Molecular Phylogenetic Analysis of *Nitrobacter* spp.

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The phylogeny of bacteria belonging to the genus *Nitrobacter* was investigated by sequencing the whole 16S rRNA gene. The average level of similarity for the three *Nitrobacter* strains examined was high (99.2%), and the similarity level between *Nitrobacter winogradskyi* and *Nitrobacter* sp. strain LL, which represent two different genomic species, was even higher (99.6%). When all of the *Nitrobacter* strains and their phylogenetic neighbors *Bradyrhizobium* and *Rhodopseudomonas* species were considered, the average similarity level was 98.1%. When complete sequences were used, *Nitrobacter hamburgensis* clustered with the two other *Nitrobacter* strains, while this was not the case when partial sequences were used. The two *Rhodopseudomonas palustris* strains examined exhibited a low similarity level (97.6%) and were not clustered.

Bacteria belonging to the genus *Nitrobacter* constitute a key part of the nitrification process as they are the only nitrite-oxidizing microorganisms known to occur in soil. Until now, this chemical process has been studied more than the microorganisms themselves because of difficulties in isolating and cultivating the bacteria (4, 13, 17).

Nevertheless, workers have performed taxonomic studies to classify the few strains that have been isolated and to relate serological and physiological characteristics to genomic differences. Initially, taxonomic studies started with observations of morphological features, and currently the taxonomy of the genus *Nitrobacter* is still largely based on such criteria (24). However, morphological criteria are often considered inappropriate for defining natural relationships, particularly when microorganisms are concerned. Because of such limitations, genomic methods, such as DNA-DNA hybridization and 16S rRNA oligonucleotide cataloging, have been used (13, 20). These methods permitted demonstration that the genus *Nitrobacter* belongs to the alpha subdivision of the *Proteobacteria* (21), which includes, among other taxa, the genus *Bradyrhizobium* and the species *Rhodopseudomonas palustris*, which are known to be phylogenetically very closely related to the genus *Nitrobacter* (5, 8, 20). Recent DNA-DNA hybridization data permitted division of the genus *Nitrobacter* into three genomic species; genomic species 1 contains type strain of *Nitrobacter winogradskyi*, genomic species 3 corresponds to *Nitrobacter hamburgensis*, and genomic species 2 contains proposed reference strain LL (13).

Among the genomic methods used, nucleic acid sequencing is certainly the technique which has most revolutionized microbiology; this technique has been shown to be a powerful tool for exploring the phylogeny of both prokaryotes and eukaryotes (27). As a result, microorganisms are now characterized not only on the basis of their phenotypic features but also with an evolutionary perspective. The phylogenetic aspect of bacterial characterization is becoming an increasingly important step, even a necessary one for

any microbial taxonomic study. Several molecules have been used as a molecular clock, but the 16S rRNA gene has received most attention because of its highly conserved and ubiquitous nature (27).

The aim of this work was to sequence the 16S rRNAs of *Nitrobacter* spp. strains, primarily to investigate the phylogeny of the genus *Nitrobacter* and its relatives. Recently, Young et al. (28) have confirmed, by using partial 16S rRNA gene sequences, the phylogenetic relatedness of *R. palustris* and *Bradyrhizobium japonicum*. As this conclusion agreed with previous results and as data from other work hinted that sequencing only a part of the 16S rRNA molecule could be sufficient to establish a correct phylogeny (7, 11, 22), we determined the sequence of a 16S rRNA gene segment for the type strains of the three *Nitrobacter* genomic species and for the type strain of *R. palustris*. However, the partial sequences revealed too few substitutions in the different strains for oligonucleotide probe construction, the ultimate aim of our work. We therefore determined the whole 16S rRNA sequence and compared the resulting phylogeny with the DNA-DNA hybridization data published recently (13).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. The strains were grown and DNA was extracted as described previously (14).

DNA amplification. The 16S rRNA genes were amplified by the PCR (12) by using the following two prokaryote-specific primers: primer FGPS6-63 (5'-GGAGAGTTAGATC TTGGCTCAG-3') and primer FGPS1509'-153 (5'-AAGGAG GGGATCCAGCCGCA-3'), which contain *Bgl*II and *Bam*HI restriction sites (underlined), respectively. The amplified fragments were purified with a GeneClean kit (Bio 101, La Jolla, Calif.) according to the manufacturer's instructions.

Cloning. The fragments were cleaved with endonucleases *Bgl*II and *Bam*HI and ligated into *Bam*HI-restricted vector Bluescript SK⁻ (Stratagene, La Jolla, Calif.). Plasmid purification from host strain DH5αF' was performed with QUI-AGEN midi-prep columns (Diagen, Düsseldorf, Germany). For most strains eight or more clones were obtained, and for *R. palustris* five clones were used; the clones were pooled for the sequencing reaction in order to obscure possible errors due to the *Taq* polymerase.

Sequencing. The designations of the primers used for sequencing refer to the small subunit ribosomal gene; the number is the coordinate of the 5' end in the *Frankia*

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TABLE 1. Bacterial strains used in this study

Strain	Source or reference ^a	GenBank nucleotide sequence accession no.
<i>Nitrobacter</i> genomic species 2 strain LL	9	L11661
<i>N. winogradskyi</i> W ^T (= ATCC 25391 ^T) ^b	ATCC	L11662
<i>N. hamburgensis</i> X14 ^T	1	L11663
<i>R. palustris</i> ^T (= DSM123 ^T)	DSM	L11664

^a ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany.

^b T = type strain.

sequence (15), and the prime refers to the primer in the opposite direction of the transcription. Twelve conserved or specific primers (FGPS6-63 and FGPS305'-78, CCAAGTGTG GCCGTCGCCCTCTC; FGPS310-20, GAGACACGGCCC AGACTCCT; FGPS594-171, GAACTGCCTTTGATACTG; FGPS700'-190, AG/ATCCACTCA/TCCTCT; FGPS872-61, CTAAGAACTCAAAGGAATTGA; FGPS950'-184, GTA CTCCCCAGGCGGA; FGPS1093'-23, GCAACATAGGAG GAGGGTTG; FGPS1156-39, GACGTCAAAGTCACATGC CC; FGPS1445'-182, GCGATTCCAACCTTCA; and primers T3 and T7 supplied by Pharmacia) were necessary to determine all of the 16S rRNA molecule on both strands. The dideoxy chain termination sequencing procedure (19) was performed by using T7 sequencing kits (Pharmacia LKB, Uppsala, Sweden).

Data analysis. Sequences were aligned by using the Clustal program (6). Previously published sequences for *B. japonicum* (26) (GenBank accession number X66024) and for strain B of *R. palustris* (27a) (GenBank accession number M59068) were added to the analysis, together with sequences of *Agrobacterium tumefaciens* (GenBank accession number M11223) and *Escherichia coli* (GenBank accession number J01695) as outgroups. Matrix pairwise comparisons were corrected for multiple base substitutions by the method of Jukes and Cantor (10). Phylogenetic trees were constructed by the neighbor-joining method (18). Gaps and regions of ambiguous sequence alignment were excluded from subsequent analyses. A bootstrap confidence analysis was performed with 1,000 replicates to determine the reliability of the tree topologies obtained (3).

RESULTS

Phylogenetic analysis. Partial 16S rRNA sequences were determined for all of the bacteria listed in Table 1. The resulting dendrogram (data not shown) clustered *N. winogradskyi*, *Nitrobacter* sp. strain LL, and *B. japonicum* but excluded *N. hamburgensis*. This finding led us to determine the complete 16S rRNA sequences for the three *Nitrobacter* strains and the *R. palustris* strain.

All of the complete sequences have been deposited in the GenBank data base (accession numbers are shown in Table 1). The intersequence similarity matrix and the phylogenetic tree deduced from the complete sequences are shown in Table 2 and Fig. 1, respectively. The average level of similarity determined for the *Nitrobacter* strains is 99.2%, and the level of similarity between *N. winogradskyi* and *Nitrobacter* sp. strain LL is 99.6%. When all of the *Nitrobacter*, *Bradyrhizobium*, and *Rhodopseudomonas* strains (but not the two outgroup sequences) were compared, the average level of similarity was 98.1%. When

TABLE 2. Homology matrix for complete 16S rRNA sequences

Organism	No. of differences and % homology ^a						
	<i>Nitrobacter</i> sp. strain LL	<i>N. winogradskyi</i>	<i>N. hamburgensis</i>	<i>R. palustris</i>	<i>R. palustris</i> B	<i>B. japonicum</i>	<i>A. tumefaciens</i>
<i>Nitrobacter</i> sp. strain LL		5	13	33	32	21	144
<i>N. winogradskyi</i>	99.6		14	34	35	22	143
<i>N. hamburgensis</i>	99.0	99.0		31	33	26	146
<i>R. palustris</i>	97.6	97.5	97.7		32	31	140
<i>R. palustris</i> B	98.5	97.3	98.5	97.6		21	146
<i>B. japonicum</i>	98.5	98.4	98.1	97.7	98.4		147
<i>A. tumefaciens</i>	88.7	88.0	88.5	89.0	88.1	88.4	
<i>E. coli</i>	77.4	77.4	77.2	77.9	76.5	77.0	78.5

^a The values on the upper right are the numbers of observed differences, and the values on the lower left are the percentages of homology corrected for multiple base changes by the method of Jukes and Cantor (10).

complete sequences were used, *N. hamburgensis* clustered with the two other *Nitrobacter* strains, and this position was corroborated by a bootstrap analysis; *N. hamburgensis* clustered with *N. winogradskyi* and *Nitrobacter* sp. strain LL in 96% of the replicates. The two *R. palustris* strains exhibited a relatively low similarity level (97.6%) and were not clustered.

DISCUSSION

The high level of sequence homology found within the genus *Nitrobacter* (99.2%) confirms the proposal of Seewaldt et al. (20) that this genus is a recent group. These results also establish the fact that the genus *Nitrobacter* is an evolutionarily coherent group as the phylogenetic relationships agree with the relationships determined in phenotypic and genomic studies. *N. winogradskyi* and *Nitrobacter* sp. strain LL are more closely related than *N. hamburgensis*, a conclusion reached previously on the basis of the results of phenotypic characterization, rRNA gene polymorphism, and DNA-DNA hybridization studies (13). Phenotypically, *N. hamburgensis* is characterized by faster growth heterotrophically than autotrophically.

B. japonicum is more closely related to the genus *Nitrobacter* than the *R. palustris* strains are, whereas we expected the converse on the basis of rRNA-DNA hybridization data (13) and the fact that *R. palustris* cells develop an important intracytoplasmic membrane system reminiscent of the system in *Nitrobacter* cytoplasm. *B. japonicum* shares phenotypic characteristics with members of the genus *Nitrobacter* (for instance, its ability to denitrify, although this is a widespread characteristic). Perhaps the main difference between *B. japonicum* and *Nitrobacter* species is fixation of nitrogen, which is also a property of *R. palustris*, except that *B. japonicum* forms nitrogen-fixing nodules on leguminous plants while *R. palustris* is a free-living nitrogen-fixing microorganism.

The relatedness of *B. japonicum* and *R. palustris* was confirmed in this study by examining complete sequence data. The close relationship of these two species has been described previously on the basis of 16S rRNA oligonucleotide catalogs (5), rRNA-DNA hybridization data (8) and

FIG. 1. Phylogenetic neighbor-joining tree based on complete 16S sequences. Bar = 0.012 substitution per site. The numbers in the boxes are the percentages of bootstrap replicates in which the cluster was found.

partial 16S rRNA sequences (28). The phylogenetic relatedness of *R. palustris* and *B. japonicum* led Young et al. (28), as well as Jarvis et al. (8), to suggest that it would be appropriate to group these two organisms in a single genus. However, *B. japonicum* differs phenotypically from *R. palustris*. According to the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (25), when phylogenetic data are not consistent with phenotypic data, priority should be given to the latter (25). Young et al. argued that an important characteristic such as nitrogen fixation is shared with *R. palustris* but is not taken into account as *B. japonicum* is always compared with the genus *Rhizobium* and not with *R. palustris*. If a revision does reclassify *B. japonicum* and *R. palustris* as members of a single taxonomic unit (genus or family), then the genus *Nitrobacter* should be clustered with them too, since the average level of homology between *B. japonicum* and the three *Nitrobacter* species is 98.3%, whereas the average level of homology between *R. palustris* and the three *Nitrobacter* species is 97.8%. The genera *Nitrobacter*, *Rhodopseudomonas*, and *Bradyrhizobium* are phylogenetically related but do not form a phenotypically coherent group; *Nitrobacter* species have features in common with species belonging to the two other genera but do not fix nitrogen, which is considered a discriminative characteristic. This lack of consistency between phylogenetic and phenotypic data probably stems from the fact that some of the phenotypic characteristics taken into consideration for taxon description (nitrogen fixation, photosynthesis) are given undue weight, especially given the recent description of a photosynthetic *Bradyrhizobium* strain (2).

The proposal of Seewaldt et al. (20) that the emergence of the genus *Nitrobacter* coincided with the onset of the aerobic atmosphere on earth (1×10^9 to 1.2×10^9 years ago) is in conflict with the value of 50×10^6 to 100×10^6 years ago obtained applying the 16S rRNA molecular clock of Ochman and Wilson (16) to *Nitrobacter* sequences, given a "ticking" rate for the 16S rRNA gene of 50×10^6 years for each 1% substitution per site. Considering the close relationship between *Bradyrhizobium* and *Nitrobacter* species, we speculate that the genera *Nitrobacter* and *Bradyrhizobium*

evolved from a *Rhodopseudomonas*-like photosynthetic, nitrogen-fixing ancestor at about the time of differentiation of land plant families. One evolutionary line retained the widespread characteristic of fixing nitrogen and lost for the most part the ability to photosynthesize, while the other evolutionary line acquired the ability to nitrify and seemingly lost the ability to fix nitrogen and the ability to photosynthesize. In this context the ability of nitrogenase to reduce nitrite (23) could be a remnant of some *nif* gene function.

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