phenotype.

Biologists have been asking questions concerning the evolutionary origins of, and phylogenetic relationships among, chemolithotrophic microorganisms for well over a century. However, it has been only in the last decade that comparative molecular studies have provided the basis to shape an understanding of their phylogeny. Most notably, the use of comparative rRNA sequencing has provided an all-encompassing phylogenetic framework within which all the chemolithotrophs can be placed. The emerging phylogeny has, in turn, provided insights into their antiquity and the origins of lithotrophic metabolism. For example, sulfur oxidation and iron oxidation appear to be evolutionarily early and widespread metabolic modes that are not confined to a single phylogenetic assemblage of bacteria (27). An important group of chemolithotrophs commonly called nitrifying bacteria or nitrifiers, the nitrite- and ammonia-oxidizing bacteria, has to be reconsidered as well.

These classical chemolithotrophs are still viewed as one coherent group, the family *Nitrobacteriaceae* (45, 46), defined by their characteristic ability to grow as lithotrophs by oxidation of ammonia to nitrite or nitrite to nitrate. No organism that has been described is capable of fully oxidizing ammonia to nitrate. Consequently, the classification of nitrifying bacteria is based primarily upon oxidation of either ammonia or nitrite,

with secondary consideration of cell shape and the highly characteristic cytoplasmic membrane structures (46). All known ammonia-oxidizing bacteria are obligate chemolithoautotrophs. In contrast, some nitrite-oxidizing bacteria are mixotrophs and also can grow heterotrophically (4, 26). Although the existing taxonomy assigns these bacteria to a single family, accumulating biochemical and molecular data do not support their phylogenetic coherence.

Physiological and enzymatic data argue against a close relatedness between ammonia and nitrite oxidizers. They employ two very different key enzyme systems for the energy-gaining oxidation of ammonia and nitrite (5). Comparative sequencing studies based on 16S rRNA oligonucleotide cataloging provided the first outline of phylogenetic diversity of nitrifying bacteria (56, 56a, 57). More recently, the phylogeny of the ammonia-oxidizing bacteria has been refined on the basis of near-complete 16S rRNA sequences (17). These results are consistent with the earlier cataloging studies. However, this picture is far from complete. Many new species were not included in sequencing studies. Others have been characterized by DNA-DNA hybridization and GC content but not by sequence comparisons.

In this study, we used comparative 16S rRNA sequencing to further delineate and refine the phylogeny of ammonia- and nitrite-oxidizing bacteria. In so doing, we identify closely related nonnitrifying species and discuss the physiological and evolutionary significances of their recent common origins.

MATERIALS AND METHODS

Bacterial strains and 16S rRNA sequences. Strains sequenced in this study are listed in Table 1. Nitrifier species and strains were obtained from the American Type Culture Collection and from J. B. Waterbury, Woods Hole Marine Biology Laboratory, Woods Hole, Mass., and J. Prosser, University of Aberdeen, Aberdeen, Scotland. In addition to those sequences

* Corresponding author. Present address: Department of Civil Engineering, Northwestern University, 2145 Sheridan Rd., Evanston, IL 60208-3109. Phone: (708) 491-4997. Fax: (708) 491-4011. Electronic mail address: d-stahl@nwu.edu.

† Present address: Max Planck Institute for Marine Microbiology, D-28359, Bremen, Germany.

‡ Present address: Warzyn, Inc., Madison, WI 53705.

§ Present address: CSIRO Division of Water Resources, Perth, Western Australia, Australia 6014.

‖ Present address: Department of Civil Engineering, Northwestern University, Evanston, IL 60208-3109.

TABLE 1. Sources of bacterial strains used in this study

Species	Strain
Nitrobacter winogradskyi	ATCC 25381 type strain (46)
	ATCC 14123 (formerly designated *Nitrobacter agilis*, reassigned to the species *Nitrobacter winogradskyi* [20, 49])
Nitrobacter hamburgensis	X14 type strain (7) (from S. W. Watson and F. Valois)
Nitrobacter species	R6 (Rothamsted, United Kingdom, from J. Prosser)
Nitrobacter species[a]	ATCC 25383
Nitrobacter species[a]	ATCC 25384
Nitrobacter species[a]	ATCC 25385
Nitrococcus mobilis	ATCC 25380 type strain (46)
Nitrosolobus multiformis	ATCC 25196 type strain (46)
	ATCC 25198[a]
Nitrosomonas europae	ATCC 19718
Nitrosospira briensis	C-128 (from S. W. Watson and F. Valois)
Nitrospina gracilis	Nb-3, Pacific Ocean isolate (from S. W. Watson and F. Valois)
	Nb-211, Atlantic Ocean isolate (from S. W. Watson and F. Valois)
Nitrospira marina	Nb-295 (from S. W. Watson and F. Valois)

[a] Sequence determined by reverse transcriptase sequencing.

determined in this study, previously determined 16S rRNA sequences used for comparison were obtained from the EMBL and RDP databases (29). Unpublished 16S rRNA sequences or sequences previously determined for nitrifying species (and corresponding accession numbers) are those recently reported by Head et al. (17) (*Nitrosospira briensis* strain C-128 [M96396], *Nitrosovibrio tenuis* strain C-141 [M96397], *Nitrosococcus oceanus* strain C-27 [M96398], *Nitrosomonas europaea* strain C-31 [M96399], *Nitrosomonas marina* strain C-56 [M96400], *Nitrosolobus multiformis* strain C-71 [M96401], *Nitrosomonas eutropha* strain C-91 [M96402], *Nitrosococcus mobilis* strain Nc2 type strain [M96403], and *Nitrosovibrio tenuis* strain Nv12 [M96405]) and those available from the RDP (29) (*Nitrosomonas europaea* and *Nitrosolobus multiformis*).

Isolation of nucleic acids and sequencing. Nucleic acids were isolated by mechanical disruption of cells and phenol extraction as previously described (40). The 16S rRNA was isolated and sequenced directly, using the reverse transcriptase method of Lane et al. (28). DNA sequencing was done by using PCR and direct sequencing of PCR products. For direct sequencing, 16S rRNA genes were amplified by using two primers, 11F (5'-GTTTGATCCTGGCTCAG-3', corresponding to *Escherichia coli* positions 11 to 27) and 1512-AR (5'-ACGGT/CTACCTTGTTACGACTT-3', corresponding to *E. coli* positions 1492 to 1513). Each of the 35 cycles started with 1 min of denaturation at 95°C, continued with 2 min of annealing at 40°C, and ended with 3 min of elongation at 71°C. The reaction mix (total volume, 50 or 100 μl) contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl$_2$, 0.01% (wt/vol) gelatin, 200 μM (each) dGTP, dATP, dTTP, and dCTP, 0.5 to 1.0 U of *Taq* DNA polymerase (United States Biochemical Co., Cleveland, Ohio), and 1 μM (each) primer.

The PCR products were analyzed by electrophoresis on 1% horizontal agarose gels in TAE buffer (36), using a 1-kb DNA ladder as a size marker (Difco, Detroit, Mich.; Gibco BRL, Grand Island, N.Y.). The DNA from the region of the gel containing the PCR amplification product of appropriate size was electrophoretically recovered by cutting a small well in the gel directly in front of the selected PCR product. Electro-

phoresis was continued until the DNA migrated into the buffer-filled well. The buffer was quickly transferred to a microcentrifuge tube, and 0.1 volume of 5 M NaCl and 2.5 volumes of ethanol were added. Following incubation for 1 h at −80°C, the DNA was pelleted by centrifugation and directly used in the sequencing reactions (9), using primers complementary to highly conserved regions of 16S rRNA sequence. The previously published protocol of Böttger (9) was modified to include addition of 1 μl of aqueous 5% (wt/vol) Nonidet P-40 (Sigma Co., St. Louis, Mo.) to the template-primer mix (0.5% in a final reaction volume of 10 μl). In addition, denaturation at 94°C was extended to 10 min and the 37°C renaturation step was omitted.

The sequencing primers and corresponding positions within the *E. coli* 16S rRNA were 11F (5'-GTTTGATCCTGGCTCAG-3' [11 to 27]), 61F (5'-GCTTAACACATGCAAG-3' [46 to 61]), 343aR (5'CTGCTGCCTCCCGTA-3' [357 to 341]), 519R (5'-GWATTACCGCGGCKGCTG-3' [536 to 519]), 530F (5'-GTGCCAGC[A/C]GCCGCGG-3' [515 to 530]), 690R (5'-TCTACGCATTTCACC-3' [704 to 690]), 786R (5'-C TACT[C/G]GGGTATCTAATC-3' [803 to 786]), 802F (5'-AT TAGATACCCTGGTA-3' [787 to 802]), 922F (5'-GAAACT TAAA[G/T]GAATTG-3' [906 to 922]), 956R (5'-GGCGTT GTGTC[C/G]AATTAA-3' [974 to 956]), 1056R (5'-ACGA GCTGACGAC[A/G]GCCA-3' [1073 to 1056]), 1114F (5'-GC AACGAGCGCAACCC-3' [1099 to 1114]), 1100R (5'-AGGG TTGCGCTCGTTG-3' [1115 to 1100]), 1240F (5'-ACACGC GTGCTACAAT-3' [1225 to 1240]), 1227R (5'-CCATTGTAG CACGTGT-3' [1242 to 1227]), 1406F (5'-TG[C/T]ACACAC CGCCCGT-3' [1391 to 1406]), 1392R (5'-ACGGGCGGTGT GT[G/A]C-3' [1406 to 1392]), and 1512-AR (5'-ACGG[T/C]T ACCTTGTTACGACTT-3' [1512 to 1492]).

Alignment and phylogenetic tree inference. The sequences were aligned by secondary structure according to the RDP database alignment (29). The SIMILARITY_RANK tool of the RDP database was used to search the RDP database for close evolutionary relatives of different nitrifier sequences. Phylogenetic trees were inferred, using the PAUP3.1 parsimony program package (42a) and the distance matrix method of Fitch and Margoliash as implemented by PHYLIP (Phylogeny Inference Package) version 3.5c (15). Regions of ambiguous sequence alignment were excluded from analysis.

Nucleotide sequence accession numbers. Sequences have been deposited in GenBank under accession numbers L35501 to L35514.

RESULTS AND DISCUSSION

The now recognized diversity of genera and species of the ammonia oxidizers includes *Nitrosomonas*, with at least 10 species (19, 23, 53), *Nitrosococcus* (53), with 3 species (22, 25, 53), *Nitrosospira*, with 1 recognized species (44, 54) and 4 other species indicated by DNA homology studies (24), *Nitrosovibrio*, with 1 described species (16) and 1 additional species indicated by DNA homology studies (24), and *Nitrosolobus*, with one described species (49) and 1 additional species indicated by DNA homology studies (24). The nitrite-oxidizing genera have received relatively less attention, and few have been examined by comparative sequence analysis. The four recognized genera of nitrite-oxidizing bacteria include *Nitrobacter* (53), with three species (6, 7), and *Nitrospina* (50), *Nitrococcus* (50), and *Nitrospira* (47), with one species each.

Previous comparative studies and the sequence data presented here indicate that all characterized nitrifying bacteria are members of the proteobacteria, a large bacterial group of presumed photosynthetic ancestry (55, 56). Relationships
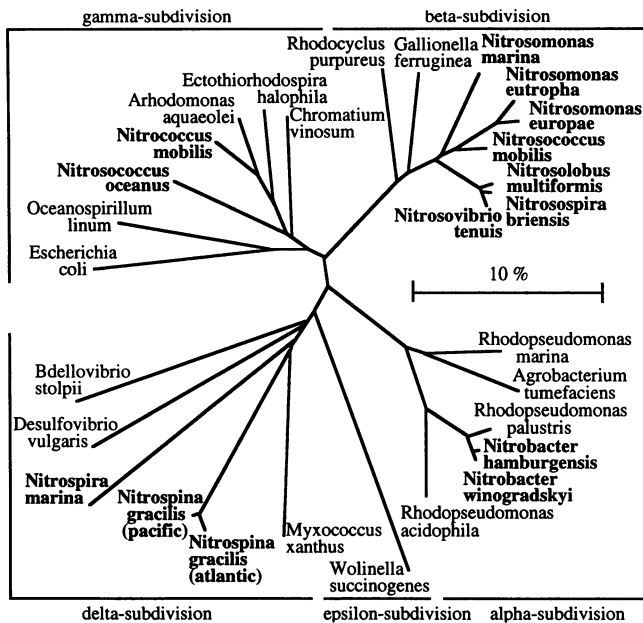
FIG. 1. Distance tree for the the proteobacteria, including the nitrifying isolates characterized in this study. The scale bar corresponds to 0.1 estimated fixed mutation per sequence position.

within the proteobacteria as inferred by comparative 16S rRNA sequence analyses are displayed in Fig. 1. The general phylogenetic distribution is briefly described here and discussed in greater detail in the following sections, which elaborate specific affiliations between nitrifying and nonnitrifying representatives of the individual proteobacterial subdivisions.

Nitrite-oxidizing genera occur in the alpha, gamma, and delta subdivisions of the proteobacteria. The genus *Nitrobacter* forms a tight group of very closely related species in the alpha subdivision, clustering together with species of the genera *Bradyrhizobium*, *Rhodopseudomonas*, and *Afipia*. *Nitrococcus mobilis* is a member of the gamma subdivision, related to

members of the family *Ectothiorhodospiraceae* and the ammonia-oxidizing bacterium *Nitrosococcus oceanus*. *Nitrospira marina*, the only described species of the genus, shows an affiliation with the delta subdivision. The two strains of *Nitrospina gracilis* (the only described species in the genus) also demonstrate a peripheral relationship to the delta subdivision but are unrelated to *Nitrospira marina*. The ammonia-oxidizing bacteria (with the exception of *Nitrosococcus oceanus*) constitute a closely related assemblage within the beta subdivision composed of the genera *Nitrosolobus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosomonas*. In contrast, the two species of the genus *Nitrosococcus* belong to different subdivisions (17, 55). *Nitrosococcus mobilis* is related to the genus *Nitrosomonas* in the beta subdivision; *Nitrosococcus oceanus* is the only recognized ammonia oxidizer in the gamma subdivision.

For a more detailed comparison of relationships, we consider each subdivision separately. This treatment is a consequence of the requirement for sequence alignment prior to phylogenetic analysis. By comparing only closely related sequences, to the exclusion of sequences from different subdivisions, it is possible to include a greater number of aligned sequence positions in the comparative analyses. Highly variable regions of sequence generally must be excluded when distantly related sequences are compared, because they cannot be unambiguously aligned. A similar treatment was recently used to characterize sulfur- and iron-oxidizing bacteria (27). The following discussion is based on an independent analysis of each of the four subdivisions.

The alpha subdivision: the genus *Nitrobacter*. The members of the genus *Nitrobacter* make up an exclusive and highly related cluster that is closely associated with *Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, and *Afipia clevelandensis* within the alpha-2 branch of the proteobacteria (Fig. 2). More distantly related sequences include those of an additional photosynthetic species (e.g., *Rhodopseudomonas acidophila*) and methylotrophs.

Near-complete sequences were determined for four *Nitrobacter* strains: *Nitrobacter winogradskyi* ATCC 14123 (formerly *Nitrobacter agilis*), *Nitrobacter winogradskyi* ATCC 25381, *Nitrobacter hamburgensis* X14, and *Nitrobacter* sp. strain R6 (isolated by J. Prosser, Rothamsted, United Kingdom). The
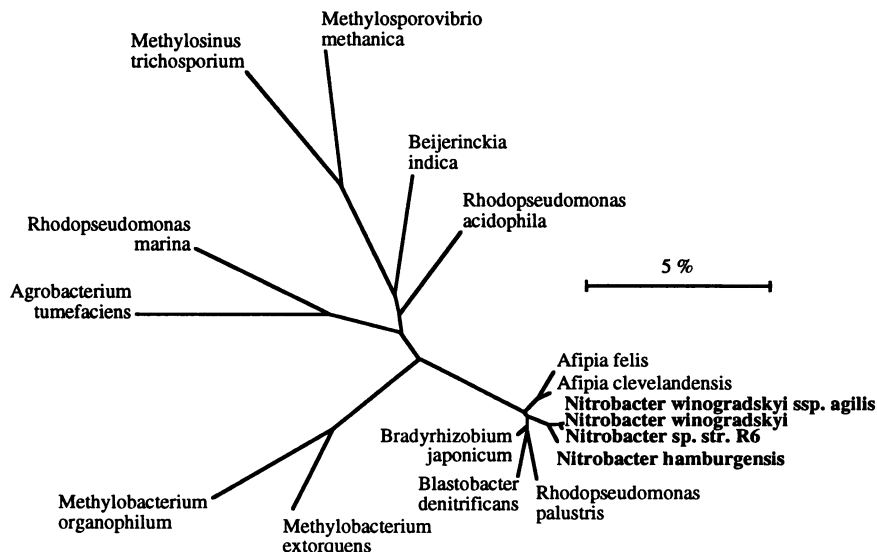

FIG. 2. Distance tree for the alpha subdivision of the proteobacteria.

16S rRNA sequences for the two strains of *Nitrobacter wino-gradskyi* (ATCC 14123 and ATCC 25381) were identical and differed from *Nitrobacter* sp. strain R6 at only one nucleotide position (*E. coli* position 1267, C/U). *Nitrobacter hamburgensis* X14 differs in at least nine nucleotide positions from these three strains. In addition to the near-complete sequences determined for these members of the genus, partial sequences of three additional *Nitrobacter* strains (ATCC 25383, ATCC 25384, and ATCC 25385) were determined by rRNA-templated sequencing with reverse transcriptase. Approximately 500 nucleotides of 16S rRNA sequence beginning at the 5' end were determined for each. The partial sequences of strain 25383 and 25384, not considering ambiguous nucleotide assignments, are identical to the near-complete sequences determined for *Nitrobacter winogradskyi* strains ATCC 25381 and 14123. However, *Nitrobacter* sp. strain 25383 differs at two nucleotide positions, *E. coli* positions 250 (A/U) and 264 (C/U). Thus, additional genetic diversity exists among available *Nitrobacter* species and may be great enough to warrant species distinction.

Within the genus *Nitrobacter*, the pairwise evolutionary distance estimates do not exceed 1%, indicating a low degree of genetic diversity. These results are consistent with the recent 16S rRNA sequence comparisons of three *Nitrobacter* isolates (*N. winogradskyi*, *Nitrobacter* sp. strain LL, and *N. hamburgensis*) by Orso et al. (34). Many other genera of bacteria show 10 to 15% 16S rRNA sequence divergence between species, although this may be considered the upper limit for genus rank and more appropriate for the definition of families (14, 37). The high degree of genetic homogeneity within the genus *Nitrobacter* was also demonstrated by an earlier 16S rRNA oligonucleotide study (38) reporting a high S_{ab} (similarity) value (0.82) between *Nitrobacter winogradskyi* and *Nitrobacter hamburgensis*. However, we do not advocate the use of rRNA sequence divergence alone for defining taxonomic rank. This information must be integrated with phenotypic variation and environmental distribution.

The phylogenetic relationships between *Nitrobacter* species were also investigated by Navarro et al. (31). On the basis of restriction fragment length polymorphism data of PCR-amplified intergenic spacer regions of the ribosomal operon, they estimated sequence divergences of intergenic spacer regions in 39 different *Nitrobacter* strains. Their results are congruent with our 16S rRNA-based phylogeny of the genus *Nitrobacter*. The highest spacer region divergences (7.5 to 7.7%) were observed between *Nitrobacter hamburgensis* X14 and *Nitrobacter winogradskyi* ATCC 14123. Lower sequence divergences, in the range of 3 to 4%, were observed between *Nitrobacter winogradskyi* ATCC 14123 and various other *Nitrobacter* spp., including strain R6. DNA-DNA reassociation studies (7) demonstrated an overall DNA homology of 36% between species, in the accepted range (20 to 60%) for species (genospecies) relationship within a genus. Thus, the 16S rRNA provides information of more general relationships at approximately the species level of discrimination, whereas DNA homology and comparison of intergenic spacer regions provide resolution of individual strains.

The tight cluster of *Nitrobacter* species and several nonnitrifying species (*Rhodopseudomonas palustris*, *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, *Afipia felis*, and *Afipia clevelandensis*) was only partially outlined by earlier studies. Oligonucleotide data demonstrated a close relationship between the 16S rRNAs of *Nitrobacter winogradskyi* and *Nitrobacter hamburgensis* to that of *Rhodopseudomonas palustris* (38). A 16S rRNA sequence analysis of *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, and *Afipia clevelandensis* and
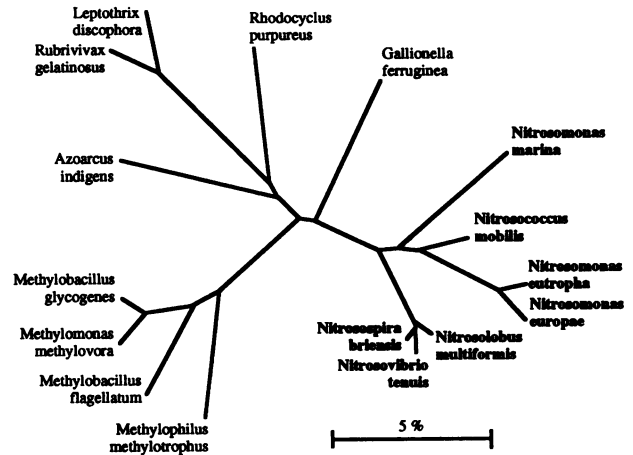

FIG. 3. Distance tree for the beta subdivision of the proteobacteria.

Afipia felis showed their close relationships (32, 52). A chemotaxonomic study (3, 30a), based on analysis of lipid A and deep core regions of cell wall lipopolysaccharides, was consistent with the sequence data presented here. The 16S rRNA sequences of *Bradyrhizobium japonicum*, *Rhodopseudomonas palustris*, and *Nitrobacter winogradskyi* form a coherent cluster, well separated from other members of the alpha subdivision (96.8 to 100% sequence similarity). Thus, the root-nodulating *Bradyrhizobium japonicum*, *Blastobacter denitrificans*, the human pathogens *Afipia felis* and *Afipia clevelandensis*, the anaerobic photosynthetic *Rhodopseudomonas palustris*, and the facultative lithoautotrophic *Nitrobacter* species all have a relatively recent common ancestor, also suggesting they have common traits. For example, the capacity for denitrification is present in *Blastobacter denitrificans*, *Nitrobacter* species, and some strains of *Rhodopseudomonas palustris* (8, 21, 43). However, nitrification has not been reported for *Blastobacter denitrificans* (43), and the need for a systematic comparison of these enzyme systems is indicated. Three members of this assemblage divide by budding, *Rhodopseudomonas palustris*, *Blastobacter denitrificans*, and *Nitrobacter* species (35, 41, 46). Thus, the other members of this assemblage should be reexamined for this characteristic. Another common phenotypic theme of related bacteria is the intracellular habitat of *Bradyrhizobium japonicum* and *Afipia* species (agents of cat scratch disease) (32). However, facultative or obligate intracellular associations are observed in a variety of the alpha-subdivision bacteria, including species of the genera *Rickettsia*, *Rochalimaea*, and *Brucella* (55), and so should not be considered unique to the specific nitrobacter lineage.

Related but physiologically distinct bacteria include the methylotrophs. Methylotrophs are affiliated with the alpha, beta, and gamma subdivisions of the proteobacteria in close peripheral relationship to both nitrite and ammonia oxidizers (Fig. 2 to 4). Within the alpha subdivision, *Methylobacterium* species are the closest methylotrophic relatives of the characterized *Nitrobacter* species. They have similar intracytoplasmic membrane structures, a feature that ties them both to the phototrophs (13). A relationship between methane-oxidizing and ammonia-oxidizing species was earlier suggested by the capacity of ammonia monooxygenase and methane monooxygenase to oxidize either ammonia or methane, but the evolutionary and ecophysiological significances of this oxidative flexibility are unresolved (18, 33). Nitrite-oxidizing genera,
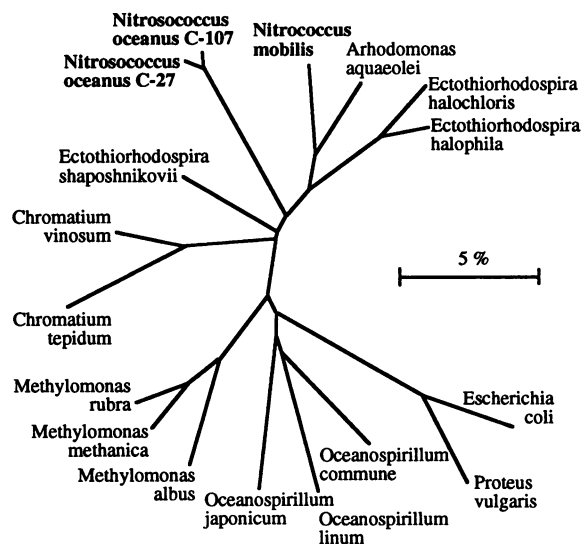
FIG. 4. Distance tree for the gamma subdivision of the proteobacteria.

such as *Nitrobacter*, *Nitrospina*, and *Nitrococcus* species, cannot oxidize methane (18). We call attention to similar peripheral affiliations of methylotrophs with nitrifiers in the beta and gamma subdivisions but will not further address these relationships.

The beta subdivision: the genera *Nitrosolobus*, *Nitrosospira*, *Nitrosovibrio*, *Nitrosomonas*, and *Nitrosococcus*. The physiological differences of ammonia-oxidizing and nitrite-oxidizing bacteria are reflected by their mutually exclusive phylogenetic positions as inferred by 16S rRNA sequence comparisons. However, since these sequences were independently reported, we restrict our discussion to general features of relationship between ammonia- and nitrite-oxidizing species and, when appropriate, differences in the independently determined sequences. The ammonia-oxidizing bacteria, with one exception (*Nitrosococcus oceanus*), constitute a monophyletic line of descent within the beta subdivision of the proteobacteria (98% bootstrap). No described nitrite oxidizer has been placed in this subdivision. Previous 16S rRNA oligonucleotide data (57) indicated a high degree of genetic homogeneity among the ammonia oxidizers, and this is now confirmed by analysis of near-complete 16S rRNA sequences (17). A recent taxonomic treatment of the genus *Nitrosomonas* by Koops et al. (23) classified eight new species of *Nitrosomonas*, including *Nitrosomonas marina*. The 5′-terminal sequence (ca. 500 nucleotides) of *Nitrosomonas europaea* ATCC 19718 is identical to that of strain C-31 (ATCC 25984). With reference to the phylogenetic relationships defined in Fig. 1 and 3, *Nitrosococcus mobilis* is encompassed by the now described genus *Nitrosomonas*. We suggest that taxonomic revision is necessary to include this additional information of phylogenetic relationship.

The closest relatives to the beta-subdivision ammonia oxidizers are the iron-oxidizing bacterium *Gallionella ferruginea*, the photosynthetic *Rhodocyclus purpureus*, and methylotrophic bacteria (Fig. 3). The close relationship to the chemolithotrophic *Gallionella ferruginea* is also reflected by an extensive intracellular membrane system, also found in some *Nitrosomonas* species, consisting (in part) of irregular tubes protruding into the cytoplasm continuous with the cytoplasmic membrane (30). The tubular membrane system of *R. purpureus* also

resembles those of *Gallionella ferruginea* and some *Nitrosomonas* species.

Nitrosospira briensis (strain C-128) is one representative of at least two, and probably five, different genospecies. Two groups of *Nitrosospira* species could be distinguished on the basis of their G+C contents (24). DNA hybridization data allowed a further categorization into five species (24). However, supporting 16S rRNA sequence data are not yet available. In this regard, there are 10 nucleotide differences between the sequence of strain C-128 determined in this study and the previously published sequence for this strain (17). Every mismatch was checked and resequenced, confirming the sequence presented here. The character of the differences suggests that the differences arose from sequencing errors in the previously published sequence rather than strain confusion. Thus, this genus is currently represented by only one 16S rRNA sequence.

Nitrosolobus multiformis C-71 (ATCC 25196, type strain) represents one of two species defined by G+C content and DNA hybridization (24, 48). The 16S rRNA sequence of C-71 was determined in this study and independently by Larsen et al. (29). These sequences are identical at all comparable positions; however, the previously published sequence (17) differs at six nucleotide positions. These differences should be noted for future determinative and taxonomic studies. In addition, partial sequencing of the 5′ termini (ca. 300 nucleotides) of *N. multiformis* ATCC 25198 revealed two differences at *E. coli* positions 122 (A/G) and 187 (C/U). However, additional sequencing is needed to determine the need for taxonomic revision.

A recent phylogenetic treatment of the ammonia oxidizers based on 16S rRNA sequence comparisons (17) recommended that the genera *Nitrosovibrio*, *Nitrosospira*, and *Nitrosolobus* be combined in a single genus. Although we concur that these genera constitute a closely related assemblage, of lesser phylogenetic depth than the described species of *Nitrosomonas*, we note that the ultrastructure of *Nitrosolobus* (cytomembranes that partially compartmentalize the cell) is distinct from those of *Nitrosospira* and *Nitrosovibrio* (both lacking an extensive cytomembrane system) (46). Thus, although *Nitrosovibrio* and *Nitrosospira* spp. could be accommodated within a single genus, we recommend reconsideration of inclusion of *Nitrosolobus* in such a revised taxonomic description.

The gamma subdivision: the genera *Nitrosococcus* and *Nitrococcus*. The gamma subdivision of the proteobacteria harbors the ammonia oxidizer *Nitrosococcus oceanus* (strains C-107 and C-27) and the nitrite oxidizer *Nitrococcus mobilis* (ATCC 25380, type strain). This is the only example of both metabolic types occurring within the same subdivision (Fig. 1 and 4). Both species are found within the purple sulfur bacterial line of descent.

Physiologically, the purple sulfur bacteria are divided between those depositing elemental sulfur inside the cell (*Chromatiaceae*) and those depositing sulfur outside (*Ectothiorhodospiraceae*). This division corresponds to phylogenetic relationships as shown earlier by oligonucleotide comparisons (39). *Nitrosococcus oceanus* and *Nitrococcus mobilis* are both members of the ectothiorhodospira branch. These associations are reminiscent of those seen in the alpha and beta subdivisions and again suggest that nitrifying bacteria are derived from photosynthetic ancestry. Similarly, the intracytoplasmic membrane system of *Nitrosococcus oceanus* resembles the stacked ectothiorhodospira type: several stacks in ectothiorhodospira and one central stack in *Nitrosococcus oceanus*. *Nitrococcus mobilis* differs in having tubes randomly arranged in the cytoplasm. Another similarity among them is common marine

origin. *Nitrococcus mobilis*, *Nitrosococcus oceanus*, and nearly all *Ectothiorhodospira* species require specific salt conditions for growth. The closest relative of *Nitrococcus mobilis* is a bacterium within the ectothiorhodospira lineage, *Arhodomonas aquaeolei*, a recently described, aerobic, halophilic bacterium isolated from subterranean brine (1). The relatively close relationship between *Nitrosococcus oceanus* and *Nitrococcus mobilis* raises the question of independent or derived origins of the corresponding ammonia- and nitrite-oxidizing enzyme systems.

The delta subdivision: the genera *Nitrospina* and *Nitrospira*. There were no precedents for assigning phylogenetic affiliations of the genera *Nitrospina* and *Nitrospira*. The 16S sequence of *Nitrospira marina* strain Nb-295 marks an early divergence within the delta subdivision of the proteobacteria. The two strains of *Nitrospina gracilis*, Nb-211 (Atlantic strain) and Nb-3 (Pacific strain), are closely related and define a second early divergence within the delta subdivision. Neither genus is closely related to any known member of the delta proteobacteria (e.g., *Myxococcus xanthus* or *Bdellovibrio bacteriovorans*) (Fig. 1).

The affiliation of *Nitrospina* and *Nitrospira* with the delta subdivision is also supported by specific nucleotide signatures (55). *Nitrospina gracilis* and *Nitrospira marina* share the sequence motif CCTGACGCAGC(G/A)ACGCCG (*E. coli* 16S rRNA positions 385 to 402) common to all delta-subgroup sulfate reducers and also *M. xanthus* (2). Their distance from *Nitrobacter* species (alpha subdivision) and *Nitrococcus mobilis* (gamma subdivision) is reflected by different nitrite-oxidizing enzymes; none of the five major protein bands of the nitrite-oxidizing membrane of *Nitrobacter hamburgensis* (42) was detected in *Nitrospira marina* (47).

In contrast to the other subdivisions, the delta subdivision contains no known phototrophs. The ancestor of this subdivision is assumed to have lost its photosynthetic ability (55). The *Nitrospina* and *Nitrospira* species probably represent a type of nitrifying metabolism that is not derived directly from immediate photosynthetic ancestry. Consistent with this view, the nitrite-oxidizing intracytoplasmic membranes common to other subdivision genera, thought to be derived from photosynthetic precursors, are lacking in *Nitrospina* and *Nitrospira* species.

Summary. *Nitrobacteriaceae* is a polyphyletic family. Nitrite- and ammonia-oxidizing bacteria are widely distributed within the proteobacteria, demonstrating a phylogenetic diversity comparable to that previously reported for photosynthetic bacteria and sulfur- and iron-oxidizing chemolithotrophs (27). Although phylogenetically diverse, there are common themes. Notably, most of the nitrifiers are closely affiliated with phototrophs: *Rhodopseudomonas palustris* with *Nitrobacter* species, *Rhodocyclus purpureus* with *Nitrosomonas* species, and *Ectothiorhodospira* species with *Nitrosococcus oceanus*. This suggests a close evolutionary link between photosynthesis and nitrite/ammonia oxidation. Both reactions are associated with intracytoplasmic membranes, and related nitrifying and photosynthetic species often share common membrane structural arrangement. Although these data are consistent with the ammonia- or nitrite-oxidizing membrane systems being derivative from photosynthetic membrane systems, the photosynthetic phenotype is widely distributed within the proteobacteria, and phylogenetic affiliation alone is only circumstantial evidence. Thus, these observations should primarily serve as an impetus to better establish the character of the postulated link.

The close connection between photosynthesis and nitrite or ammonia oxidation also suggests the possible existence of ammonia-oxidizing photosynthetic bacteria (10). Ammonia could be used by photosynthetic bacteria as an electron donor in a way analogous to sulfide: $1.3\ NH_4^+ + CO_2 = (CH_2O) + 0.65\ N_2 + H_2O + 1.3\ H^+$ ($\Delta G° = 50$ kJ). The possible existence of iron-oxidizing photosynthetic bacteria was postulated earlier on the basis of similar considerations, and representative bacteria were recently isolated (51). The suggested evolutionary progression is consistent with the conversion hypothesis (10–12). This hypothesis proposes that respiratory metabolism, both chemoorganotrophic and chemolithotrophic, has originated repeatedly via the independent conversion of an ancestral membrane-bound photosynthetic apparatus into a respiratory membrane. These conversions presumably occurred as oxidized compounds, produced by anoxygenic and oxygenic photosynthesis, became abundant (11). The purple nonsulfur bacteria and some purple sulfur bacteria are viewed as transitional forms, photosynthetic bacteria with additional (limited) capabilities for aerobic respiration and heterotrophic metabolism (12). The phylogenetic diversity of nitrite-oxidizing and ammonia-oxidizing bacteria seems to reflect the relative ease of transition from photosynthesis to ammonia- and nitrite-based chemolithotrophy. Each nitrifier retains the general structural features of the putative ancestor's photosynthetic membrane complex. Thus, the nitrifiers, as a group, are not derived from an ancestral nitrifying phenotype but appear to have arisen independently multiple times, possibly from different photosynthetic ancestors.

REFERENCES

1. **Adkins, J. P., M. T. Madigan, L. Mandelco, C. R. Woese, and R. S. Tanner.** 1993. *Arhodomonas aquaeolei* gen. nov. sp. nov., an aerobic, halophilic bacterium isolated from a subterranean brine. Int. J. Syst. Bacteriol. **43:**514–520.
2. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
3. **Blumer, M., T. Chase, and S. W. Watson.** 1969. Fatty acids in the lipids of marine and terrestrial nitrifying bacteria. J. Bacteriol. **99:**366–370.
4. **Bock, E., and H.-P. Koops.** 1991. The genus *Nitrobacter* and related genera, p. 2302–2309. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.
5. **Bock, E., H.-P. Koops, B. Ahlers, and H. Harms.** 1991. Oxidation of inorganic nitrogen compounds as energy source, p. 414–430. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.
6. **Bock, E., H.-P. Koops, U. C. Möller, and M. Rudert.** 1990. A new facultatively nitrite oxidizing bacterium, *Nitrobacter vulgaris* sp.nov. Arch. Microbiol. **153:**105–110.
7. **Bock, E., H. Sundermeyer-Klinger, and E. Stackebrandt.** 1983. New facultative lithotrophic nitrite-oxidizing bacteria. Arch. Microbiol. **136:**281–284.
8. **Bock, E., P. A. Wilderer, and A. Freitag.** 1988. Growth of *Nitrobacter* in the absence of dissolved oxygen. Water Res. **22:**245–250.
9. **Böttger, E. C.** 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **65:**171–176.
10. **Broda, E.** 1977. Two kinds of lithotrophs missing in nature. Z. Allg. Mikrobiol. **17:**491–493.

11. **Broda, E.** 1978. The evolution of the bioenergetic process. Pergamon Press, Oxford.

12. **Broda, E., and G. A. Peschek.** 1979. Did respiration or photosynthesis come first? J. Theor. Biol. **81:**201–212.

13. **Davies, S. L., and R. Wittenbury.** 1970. Fine structure of methane and other hydrocarbon utilising bacteria. J. Gen. Microbiol. **61:**227–232.

14. **Devereux, R., S.-H. He, C. L. Doyle, S. Orkland, D. A. Stahl, J. LeGall, and W. B. Whitman.** 1990. Diversity and origin of *Desulfovibrio* species: phylogenetic definition of a family. J. Bacteriol. **172:**3609–3619.

15. **Felsenstein, J.** 1993. PHYLIP (Phylogeny Inference Package) version 3.5c. Distributed by the author. Department of Genetics, University of Washington, Seattle.

16. **Harms, H., H.-P. Koops, and H. Wehrmann.** 1976. An ammonia-oxidizing bacterium, *Nitrosovibrio tenuis* nov.gen.nov.sp. Arch. Microbiol. **108:**105–111.

17. **Head, I. M., W. D. Hiorns, T. M. Embley, A. J. McCarthy, and J. R. Saunders.** 1993. The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. **139:**1147–1153.

18. **Jones, R. D., and R. Y. Morita.** 1983. Methane oxidation by *Nitrosococcus oceanus* and *Nitrosomonas europeae*. Appl. Env. Microbiol. **45:**401–410.

19. **Jones, R. D., R. Y. Morita, H.-P. Koops, and S. W. Watson.** 1988. A new marine ammonium-oxidizing bacterium, *Nitrosomonas cryotolerans* sp. nov. Can. J. Microbiol. **34:**1122–1128.

20. **Kalthoff, H., S. Fehr, H. Sundermeyer, L. Renwrantz, and E. Bock.** 1979. A comparison by means of antisera and lectins of surface structures of *Nitrobacter winogradskyi* and *N. agilis*. Curr. Microbiol. **125:**375–380.

21. **Klemme, J.-H., I. Chyla, and M. Preuss.** 1980. Dissimilatory nitrate reduction by strains of the facultative phototrophic bacterium *Rhodopseudomonas palustris*. FEMS Microbiol. Lett. **9:**137–140.

22. **Koops, H.-P., B. Bottcher, U. C. Möller, A. Pommerening-Roser, and G. Stehr.** 1990. Description of a new species of *Nitrosococcus*. Arch. Microbiol. **154:**244–248.

23. **Koops, H.-P., B. Bottcher, U. C. Möller, A. Pommerening-Roser, and G. Stehr.** 1991. Classification of eight new species of ammonia-oxidizing bacteria: *Nitrosomonas communis* sp.nov., *Nitrosomonas ureae* sp.nov., *Nitrosomonas aestuarii* sp.nov., *Nitrosomonas marina* sp.nov., *Nitrosomonas nitrosa* sp.nov., *Nitrosomonas eutropha* sp.nov., *Nitrosomonas oligotropha* sp.nov., and *Nitrosomonas halophila* sp.nov. J. Gen. Microbiol. **137:**1689–1699.

24. **Koops, H.-P., and H. Harms.** 1985. Deoxyribonucleic acid homologies among 96 strains of ammonia-oxidizing bacteria. Arch. Microbiol. **141:**214–218.

25. **Koops, H.-P., H. Harms, and H. Wehrmann.** 1976. Isolation of a moderate halophilic ammonia-oxidizing bacterium, *Nitrosococcus mobilis* nov.sp. Arch. Microbiol. **107:**277–282.

26. **Koops, H.-P., and U. C. Möller.** 1991. The lithotrophic ammonia-oxidizing bacteria, p. 2625–2637. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The procaryotes, 2nd ed. Springer-Verlag, New York.

27. **Lane, D. J., A. P. Harrison, D. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace.** 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. J. Bacteriol. **174:**269–278.

28. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA **82:**6955–6959.

29. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeck, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The ribosomal database project. Nucleic Acids Res. **21(Suppl.):**3021–3023.

30. **Lütters, S., and H. H. Hanert.** 1989. The ultrastructure of chemolithoautotrophic *Gallionella ferruginea* and *Thiobacillus ferrooxidans* as revealed by chemical fixation and freeze-etching. Arch. Microbiol. **151:**245–251.

30a.**Mayer, H., J. H. Krauss, T. Urbanik-Sypniewska, V. Puvanesarajah, G. Stacey, and G. Auling.** 1989. Lipid A with 2,3-diamino-2,3-

dideoxyglucose in lipopolysaccharides from slow-growing members of *Rhizobiaceae* and from "*Pseudomonas carboxydovorans*." Arch. Microbiol. **151:**111–116.

31. **Navarro, E., P. Simonet, P. Normand, and R. Bardin.** 1992. Characterization of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. Arch. Microbiol. **157:**107–115.

32. **O'Connor, S. P., M. Dorsch, A. G. Steigerwalt, D. J. Brenner, and E. Stackebrandt.** 1991. 16S rRNA sequences of *Bartonella bacilliformis* and cat scratch disease bacillus reveal phylogenetic relationships with the alpha-2 subgroup of the class *Proteobacteria*. J. Clin. Microbiol. **29:**2144–2150.

33. **O'Neill, J. G., and J. F. Wilkinson.** 1977. Oxidation of ammonia by methane-oxidizing bacteria and the effects of ammonia on methane oxidation. J. Gen. Microbiol. **100:**407–412.

34. **Orso, S., M. Gouy, E. Navarro, and P. Normand.** 1994. Molecular phylogenetic analysis of *Nitrobacter* spp. Int. J. Syst. Bacteriol. **44:**83–86.

35. **Pfennig, N., and H. G. Trüper.** 1989. Anoxygenic phototrophic bacteria, p. 1635–1709. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.

36. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

37. **Schleifer, K. H., and E. Stackebrandt.** 1983. Molecular systematics of prokaryotes. Annu. Rev. Microbiol. **37:**143–187.

38. **Seewaldt, E., K. H. Schleifer, E. Bock, and E. Stackebrandt.** 1982. The close phylogenetic relationship of *Nitrobacter* and *Rhodopseudomonas palustris*. Arch. Microbiol. **131:**287–290.

39. **Stackebrandt, E., V. J. Fowler, W. Schubert, and J. F. Imhoff.** 1984. Towards a phylogeny of phototrophic sulfur bacteria—the genus *Ectothiorhodospira*. Arch. Microbiol. **137:**366–370.

40. **Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery.** 1988. Use of phylogenetically based hybridisation probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. **54:**1079–1084.

41. **Staley, J. T., and J. A. Fuerst.** 1989. Budding and/or appendaged bacteria, p. 1890–1993. *In* J. T. Staley, M. P. Bryant, N. Pfennig, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 3. The Williams & Wilkins Co., Baltimore.

42. **Sundermeyer-Klinger, H., W. Meyer, B. Warninghoff, and E. Bock.** 1984. Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrite reductase system. Arch. Microbiol. **140:**153–158.

42a.**Swofford, D. L.** 1991. PAUP: phylogenetic analysis using parsimony version 3.1. Computer program distributed by the Illinois Natural Survey. Champaign, Ill.

43. **Trotsenko, Y. A., N. V. Doronina, and P. Hirsch.** 1989. Genus *Blastobacter* Zavarzin 1961, p. 1963–1968. *In* R. G. E. Murray, D. J. Brenner, M. P. Bryant, J. G. Holt, N. R. Krieg, J. W. Moulder, N. Pfennig, P. H. A. Sneath, J. T. Stanley, and S. Williams (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.

44. **Watson, S. W.** 1971. Re-isolation of *Nitrosospira briensis* S. Winogradsky and H. Winogradsky, p. 450–456. *In* R. E. Buchanaan and N. E. Gibbons (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.

45. **Watson, S. W.** 1971. Taxonomic considerations of the family *Nitrobacteriaceae* Buchanan. Requests for opinions. Int. J. Syst. Bacteriol. **21:**254–270.

46. **Watson, S. W., E. Bock, H. Harms, H.-P. Koops, and A. B. Hooper.** 1989. Nitrifying bacteria, p. 1808–1834. *In* R. G. E. Murray, D. J. Brenner, M. P. Bryant, J. G. Holt, N. R. Krieg, J. W. Moulder, N. Pfennig, P. H. A. Sneath, J. T. Stanley, and S. Williams (ed.), Bergey's manual of systematic bacteriology. The Williams & Wilkins Co., Baltimore.

47. **Watson, S. W., E. Bock, F. W. Valois, J. B. Waterbury, and U. Schlosser.** 1986. *Nitrospira marina*, gen.nov.,sp.nov.: a chemolithotrophic nitrite-oxidizing bacterium. Arch. Microbiol. **144:**1–7.

48. **Watson, S. W., L. B. Graham, C. C. Remsen, and F. W. Valois.** 1971. A lobular, ammonia-oxidizing bacterium, *Nitrosolobus multiformis* nov.sp. Arch. Microbiol. **76:**183–203.

49. **Watson, S. W., and M. Mandel.** 1971. Comparison of the morphology and deoxyribonucleic acid composition of 27 strains of nitrifying bacteria. J. Bacteriol. **107:**563–569.

50. **Watson, S. W., and J. B. Waterbury.** 1971. Characteristics of two marine nitrite oxidizing bacteria, *Nitrospina gracilis* nov.gen. nov.sp. and *Nitrococcus mobilis* nov.gen.nov.sp. Arch. Microbiol. **77:**203–230.

51. **Widdel, F., S. Schnell, S. Heising, A. Ehrenreich, B. Assmus, and B. Schink.** 1993. Ferrous iron oxidation by anoxygenic phototrophic bacteria. Nature (London) **362:**834–835.

52. **Willems, A., and M. D. Collins.** 1992. Evidence for a close genealogical relationship between *Afipia* (the causal organism of cat scratch disease), *Bradyrhizobium japonicum* and *Blastobacter denitrificans*. FEMS Microbiol. Lett. **96:**241–246.

53. **Winogradsky, S.** 1892. Contributions a la morphologie des organismes de la nitrification. Arch. Sci. Biol. (St. Petersburg) **1:**88–137.

54. **Winogradsky, S., and H. Winogradsky.** 1933. Etudes sur la microbiologie du sol. VII. Nouvelles recherches sur les organisms de la nitrification. Ann. Inst. Pasteur **50:**350–432.

55. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51:**221–271.

56. **Woese, C. R., E. Stackbrandt, W. G. Weisburg, B. J. Paster, M. T. Madigan, V. J. Fowler, C. M. Hahn, P. Blanz, R. Gupta, K. H. Nealson, and G. E. Fox.** 1984. The phylogeny of purple bacteria: the alpha subdivision. Syst. Appl. Microbiol. **5:**315–326.

56a.**Woese, C. R., W. G. Weisburg, C. M. Hahn, B. J. Paster, L. B. Zablen, B. J. Lewis, T. J. Macke, W. Ludwig, and E. Stackebrandt.** 1985. The phylogeny of purple bacteria: the gamma subdivision. Syst. Appl. Microbiol. **6:**25–33.

57. **Woese, C. R., W. G. Weisburg, B. J. Paster, C. M. Hahn, R. S. Tanner, N. R. Krieg, H.-P. Koops, H. Harms, and E. Stackebrandt.** 1984. The phylogeny of purple bacteria: the beta subdivision. Syst. Appl. Microbiol. **5:**327–336.