The Phylogeny of the Genera *Chryseomonas*, *Flavimonas*, and *Pseudomonas* Supports Synonymy of These Three Genera

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The 16S rRNA sequences of *Chryseomonas luteola*, the type species of the genus *Chryseomonas*, and *Flavimonas oryzihabitans*, the type species of the genus *Flavimonas*, were determined. These sequences were compared with the sequences of 27 representative strains of the genus *Pseudomonas*. *C. luteola* and *F. oryzihabitans* were located in the cluster that contains *Pseudomonas aeruginosa*, the type species of genus *Pseudomonas* Migula 1894, and the levels of 16S rRNA sequence homology between *P. aeruginosa* and the other two species were more than 93.9%. All of the strains of the genus *Pseudomonas sensu stricto* whose sequences have been determined were included in the *P. aeruginosa* cluster. These results suggested that *Chryseomonas*, *Flavimonas*, and *Pseudomonas* are synonymous, and we concluded that *Chryseomonas* and *Flavimonas* are junior subjective synonyms of *Pseudomonas*.

Chryseomonas polytricha was established by Holmes et al. in 1986 for members of group Ve-2 (3). However, *C. polytricha* appeared to be a junior subjective synonym of *Pseudomonas luteola* Kodama et al. 1985 (6), and in 1987 Holmes et al. established a new combination, *Chryseomonas luteola*, because of this synonymous taxonomic status (4). *Pseudomonas oryzihabitans* was established by Kodama et al. in 1985 (6). Holmes et al. proposed that *P. oryzihabitans* should be transferred to a new genus, the genus *Flavimonas*, in 1987 and established a new combination, *Flavimonas oryzihabitans* (4). Holmes et al. recognized that the genera *Chryseomonas* and *Flavimonas* are close relatives of the genus *Pseudomonas*. The establishment of the genera *Chryseomonas* and *Flavimonas* by Holmes et al. in 1987 was grounded on low levels of DNA-DNA homology between *Chryseomonas* and *Flavimonas* strains and *Pseudomonas* strains. However, DNA-DNA homology has been used to distinguish bacterial species and has never been powerful enough to reveal phylogenetic relationships. In recent years, it has been recognized that phylogenetic analysis based on 16S rRNA sequences is necessary to draw boundaries between bacterial taxa.

In the present study the phylogenetic relationships among the genera *Pseudomonas*, *Chryseomonas*, and *Flavimonas* were revealed based on the almost complete 16S rRNA sequences of 24 selected *Pseudomonas* strains and the type strains of *C. luteola* (the type species of the genus *Chryseomonas*) and *F. oryzihabitans* (the type species of the genus *Flavimonas*). Based on the phylogenetic analysis, we concluded that *Chryseomonas* and *Flavimonas* are junior subjective synonyms of *Pseudomonas*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are shown in Table 1. For the sequencing study, these strains were cultured in nutrient broth (Difco) for 18 to 24 h at 30°C.

Sequencing of 16S rRNA. Total DNA was extracted by the phenol method from cells cultured in liquid medium by using sodium dodecyl sulfate followed by RNase treatment. The 16S rRNA-encoding region of the DNA was amplified from the total DNA by using *Taq* DNA polymerase (Boehringer, Mannheim, Germany) and two primers that attached to positions 10 to 25 (5'-TCGAATT

CGGATCCAGTTTGATCCTGGCTC-OH-3') and positions 1541 to 1525 (5'-TCGAATTCGGATCCAAGGAGGTGATCCAGCC-OH-3') (*Escherichia coli* numbering) and had *Eco*RI and *Bam*HI restriction sites in their 5'-terminal sequences. The 16S ribosomal DNA (rDNA) sequences were determined by two methods, the cloning method and the direct sequencing method. In the cloning method, after digestion with a restriction enzyme, amplified DNA was purified by electrophoresis on 1.0% agarose gels and was isolated from the agarose gels with GELase (Epicentre Technologies Co., Madison, Wis.). Isolated rDNA was ligated into M13mp18 or mp19 DNA, and ligated plasmids were then transformed into *E. coli* JM109. Then single- or double-stranded plasmid DNAs extracted from the transformants were sequenced with a cycle sequencing system (Dye Primer cycle sequencing kit; Applied Biosystems, Inc., Foster City, Calif.) as specified by the manufacturer. Direct sequencing of amplified 16S rDNA was carried out as follows. Amplified DNA was purified by passing it twice through a MicroSpin S-400 HR column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and the resulting DNA was used as a template. Sequencing gel electrophoresis was performed and nucleotide sequences were automatically obtained by using a model 373A or 310 DNA sequencer (Applied Biosystems, Inc.) and the protocol and software recommended by the manufacturer. Dye-labeled primers M13RP1 and -21M13 were purchased from Applied Biosystems, Inc., and the following dye-labeled primers, with five nucleotides (5'-CAGGA-OH-3') added to the 5' ends of the oligonucleotides so that they were suitable for the autosequencer system, were obtained from Takara Shuzo Co., Kyoto, Japan: 520F (5'-CAGGAGTGCCAGCAGCCGCGG-OH-3'; same as positions 515 to 530), 520R (5'-CAGGAACCGCGGCTGCTGGC-OH-3'; complementary to positions 531 to 517), 800F (5'-CAGGAATTAGATACCTGGTA-OH-3'; same as positions 787 to 802), 800R (5'-CAGGACTACCAGGGTATCTAAT OH-3'; complementary to positions 803 to 787), 1100F (5'-CAGGAGCAACG AGCGCAACCC-OH-3'; same as positions 1099 to 1114), and 1100R (5'-CAG GAAGGGTTGCGCTCGTTG-OH-3'; complementary to positions 1115 to 1100). The sequencing method used for each organism is indicated in Table 1.

Phylogenetic analysis. The sequences of the 16S rRNAs of *E. coli* (accession number V00348), *Pseudomonas aeruginosa* DSM 50071^T (T = type strain) (X06684), *Pseudomonas fluorescens* NCPPB 3063^T (U01916), and *Pseudomonas mendocina* ATCC 25411^T (M59154) were obtained from the EMBL database for comparison. The genetic distances between sequences were estimated by using K_{nuc} values (5). Then a phylogenetic tree was constructed by the neighbor-joining method (12), an evaluation of the tree was carried out by using the bootstrap method and the Clustal V program, and a total of 1,000 bootstrapped trees were generated (1, 2). Deleted and unknown positions were eliminated for the comparison of sequences. Positions (*E. coli* numbering) 70 to 100, 181 to 219, 447 to 487, 1004 to 1036, 1133 to 1141, and 1446 to 1456 were eliminated from the comparison because the secondary structures of these regions differed between strains.

GenBank, EMBL, and DDBJ accession numbers. The 16S rRNA gene sequences which we determined have been deposited in the DDBJ (DNA Data Base of Japan, Mishima, Japan), and these sequences are available from GenBank, EMBL, and DDBJ under the accession numbers shown in Table 1.

RESULTS AND DISCUSSION

The genus *Pseudomonas* Migula 1894 was described so that it included polarly flagellated strictly aerobic rods with a respi-

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TABLE 1. Bacterial strains examined and their 16S rRNA accession numbers and the sequencing methods used

Species	Strain ^a	Accession no.	Method used for sequencing
<i>Chryseomonas luteola</i>	IAM 13000 ^T	D84002	Cloning
<i>Chryseomonas luteola</i>	ATCC 43330	D84003	Direct
<i>Flavimonas oryzae</i>	IAM 1568 ^T	D84004	Cloning
<i>Pseudomonas agarici</i>	ATCC 25941 ^T	D84005	Direct
<i>Pseudomonas alcaligenes</i>	IAM 12411 ^T	D84006	Direct
<i>Pseudomonas amygdali</i>	ATCC 33614 ^T	D84007	Direct
<i>Pseudomonas aureofaciens</i>	IAM 12353 ^T	D84008	Direct
<i>Pseudomonas azotoformans</i>	IAM 1603 ^T	D84009	Cloning
<i>Pseudomonas caricapapayae</i>	ATCC 33615 ^T	D84010	Direct
<i>Pseudomonas chlororaphis</i>	IAM 12354 ^T	D84011	Direct
<i>Pseudomonas corrugata</i>	ATCC 29736 ^T	D84012	Direct
<i>Pseudomonas fluorescens</i>	IAM 12022 ^T	D84013	Cloning
<i>Pseudomonas fragi</i>	IAM 12402	D84014	Cloning
<i>Pseudomonas fulva</i>	IAM 1529 ^T	D84015	Cloning
<i>Pseudomonas mendocina</i>	NCIB 10541	D84016	Cloning
<i>Pseudomonas mucidolens</i>	IAM 12406 ^T	D84017	Cloning
<i>Pseudomonas nitroreducens</i>	IAM 1439 ^T	D84021	Direct
<i>Pseudomonas nitroreducens</i> subsp. <i>thermotolerans</i>	IAM 12401	D84022	Cloning
<i>Pseudomonas oleovorans</i>	IAM 1508 ^T	D84018	Cloning
" <i>Pseudomonas pavonaceae</i> "	IAM 1155	D84019	Cloning
<i>Pseudomonas putida</i>	IAM 1236 ^T	D84020	Cloning
<i>Pseudomonas straminea</i>	IAM 1598 ^T	D84023	Cloning
<i>Pseudomonas stutzeri</i>	IAM 12668 ^T	D84024	Direct
<i>Pseudomonas synxantha</i>	IAM 12356 ^T	D84025	Cloning
<i>Pseudomonas syringae</i>	ATCC 19310 ^T	D84026	Direct
<i>Pseudomonas taetrolens</i>	IAM 1653 ^T	D84027	Cloning
<i>Pseudomonas tolaasii</i>	ATCC 33618 ^T	D84028	Direct

^a IAM, Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Rockville, Md.; NCIB, National Collection of Industrial Bacteria, Aberdeen, United Kingdom.

ratory type of metabolism in which oxygen is used. Defined in this way, the genus was very heterogeneous, and several subgroups of species have been recognized, including five subgroups based on rRNA homology data (rRNA groups I to V) (9) and groups of species based on cellular fatty acid compositions and quinone systems (groups 1 to 9) (8). There was a good correlation between rRNA groups I to V and groups 1 to 5 based on cellular fatty acids and quinone systems. In the past two decades *Pseudomonas* species belonging to rRNA groups II to V or fatty acid and quinone groups 2 to 9 have been transferred to other genera (10, 13, 14, 16, 17, 19, 20). At this time, the genus *Pseudomonas* is restricted to Palleroni rRNA group I, and only the group I strains belong to the genus *Pseudomonas* sensu stricto. Therefore, in the present study only the strains belonging to Palleroni rRNA group I were used for phylogenetic analyses.

Several subgroups could be differentiated in Palleroni rRNA group I. *P. aeruginosa* and *Pseudomonas fluorescens* were placed in different clusters as a result of oligonucleotide cataloging of 16S rRNA (18). Based on the results of rRNA-DNA hybridization, rRNA group I was divided into three groups, whose representative species were *P. aeruginosa*, *P. fluorescens*, and *Pseudomonas syringae* (9). In this study, the almost complete 16S rRNA sequences of 27 strains of the genus *Pseudomonas* were determined, and a phylogenetic analysis was carried out. A phylogenetic tree was drawn on the basis of these new sequences and four sequences obtained from the database (Fig. 1). The total number of nucleotides compared was 1,073. This tree had two main clusters, although the bootstrap values

for two branches were not high enough (66 and 67%) to give a high level of confidence. The first cluster contained 16 strains and included *P. aeruginosa* DSM 50071^T, *Pseudomonas alcaligenes* IAM 12411^T, *P. mendocina* ATCC 25411^T and NCIB 10541, *Pseudomonas stutzeri* IAM 12668^T, and *Pseudomonas putida* IAM 1236^T; all of these strains except *P. putida* IAM 1236^T belonged to the *P. aeruginosa* subgroup of Palleroni rRNA group I. The second cluster contained 14 strains and included *Pseudomonas agarici* ATCC 25941^T, *Pseudomonas chlororaphis* IAM 12354^T, *Pseudomonas aureofaciens* IAM 12353^T, *P. syringae* ATCC 19310^T, and *P. fluorescens* IAM 12022^T, which belonged to the *P. fluorescens* or *P. syringae* subgroup of Palleroni rRNA group I. The results of this phylogenetic analysis based on 16S rRNA sequences were compared with the results obtained by oligonucleotide cataloging of 16S rRNA and the rRNA-DNA hybridization studies.

In this study we determined the 16S rDNA sequence of "*Pseudomonas pavonaceae*" IAM 1155, and this strain was included on the branch that contained the *Pseudomonas* sensu stricto group. However, Van Landschoot et al. (15) reported that "*P. pavonaceae*" NCTC 10693 belongs on the *Acinetobacter* rRNA branch. The genus *Acinetobacter* is a genus of the family *Moraxellaceae* (11), and the *Acinetobacter* rRNA branch has been reported to be far from the *P. fluorescens* complex branch (11, 15). Strain IAM 1155 was received from H. Takahashi as ATU 224 (Laboratory of Fermentation and Microbiology, Faculty of Agriculture, University of Tokyo, Tokyo, Japan) in 1964. The history of the isolation of strain IAM 1155

FIG. 1. Phylogenetic tree for strains of the genus *Pseudomonas* derived from 16S rDNA sequence similarities. The value on each branch is the estimated confidence limit (expressed as a percentage) for the position of the branch as determined by bootstrap analysis. *E. coli* was used as the root organism.

was not available, but this strain was apparently isolated in the Laboratory of Fermentation and Microbiology, Faculty of Agriculture, University of Tokyo. In contrast, NCTC 10693 is the strain which was originally characterized as "*P. pavonaceae*" by Levine and Soppeland (7). Therefore, the difference between our phylogenetic analysis results and the results reported by Van Landschoot et al. (15) can be attributed to the difference in the strains used.

C. luteola (4) and *F. oryzihabitans* (4) were originally described as *P. luteola* and *P. oryzihabitans*, and the type strains were isolated from a human clinical specimen and a rice paddy, respectively (6). The transfers of *P. luteola* and *P. oryzihabitans* from the genus *Pseudomonas* were grounded on low levels of DNA-DNA hybridization (1 to 5%) with the other *Pseudomonas* species (4), although the physiological and chemotaxonomic characteristics of these organisms were similar to those of the genus *Pseudomonas*. In our 16S rRNA sequence analysis, *C. luteola* IAM 13000^T and ATCC 43330 and *F. oryzihabitans* IAM 1568^T were found in the *P. aeruginosa* cluster (Fig. 1). The levels of homology between *C. luteola* IAM 13000^T and *P. aeruginosa* DSM 50071^T and between *F. oryzihabitans* IAM 1568^T and *P. aeruginosa* DSM 50071^T (*P. aeruginosa* is type species of the genus *Pseudomonas*) were 94.0 and 93.9%, respectively. In contrast, the levels of homology between *P. fluorescens* IAM 12022^T and *P. aeruginosa* DSM 50071^T and between *P. syringae* ATCC 19310^T and *P. aeruginosa* DSM 50071^T were 92.8 and 93.1%, respectively. Thus, according to our phylogenetic analysis *C. luteola* and *F. oryzihabitans* are more closely related to *P. aeruginosa*, the type species of the genus *Pseudomonas*, than *P. fluorescens* and *P. syringae* are. Therefore, if *P. fluorescens* and *P. syringae* are included in the genus *Pseudomonas*, it is not reasonable to exclude *P. luteola* and *P. oryzihabitans* from the genus *Pseudomonas*. Consequently, we conclude that *Chryseomonas* and *Flavimonas* are junior subjective synonyms of *Pseudomonas* and that the names *P. luteola* and *P. oryzihabitans* should be used.

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