16S Ribosomal DNA Sequence Analysis Confirms the Close Relationship between the Genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter* and Reveals a Lack of Phylogenetic Coherence among *Xanthobacter* Species

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A comparative 16S ribosomal DNA (rDNA) sequence analysis was used to investigate the phylogenetic position of members of the genus *Xanthobacter*. We determined 16S rDNA sequence data for the type strains of the three *Xanthobacter* species and five additional *Xanthobacter* strains. The close relationship between the genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter* previously demonstrated by DNA-rRNA hybridization studies was confirmed. The results of our phylogenetic analysis indicate that members of the genera *Xanthobacter*, *Azorhizobium*, and *Aquabacter* are intermixed and that there is no clear generic cluster consisting of the *Xanthobacter* species. A comparison of the *Xanthobacter* sequences with the 16S rDNA sequences available from environmental clone studies indicated that members of this genus have not been detected by nonculturing approaches.

Phylogenetic analysis based on comparisons of 16S ribosomal DNA (rDNA) sequence data is now routinely used in the determination of taxonomic relationships between microorganisms and in the design of taxon-specific probes for use in microbial identification and molecular ecology studies (1, 10, 15, 21).

One genus for which no full 16S rDNA sequence data are currently available is the genus *Xanthobacter*. The genus *Xanthobacter* consists of three species, *Xanthobacter agilis* (5), *Xanthobacter autotrophicus* (23), and *Xanthobacter flavus* (6, 12, 17). These organisms are gram-negative, pleomorphic rods that are able to obtain energy by the oxidation of hydrogen and can fix dinitrogen (22). *Xanthobacter* species have been isolated from soils, marine and freshwater sediments, the soil of rice fields, and the rhizosphere of rice plants (22).

DNA-rRNA hybridization studies have shown that the three species of the genus *Xanthobacter* fall within the alpha-2 subclass of the *Proteobacteria* and are the closest relatives of *Aquabacter spiritensis* and *Azorhizobium caulinodans* (2–4).

In order to investigate the relationships of the genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter*, we determined the complete 16S rDNA sequences of the type strains of the three *Xanthobacter* species and additional strains. The 16S rDNA sequence of the type strain of *Azorhizobium caulinodans* was redetermined in order to clarify some ambiguous nucleotide positions in the sequence currently available from the public databases.

The strains were grown on nutrient agar. 16S rDNA sequences were determined by directly sequencing the 16S rRNA genes amplified by PCR (16). The PCR products were directly sequenced by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Inc., Foster City, Calif.), and the purified sequence reaction mixtures were electrophoresed by using a model 373A automatic DNA sequencer (Applied Biosystems). The sequences were manually aligned with

the sequences of representatives of the alpha-2 subclass of the *Proteobacteria*. A phylogenetic analysis was performed by using the "ARB-A Software Environment for Sequence Data" programs (9). The methods of analysis used included distance methods, as well as maximum-likelihood, parsimony, and neighbor-joining methods.

The strains whose sequences we determined, their sources, and the nucleotide sequence accession numbers of their 16S rDNAs are shown in Table 1.

The 16S rDNA sequences obtained for all of the strains which we studied consisted of 1,410 to 1,414 nucleotides. The 16S rRNA sequence of *Azorhizobium caulinodans* ORS571^T (T = type strain) that was available from the Ribosomal Database Project database (11) was found to differ at seven nucleotide positions from the sequence of *Azorhizobium caulinodans* DSM 5975^T (= ORS571^T) which we determined. The sequence determined in this study was used in all analyses. The 16S rDNA sequence of *Aquabacter spiritensis* used in the phylogenetic analyses was the sequence available from the Ribosomal Database Project database (11). The results of our phylogenetic analysis based on 16S rDNA sequence comparisons indicated that the genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter* are closely related and represent a distinct cluster within the alpha-2 subclass of the *Proteobacteria*. All methods of phylogenetic analysis used in this study resulted in similar trees or dendrograms. In all cases the *Aquabacter*-*Azorhizobium*-*Xanthobacter* cluster of the alpha-2 subclass was recovered with the same internal branching order. Some slight rearrangements in the branching orders of other clusters within the alpha-2 subclass were observed when different methods were used. The phylogenetic dendrogram shown in Fig. 1 was reconstructed by the neighbor-joining method (20) from a distance matrix calculated by using the correction of the algorithm of Jukes and Cantor (7, 14).

Figure 1 shows the close relationship among the *Xanthobacter* species and strains, *Aquabacter spiritensis*, and *Azorhizobium caulinodans*. The representatives of the genera *Xanthobacter*, *Aquabacter*, and *Azorhizobium* form a distinct cluster

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Strain	Source	Nucleotide sequence accession no.
<i>Xanthobacter flavus</i> 301 ^T	J. Wiegel	X94199
<i>Xanthobacter flavus</i> JW/KR-1	J. Wiegel	X94204
<i>Xanthobacter flavus</i> H4-14	J. Wiegel	X94202
<i>Xanthobacter flavus</i> JW/KR-E1	J. Wiegel	X94206
<i>Xanthobacter flavus</i> JW/KR-2	J. Wiegel	X94205
<i>Xanthobacter agilis</i> SA35 ^T	J. Wiegel	X94198
<i>Xanthobacter autotrophicus</i> 7C ^T	J. Wiegel	X94201
<i>Xanthobacter autotrophicus</i> JW33	J. Wiegel	X94203
<i>Azorhizobium caulinodans</i> ORS571 ^T	DSMZ ^a	X94200

The three *Xanthobacter* species represent individual branches within the *Aquabacter*-*Azorhizobium*-*Xanthobacter* cluster, as do *Aquabacter spiritensis* and *Azorhizobium caulinodans*. The level of 16S rDNA sequence similarity for two strains of *X. autotrophicus* was 99.9%. The same high level of 16S rDNA sequence similarity was found with the *X. flavus* strains studied, which included marine isolate H4-14 (8) and wetland rice isolates JW/KR-1, JW/KR-2, and JW/KR-E1 (18, 19). Both the phylogenetic tree and the 16S rDNA similarity values show that in some cases the *Xanthobacter* species are

In the Ribosomal Database Project release of 17 May 1995 (11) under the section labelled RPS.VIR_SUBGROUP three environmental clone sequences are listed. The Ribosomal Database Project listing indicates that environmental clone sequence MC24 was from an Australian forest soil, while two environmental clone sequences env.PAD53 and env.PAD54 were from rice paddy field soil (11). As this study revealed that the *Xanthobacter* strains studied are most closely related to the strains in the same Ribosomal Database Project group, these environmental clone sequences were compared with the new *Xanthobacter* strain sequences determined in this study. Environmental clone sequences env.PAD53 and env.PAD54 consist of only 275 nucleotides (*Escherichia coli* positions 1115 to 1391), and a comparison showed that they are most similar to the sequences of *Rhodoplanes roseus* and *Rhodoplanes elegans* (levels of similarity, 96.9 and 96.2%, respectively) and thus do not fall in the *Aquabacter-Azorhizobium-Xanthobacter* cluster, to which they are less than 91.5% similar. The small number of nucleotides available for these environmental clone sequences did not allow an exact phylogenetic position to be determined. Clone MC24 seems to represent a chimeric structure since a comparative analysis showed that the 3' end of the sequence is similar to the 3' ends of the sequences of members of the alpha-2 subclass, while the 5' end shows little similarity to the 5' ends of the sequences of members of this group. Although *Xanthobacter* strains have been isolated from rice paddy field soil (18), the two clones recovered from paddy field soil are not representatives of the genus *Xanthobacter*.

The results of our 16S rDNA sequence analysis of the genus *Xanthobacter* are consistent with the DNA-rRNA hybridization data of Dreyfus et al. (2) and Gillis et al. (3), who showed that the three *Xanthobacter* species are closely related to *Azorhizobium caulinodans*. The relatedness of the genera *Xanthobacter*, *Aquabacter*, and *Azorhizobium* previously demonstrated by DNA-rRNA hybridization (2-4) was also confirmed by our 16S rDNA sequence analysis. It should be noted that the denaturation temperature of the hybrid of *Aquabacter spiritensis* DNA and labelled *Azorhizobium caulinodans* rRNA was 72.9°C (4), while the denaturation temperatures of the hybrid of the DNAs of the three *Xanthobacter* species and *Azorhizobium caulinodans* rRNA ranged from 77 to 78°C (2, 3). These data indicate that *Aquabacter spiritensis* is less closely related to the *Xanthobacter* species than to *Azorhizobium caulinodans*, a fact not clearly shown by the 16S rDNA sequence analysis.

Our 16S rDNA sequence comparison showed that the three *Xanthobacter* species are distinct, as was previously established on the basis of DNA-DNA hybridization data (6). Our phylogenetic analysis indicated that the members of the genera *Aquabacter*, *Azorhizobium*, *Xanthobacter* are intermixed and that there is not a clear generic cluster, especially for the *Xanthobacter* species and strains. The low bootstrap values obtained for some of the internal clusters mean that we should be cautious in interpreting the data and the phylogenetic dendrogram resulting from the data analysis. There are three ways

TABLE 2. Levels of 16S rDNA similarity between strains belonging to the *Aquabacter-Azorhizobium-Xanthobacter* cluster and related taxa

Strain	% 16S rDNA similarity											
	<i>X. flavus</i> 301 ^T	<i>X. flavus</i> JW/KR-1	<i>X. flavus</i> H4-14	<i>X. flavus</i> JW/KR-E1	<i>X. flavus</i> JW/KR-2	<i>Azorhizobium caulinodans</i> ORS571 ^T	<i>X. agilis</i> SA35 ^T	<i>X. autotrophicus</i> 7C ^T	<i>X. autotrophicus</i> JW33	<i>Aquabacter spiritensis</i> SPL-1 ^T	<i>Rhodopseudomonas viridis</i> ATCC 19567 ^T	<i>Rhodoplanes roseus</i> DSM 5909 ^T
<i>X. flavus</i> JW/KR-1	100.0											
<i>X. flavus</i> H4-14	99.9	99.9										
<i>X. flavus</i> JW/KR-E1	99.9	99.9	100.0									
<i>X. flavus</i> JW/KR-2	99.9	99.9	100.0	100.0								
<i>Azorhizobium caulinodans</i> ORS571 ^T	98.2	98.2	98.1	98.1	98.1							
<i>X. agilis</i> SA35 ^T	98.0	98.0	98.0	98.0	98.0	97.5						
<i>X. autotrophicus</i> 7C ^T	97.5	97.5	97.5	97.5	97.5	96.8	96.8					
<i>X. autotrophicus</i> JW33	97.5	97.5	97.4	97.4	97.4	96.7	96.7	99.9				
<i>Aquabacter spiritensis</i> SPL-1 ^T	97.0	97.0	96.9	96.9	96.9	96.9	97.0	97.2	97.2			
<i>Rhodopseudomonas viridis</i> ATCC 19567 ^T	92.8	92.8	92.7	92.7	92.7	93.2	92.9	92.9	92.9	93.7		
<i>Rhodoplanes roseus</i> DSM 5909 ^T	91.9	91.9	91.8	91.8	91.8	92.0	92.2	92.3	92.2	91.4	94.0	
<i>Rhodoplanes elegans</i> JCM 9224 ^T	92.0	92.0	91.9	91.9	91.9	92.4	92.1	92.4	92.4	91.9	94.0	98.1

to view or interpret this analysis. First, it is possible that while the 16S rDNA sequence data clearly indicate that the three genera are closely related, it is not possible to determine the true intergeneric relationships because of the high levels of sequence similarity, a problem which cannot be resolved by current methods of phylogenetic analysis. Second, the data could be interpreted to show that the genus *Xanthobacter* is not coherent, as previously published physiological data have suggested (22), and that each of the three *Xanthobacter* species represents an individual genus. Third, all strains within the *Aquabacter-Azorhizobium-Xanthobacter* cluster could be viewed as members of one genus, the three genera could be combined, and an emended description of the genus *Xanthobacter* could be proposed.

In systematic microbiology, it is still thought that descriptions of new taxa should be based on polyphasic data rather than just 16S rDNA sequence similarity values (13). The genera *Aquabacter*, *Azorhizobium*, and *Xanthobacter* can be clearly differentiated from each other on the basis of their current descriptions (2, 4, 22). *Aquabacter spiritensis* differs from both *Azorhizobium caulinodans* and *Xanthobacter* species in that it does not fix nitrogen, contains gas vacuoles, and produces chalky white colonies (4). The stem-nodulating ability of *Azorhizobium caulinodans* distinguishes this species from all *Xanthobacter* species (2). Only the species of the genus *Xanthobacter* have been shown to contain the water-insoluble yellow pigment zeaxanthin dirhamnoside (22), although the possible presence of this pigment in members of the other two genera has not been investigated, probably because of the lack of yellow colony pigmentation in these organisms. The data currently available for comparisons of the three *Xanthobacter* species are morphological and physiological data (22). These data do not include good differential characteristics that would aid in the description of new genera. Therefore, it would be unwise to make taxonomic rearrangements in this group on the basis of our current knowledge. Chemotaxonomic data for these genera should be obtained, and such information may support the differences observed in the phylogenetic analysis.

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