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Genotypic Characterization and Phylogenetic Relations of *Pseudomonas* sp. (Formerly *P. stutzeri*) OX1

Francesca Radice,[1] Viviana Orlandi,[1] Valentina Massa,[1] Lucia Cavalca,[2] Antonella Demarta,[3] Thomas K. Wood,[4] Paola Barbieri[1]

[1]Dipartimento di Biologia Strutturale e Funzionale, Università dell'Insubria, via Dunant 3, 21100 Varese, Italy
[2]Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, Università degli Studi di Milano, via Celoria 2, 20133 Milano, Italy
[3]Istituto Cantonale di Microbiologia, via Mirasole 22A, 6500 Bellinzona, Switzerland
[4]Departments of Chemical Engineering and Molecular and Cell Biology, University of Connecticut, 191 Auditorium Road, U-3222, Storrs, CT 06269-3222, USA

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Abstract. *Pseudomonas* sp. OX1, an aromatic compound-degrading bacterium that was tentatively identified by conventional biochemical methods as *P. stutzeri*, has now been investigated at the molecular level to clarify its taxonomic position. Amplified ribosomal DNA restriction analysis and multiple enzyme restriction fragment length polymorphism (MERFLP) analysis suggested that *Pseudomonas* sp. OX1 could not be classified as *P. stutzeri*. Phylogenetic analyses based on 16S rRNA and *gyr*B genes further confirmed that this strain belongs to the *Pseudomonas* (sensu stricto) genus, but not to the *stutzeri* species. The data obtained demonstrated that *Pseudomonas* sp. OX1 belongs to intrageneric cluster II and is related to the *P. fluorescens–P. syringae* complex.

Bacteria belonging to the genus *Pseudomonas* are widely distributed motile rod-shaped gamma-Proteobacteria, and they have functions of ecologic, economic, and health-related importance. Some species or strains are relevant for their metabolic versatility, which makes them attractive for bioremediation purposes or as a source of new enzymes for biocatalysis and bioconversions [9].

Members of the genus *Pseudomonas* display a great phenotypic diversity and, since the first description by Migula in 1894 [14], the genus underwent repeated taxonomic revisions. After a first revision based on DNA–rRNA reassociation data [13], molecular approaches based on phylogenetic analysis of 16S rRNA sequence data led to the decrease of the high degree of heterogeneity that characterized the old genus of *Pseudomonas* by relocating some species within a group referred to as *Pseudomonas* sensu stricto and placing some distantly related species into new genera of the alpha-, beta-, and gamma-Proteobacteria [6, 12, 23].

Correspondence to: P. Barbieri; *email:* paola.barbieri@uninsubria.it

Within the *Pseudomonas* (sensu stricto), both Moore [12] and Anzai [2] identified two intrageneric clusters (IGs): IG-I, comprising *aeruginosa, resinovorans, mendocina*, and *flavescens* lineages, and IG-II, comprising the *fluorescens, chlororaphis, putida*, and *syringae* lineages. There was a discrepancy regarding the location of the *stutzeri* lineage, which was placed in IG-I and IG-II, respectively.

Analyses of *gyr*B and *rpo*D sequence data confirmed subdivision into two intrageneric clusters: intrageneric cluster I, comprising the *aeruginosa* and *stutzeri* complexes, and intrageneric cluster II, comprising the *putida, syringae*, and *fluorescens* complexes [25]. A recent report based on the combined analysis of *atp*DA, *car*A, *rec*A, and 16S rRNA genes further confirmed the subdivision into the *aeruginosa* and the *fluorescens* intrageneric clusters [10]. The two intrageneric clusters proposed were also well supported by chemotaxonomic analysis of fatty-acid methyl ester and phospholipid fatty-acid profiles [21].

The high degree of heterogeneity because of the extreme phenotypic diversity of *Pseudomonas* is also

reflected at the species level: Several studies have demonstrated that *P. stutzeri* consists of a complex collection of strains that might be placed in more than one species [17, 19].

Pseudomonas sp. OX1 was isolated from the sludge of a wastewater treatment plant for its ability to use *o*-xylene as sole carbon and energy source and tentatively classified as *P. stutzeri* by conventional biochemical methods [3]. However, although previously published studies referred to it as *P. stutzeri* [4, 5, 18, 20], no exhaustive studies have been performed to elucidate its taxonomic status. In this article, we report molecular data suggesting that *Pseudomonas* sp. OX1 is related to members of the *P. fluorescens–P. syringae* complex rather than to *P. stutzeri*.

Materials and Methods

Bacterial strains and growth media. *Pseudomonas* sp. OX1 (American Type Culture Collection (ATCC) no. BAA-172) was grown on minimal medium M9 [7] supplemented with *o*-xylene as sole carbon and energy source. The other *Pseudomonas* strains used for amplified ribosomal DNA restriction analysis (ARDRA) and multiple enzyme restriction fragment length polymorphism (MERFLP) were grown aerobically in liquid and/or solid Luria Bertani medium at 30°C. The collection number of the *Pseudomonas* strains used in this study, together with the 16S rDNA and *gyr*B sequence accession numbers, are reported in the Figs. 1 and 2.

16S rRNA gene restriction analysis. The template DNAs were extracted using the hexadecyltrimethylammonium bromide (CTAB) Miniprep protocol [22]. 16S rDNA amplicons were obtained as described later, purified with the QIA-quick PCR Purification Kit (Qiagen), and, for ARDRA analyses, digested with the enzymes HpaII, AluI, and RsaI. The restriction fragments were separated by agarose gel electophoresis (3.0% [w/v]; Sigma) at 7 V cm^{-1}.

For MERFLP analysis, 40 μL sample 16S rRNA gene PCR product, 5 μL SuRE/Cut buffer A, and 5 U enzymes AluI, HinfI, RsaI, and Tru9I (Roche Molecular Biochemicals) were combined in one reaction tube and incubated for at least 12 hours at 37°C. Digestion products were electrophoresed through 3% (w/v) MetaPhor agarose (FMC Bioproducts) gel with ethidium bromide for 3 hours at 75 V (5 V cm^{-1}).

After ethidium bromide staining, the gels were visualized under ultraviolet light (260 nm) and analyzed using the Kodak ID Image Analysis Software. The restriction patterns were used for cluster analysis: A binary matrix was constructed that included all of the fragments >80 bp and assigning a 0/1 value, respectively, to the absence or presence of each fragment in the restriction patterns. Fragments <80 bp were not included in the numeric analysis because of the possible interference of residual primers and primer dimers. A phylogenetic tree was obtained using the program FreeTree [15], which converts the matrix data in distance values by applying the unweighted pair group method with arithmetic mean (UPGMA) algorithm.

16S rDNA and *gyr*B sequence analyses. For 16S rDNA amplification, *Pseudomonas* sp. OX1 chromosomal DNA was run in triplicate through polymerase chain reaction (PCR) using two sets of universal primers: HK12 (5′-GAGTTTGATCCTGGCTCAG)/HK13 (5′-TACCTTGTTACGACTT) and JCR14 (5′-ACGGGCGGTGTGTAC)/JCR15(5′-GCCAGCAGCCGCGGTA). The HK12/HK13 primers amplify the entire 16S region, whereas the JCR14/JCR15 primers amplify an internal region of the 16S. The PCR products were purified using the QIA-Quick PCR Purification Kit (Qiagen) and sequenced twice in each direction using an automated Applied Biosystem ABI Prism® sequencer. These four sequences were aligned using ClustalW multiple sequence alignment software (http://www.ebi.ac.uk/clustalw) to obtain the consensus of the *Pseudomonas* sp. OX1 16S rDNA, which received European Molecular Biology Laboratory (EMBL) accession no. AJ920030.

PCR amplification and sequencing of *gyr*B gene was performed according to the method described by Yamamoto and Harayama [24]. The 3′ regions of primers UP-1 and UP-2r, used for amplification, are degenerate sequences allowing amplification of *gyr*B from a large spectrum of bacteria, whereas the 5′ regions unique sequence are used as tag sequences for sequencing with primers UP-1S and UP-2Sr [24]. The partial *gyr*B sequence of *Pseudomonas* sp. OX1 (850-bp long from position 355 to 1205 in *Escherichia coli* K12) was assigned EMBL accession no. AJ920031.

rDNA and *gyr*B sequences of the type strains analyzed in this study, as well as of some other *Pseudomonas* strains, were retrieved from the GenBank database. The phylogenetic and molecular evolutionary analyses of 16S rDNA and *gyr*B nucleotide sequences were conducted using MEGA version 3.0 [11]. Phylogenetic trees were constructed using the neighbor-joining method, with genetic distances computed by employing Kimura's 2-parameter distance. The reliability of the inferred trees was established by the Felsenstein bootstrap (1000 replicates) test [8].

Results and Discussion

16S rDNA restriction analysis. During the course of ARDRA analyses aimed at discriminating a number of *Pseudomonas* isolates, it was observed that the *Pseudomonas* sp. OX1 16S rDNA restriction patterns clearly differed from those of the *P. stutzeri* DSMZ 5190^T–type strain.

MERFLP analysis, a newly described approach for high-resolution distinction of *Pseudomonas* 16S rRNA gene [16] that exploits multiple digestions of the 16S rDNA amplicons, was then applied to compare *Pseudomonas* sp. OX1 with nine *Pseudomonas*-type strains available in our laboratory. The data from multiple restrictions were cluster analyzed, and the resulting dendrogram (not shown) revealed that *Pseudomonas* sp. OX1 grouped with *P. cichorii* DSMZ 50259^T, *P. fluorescens* DSMZ 50090^T, *P. tolaasii* LMG 2342^T, *P. aeruginosa* DSMZ 50071^T, *P. alcaligenes* DSMZ 50342^T, and *P. oleovorans* DSMZ 1045^T but not with *P. mendocina* DSMZ 50017^T, *P. putida* DSMZ 291^T, and *P. stutzeri* DSMZ 5190^T.

These data revealed a taxonomic distance between *Pseudomonas* sp. OX1 and *P. stutzeri*, suggesting that *Pseudomonas* sp. OX1 could be located in the intrageneric cluster II and not in the intrageneric cluster I that comprises the *P. stutzeri* complex [26], prompting us to further investigate its taxonomic position and phylogenetic relations.

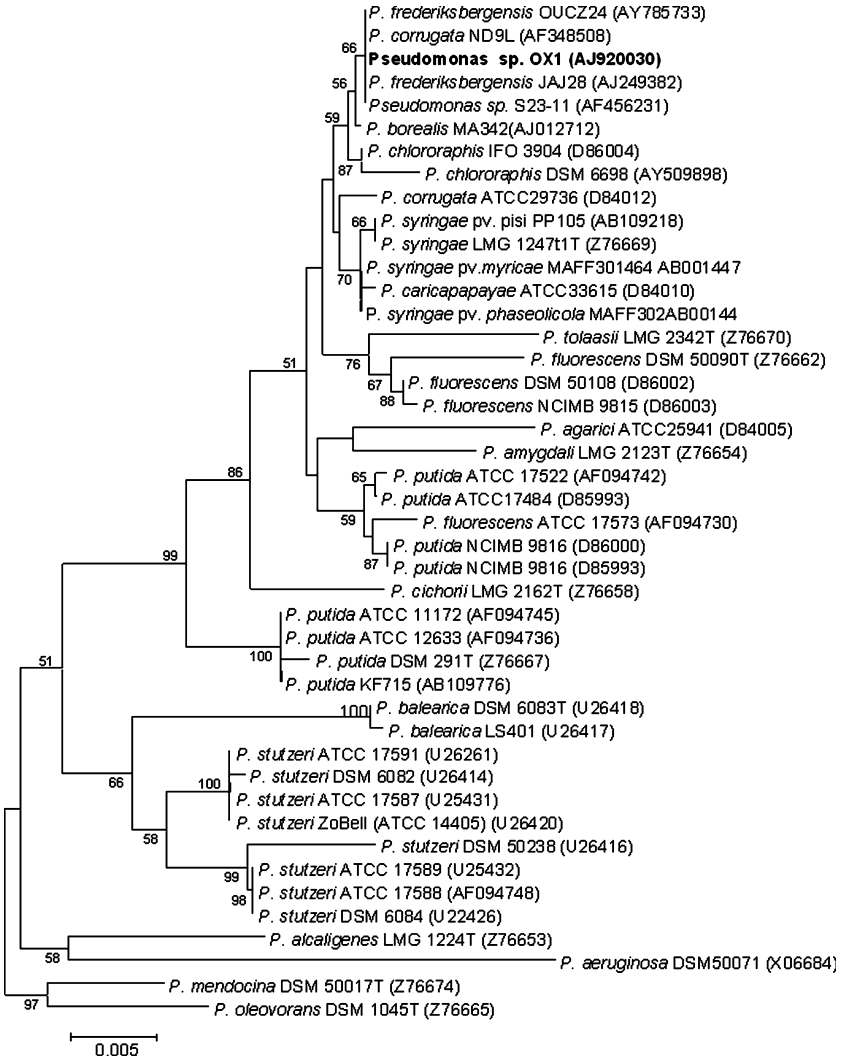

Fig. 1. Phylogenetic relations of *Pseudomonas* sp. OX1 on the basis of 16S rDNA sequences excluding the variable regions V1 and V8 (1176 bp). The tree was obtained using the Kimura-2-parameter distance and neighbor-joining methods. Numbers indicate percentage bootstrap values of 1000 replicates >50%. Scale bar = genetic distance of 0.005. Brackets = sequence accession numbers.

16S rRNA and *gyr*B gene sequencing and analysis. Sequence analysis of the small ribosomal RNA subunit gene (16S rDNA) is generally considered a valuable tool for assigning bacterial strains to a genus or species. To study the phylogenetic position of *Pseudomonas* sp. OX1 among the genus *Pseudomonas*, its 16S rDNA sequence was compared with the sequences of both of the *Pseudomonas*-type strains used for MERFLP analyses and several other *Pseudomonas* strains retrieved from BLAST search. According to Yamamoto et al. [26], in the phylogenetic tree based on the nearly complete 16S rDNA sequences (not shown), the division in IG-I and IG-II was clearly distinguishable; *Pseudomonas* sp. OX1 distinctly grouped with the species belonging to IG-II. These results were confirmed by the analysis performed, which excluded the sequences of the variable regions, V1 and V8 (Fig. 1), which has been suggested as a way to improve the reliability of the 16S rDNA analysis in *Pseudomonas* [25]. The strains identical or closest to *Pseudomonas* sp. OX1 were a strain isolated from cotton roots (*Pseudomonas* sp. S23-11), *P. borealis* MA342, whose taxonomic name, to the best of our knowledge, was not validly published, *P. corrugata* ND9L, and the recently described new species, *P. frederiksbergensis* [1]. This group of strains was related to *P. chlororaphis* sequences and to the *P. syringae* complex; however, the bootstrap analysis did not support the differentiation between the two. It is interesting to note that most of these strains, with the exception of *P. frederiksbergensis*, which was isolated from soil at a coal gasification site for polyaromatic hydrocarbon–degrading ability, were associated with plants. The nearly complete 16S rDNA sequence (1436 bp) of the strains included in the group of *Pseudomonas* sp. OX1 showed 99.89% similarity, those included in the *P. chlororaphis* group shared 98.82% similarity, and

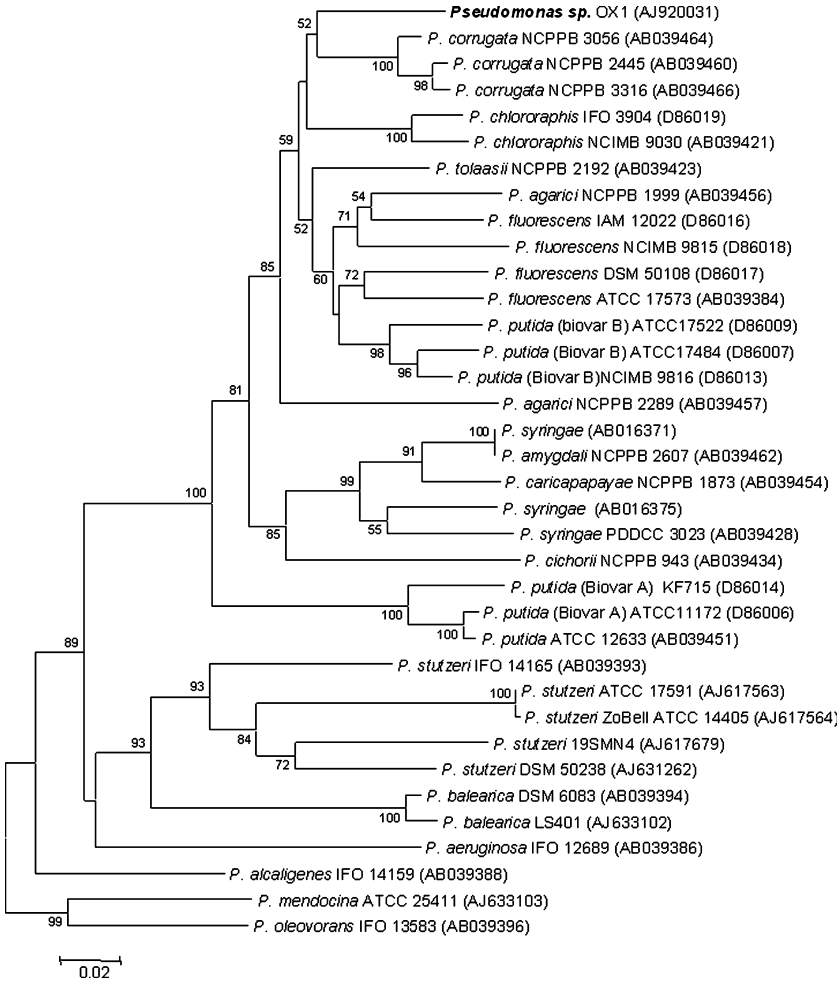

Fig. 2. Phylogenetic relations of *Pseudomonas* sp. OX1 based on *gyr*B sequences (579 bp). The tree was obtained using the Kimura-2-parameter distance and neighbor-joining methods. Numbers indicate percentage bootstrap values of 1000 replicates >50%. Scale bar = genetic distance of 0.02. Brackets = sequence accession numbers.

those of the *P. syringae* group showed 99.51% similarity. A mean of 10.2 different nucleotides differentiated *Pseudomonas* sp. OX1 group from the *syringae* group, whereas 22.82 positions on average differentiated it from the *chlororaphis* cluster.

To strengthen the phylogenetic analysis, partial *gyr*B sequence of *Pseudomonas* sp. OX1 was obtained and used to confirm the delineation of its phylogenetic position in the genus *Pseudomonas*. *gyr*B genes, which encode the subunit B proteins of DNA gyrase (topoisomerase type II), were in fact demonstrated to be suitable for bacterial phylogeny and have already been used to study the intrageneric structure of the genus *Pseudomonas* [26]. Where available, partial *gyr*B sequences of the same strains used for the 16S rRNA gene sequence analysis were retrieved from GenBank and used to draw a phylogenetic tree (Fig. 2) that confirmed the affiliation of *Pseudomonas* sp. OX1 to IG-II. Moreover, *gyr*B sequence analysis allowed for good differentiation between members of the *P. syringae* complex and those of the *P. fluorescens* complex. *Pseudomonas* sp. OX1 represented a unique branch that grouped with *P. agarici, P. putida, P. fluorescens, P. tolasii, P. chlororaphis*, and *P. corrugata* and formed with the latter two a subgroup supported by a bootstrap value of 59%. Unfortunately, *gyr*B sequences of the strains that showed identity in 16S rDNA were not available.

The partial amino acid sequence derived from the nucleotide sequences demonstrated that GyrB of *Pseudomonas* sp. OX1 is similar to that of *P. chlororaphis* and *P. corrugata*. Over a stretch of 195 amino acids, the difference between the sequence of *Pseudomonas* sp. OX1 and those of the strains belonging to the *P. syringae* group amounted to 10 to 12 amino acids, whereas only two positions diverged from those of *P. corrugata* and *P. chlororaphis*. The position 142 (with respect to the *E. coli* ATCC 8735 GyrB sequence) was found to be Asp in *Pseudomonas* sp. OX1 and Glu in the other two species. Moreover, the Val in position

140 in *Pseudomonas* sp. OX1 was replaced by Ile in *P. corrugata* and the Leu in position 164 by Gln in *P. chlororaphis*. However, these changes have no effects on the calculated charge or the theoretical isoelectric point value of the partial proteins.

In conclusion, although the polyphasic taxonomic investigation based on different genotypic methods did not allow assignment of *Pseudomonas* sp. OX1 to a listed species, it clearly demonstrated that this strain does not belong to the species *P. stutzeri* despite its previous classification obtained with conventional biochemical methods [3]. All of the analyses carried out consistently showed that *Pseudomonas* sp. OX1 would be better placed in the IG-II group, within a subgroup that includes *chlororaphis, corrugata*, and related species. Further investigations on additional phenotypic features and genomic DNA–DNA similarity are needed to establish whether or not *Pseudomonas* sp. OX1 could represent a new species.

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