Identification of Xenobiotic-Degrading Isolates from the Beta Subclass of the *Proteobacteria* by a Polyphasic Approach Including 16S rRNA Partial Sequencing

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Nineteen gram-negative, aerobic, biodegradative isolates were identified by using a polyphasic taxonomic approach. The presence of the specific polyamine 2-hydroxyputrescine and the presence of a ubiquinone with eight isoprenoid units in the side chain (ubiquinone Q-8) allowed allocation of these organisms to the beta subclass of the *Proteobacteria*. On the basis of the results of additional characterization experiments (i.e., API 20NE tests, determinations of soluble protein patterns, and DNA-DNA hybridization experiments), we classified six isolates as either *Comamonas testosteroni*, *Comamonas acidovorans*, or *Alcaligenes xylooxidans* subsp. *denitrificans*. By using the same criteria we allocated two additional isolates to the genus *Alcaligenes*. A comparison of a 16S rRNA fragment (positions 1220 to 1377; *Escherichia coli* nomenclature) indicated that the remaining isolates should be allocated as follows: one is a member of *C. testosteroni* and one is a member of *Acidovorax facilis*, as confirmed by the results of additional DNA-DNA hybridizations; two others probably belong to the family *Alcaligenaceae*; six are related to "*Alcaligenes eutrophus*"; and one, strain NRRL 12228, occupies an isolated position.

Because of growing interest in environmental pollution, numerous biodegradative bacteria have been isolated in the past, and their physiology, biochemistry, and genetics have been intensively studied. However, the taxonomy of these organisms has been neglected despite their growing importance, e.g., for ecological studies on the fate of biodegradable strains introduced into sites for bioremediation. For studies on the genetics of biodegradation it is also essential to know the relationships of the organisms being investigated to the organisms described previously. Gram-negative, aerobic, rod-shaped bacteria which have been assigned to the pseudomonad group are very prominent because of their ability to degrade substituted aromatic and other xenobiotic compounds (8, 55). However, bacteria belonging to other genera can compete with these organisms in efficiency (7, 24, 25, 31, 45, 46). In addition, reassessment of the bacterial classification system based on the availability of phylogenetic data has led to the reduction of the very heterogeneous genus *Pseudomonas* to its phylogenetic nucleus, mainly on the basis of the research conducted in the laboratory of J. De Ley (14, 17, 18, 20), with the consequent creation of new genera in the beta subclass of the *Proteobacteria* (19, 65, 66). Irrespective of these new developments, little work has been carried out on the identification of xenobiotic compound-degrading bacteria (1, 34, 44), and the taxonomic characterization of these organisms is at best restricted to some phenotypic data, which are often evaluated by using commercial multiple-test systems (70). However, in contrast to their wide application in clinical microbiology, multiple-test systems are of limited value for the identification of biodegradative bacteria, especially isolates that are members of the beta subclass of the *Proteobacteria* (6a, 21a). Furthermore,

although there has recently been excitement over gene probes for infectious diseases, there is a great lag in the development of diagnostic rRNA probes as they are, at present, not commercially available for biodegradative isolates (39, 58).

A number of gram-negative, aerobic bacteria that degrade xenobiotic compounds were received from different laboratories as pseudomonads. As only one-half of these bacteria (8) were allocated to the phylogenetically defined genus *Pseudomonas* (17), 19 isolates had to be subjected to additional tests for identification. Recently, a polyphasic approach was recommended by the Ad Hoc Committee on Approaches to Taxonomy within the *Proteobacteria* (43). In this paper we describe a polyphasic study that included chemotaxonomy and arbitration of data obtained from partial sequencing of 16S rRNA by DNA-DNA hybridization. An rRNA approach was necessary because we recognized that some of the xenobiotic compound-degrading isolates could not be allocated to previously described species and were very heterogeneous. For this purpose we preferred sequencing of one rRNA fragment as an essential step for identification instead of rRNA-DNA hybridization. The latter method would have required the preparation of several radioactively labeled reference rRNAs.

MATERIALS AND METHODS

Organisms and growth conditions. The isolates which we used in this study are listed in Table 1. Cells were grown in PYES medium (8). The biomass used for rRNA extraction was grown on the same medium except that the medium contained Polypeptone (Wako Pure Chemicals, Ltd., Osaka, Japan) instead of meat peptone.

Nutritional properties and biochemical characteristics. The biochemical characteristics (as determined by API 20NE tests) were examined and evaluated by following the instructions of the manufacturer (API bioMérieux GmbH, Nürtingen, Germany).

Chemotaxonomic methods. Analysis of quinones, analysis of polyamines, and sodium dodecyl sulfate-polyacrylamide

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TABLE 1. G+C contents and identities of the biodegradative isolates as determined by API ZONE tests

Strain	Reference ^a	Component(s) degraded	G+C content (mol%) ^b	Identification (% probability) ^c
<i>Pseudomonas</i> sp. strain D7-3 ^d	56	3-Chlorobenzoate, 3-methylbenzoate, 2-methyl-2-en-lactone ^e	65.1 ± 0.3	CDC group IVc-2 (94.4)
<i>Pseudomonas</i> sp. strain D7-4 ^d	56	3-Chlorobenzoate, 3-methylbenzoate, 2-methyl-2-en-lactone	63.9 ± 0.2	CDC group IVc-2 (94.4)
A7-2 ^d		Chlorobenzoate, chlorophenols	63.3 ± 0.7	CDC group IVc-2 (94.4)
PSB 1 ^f	59	4-Sulfobenzoate	66.4 ± 0.3	CDC group IVc-2 (91.4)
KTW11 ^d	56	2-Methyl-2-en-lactone	64.0 ± 0.3	" <i>P. diminuta</i> " (47.4) or <i>Moraxella</i> spp. (39.4)
KTW13 ^d	56	2-Methyl-2-en-lactone	65.4 ± 0.2	" <i>P. diminuta</i> " (47.4) or <i>Moraxella</i> spp. (39.4)
A 3-C ^d	4	Naphthylene sulfonic acid	65.9 ± 0.2	<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> or <i>C. acidovorans</i> (19.9)
26-8/1 ^d	6	2,6-Dinitrophenol	64.0 ± 0.6	<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> (69.9) or <i>C. acidovorans</i> (19.9)
P-3 ^f	59	Phenol sulfonic acid	62.1 ± 0.3	<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> (54.8) or <i>C. testosteroni</i> - <i>P. alcaligenes</i> (37.6)
PSB 4 ^f	59	4-Sulfobenzoate	62.2 ± 0.1	<i>C. testosteroni</i> - <i>P. alcaligenes</i> (90.9)
T 2 ^f	59	Toluene sulfonic acid	61.8 ± 0.5	<i>C. testosteroni</i> - <i>P. alcaligenes</i> (62.5) or <i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> (17.9)
NRRL B-12228 ^f	12	s-Triazines	61.5 ± 0.4	" <i>P. cepacia</i> " (99.9)
<i>C. acidovorans</i> DSM 1621 ^f	11	Phenylphosphonic acid	66.3 ± 0.5	<i>C. acidovorans</i> (99.2)
<i>C. testosteroni</i> DSM 1622 ^f	11	Alkylphosphonic acid	61.6 ± 0.4	<i>C. testosteroni</i> - <i>P. alcaligenes</i> (90.9)
B 1 ^f	59	Toluene sulfonic acid	66.4 ± 0.4	<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> (75.4)
O-2 ^f	59	Orthanilic acid	65.2 ± 0.2	<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> (69.9)
BN9 ^g	44	Salicylic acid derivatives	61.8 ± 0.2	Not identified
A 3 ^d	4	Naphthylene sulfonic acid	64.2 ± 0.5	Not identified
NRRL B-12227 ^f	12	s-Triazines	67.2 ± 0.2	Not identified

^a The sources of isolation are given in the references.

^b G+C contents were calculated by using the equation of De Ley (13). The values are averages of three measurements.

^c The percentages in parentheses indicate the probabilities of identification according to the API ZONE analytical index. The names given are names that are currently in usage or are different from the nomenclature used by API.

^d Supplied by H.-J. Knackmuss, Universität Stuttgart, Stuttgart, Germany.

^e 2-Methyl-2-en-lactone is 2-methyl-4-carboxymethylbut-2-en-4-olide.

^f Supplied by A. Cook, ETH-Zürich, Zürich, Switzerland.

^g Supplied by B. Nörtemann, Universität GH Paderborn, Paderborn, Germany.

gel electrophoresis of soluble proteins were carried out as described previously (3, 8, 9).

DNA and RNA methods. For isolation of DNA, determination of G+C contents, and DNA-DNA hybridization we used the procedures of Auling et al. (3). RNA was extracted from approximately 10 mg of wet biomass that was washed twice with saline. The cells were resuspended in 0.8 ml of TMK buffer (50 mM Tris-HCl [pH 7.6], 10 mM magnesium acetate, 25 mM KCl) to which 0.16 ml of Macaloid solution (40) was added. Cells were lysed by adding 80 µl of sodium dodecyl sulfate (10%) and incubated for 30 min at 60°C. Crude RNA was purified by repeated extraction with a solution containing 320 µl of phenol (water saturated with 0.1% 8-hydroxyquinoline) and 100 µl of chloroform-isoamyl alcohol (24:1) in the presence of Macaloid solution. The RNA was precipitated with 1.5 volumes of isopropanol at -18°C and was stored at -18°C for 10 min. The precipitate was washed twice with 1.2 ml of 70% ethanol at -18°C and dried. The pellet was dissolved in 0.4 ml of TM buffer (20 mM Tris-HCl [pH 7.5], 10 mM MgCl₂), incubated at 27°C in

the presence of DNase I (120 U) for 30 min, and purified further by several phenol extractions. The extract was mixed with 75 µl of 10 M ammonium acetate, precipitated as described above except that 375 µl of isopropanol was added, and washed twice with ethanol. The pellet was dried and dissolved in 20 to 30 µl of 10 mM Tris-HCl (pH 8.5). As a control 1 µl of the RNA solution was separated on a 1% agarose gel by using GGB buffer (40).

To sequence the 16S rRNA fragment, we used the reverse transcriptase primer extension method, with slight modifications (38), and the primer complementary to the universally conserved region of the 16S rRNA molecule at positions 1392 to 1406. A 5-µl portion of primer (2.5 µg/ml) was hybridized with 1 to 3 µl of high-molecular-weight RNA by heating the preparation at 95°C for 5 min and cooling it to room temperature within 5 min. The sequencing reaction was carried out by using 12 U of reverse transcriptase, 62.5 µM [α-thio]dATP, 40 µM ddCTP, 40 µM ddTTP, 25 µM ddGTP, or 2.5 µM ddATP, and 15 µCi of [α-³⁵S]dATP.

Nucleotide sequence accession numbers. Sequences were

TABLE 2. Quinone and polyamine patterns of the biodegradative strains

Strain	Quinone	Polyamine content ($\mu\text{mol/g}$ [dry wt])					
		2-Hydroxy-putrescine	1,3-Diamino-propane	Putrescine	Cadaverine	Spermidine	Spermine
<i>Pseudomonas</i> sp. strain D7-3	Q-8	52.4		43.3		0.4	tr
<i>Pseudomonas</i> sp. strain D7-4	Q-8	66.1		59.6		1.0	0.3
KTW11	Q-8	35.0		74.9		0.6	0.6
KTW13	Q-8	23.8		73.6		1.9	0.7
A7-2	Q-8	34.2		49.4		0.4	tr
BN9	Q-8	15.4		40.2	6.5	16.8	4.1
A 3	Q-8	31.1		43.0		10.1	3.8
A 3-C	Q-8	46.8		46.1		4.1	0.4
26-8/1	Q-8	19.9		69.0		5.6	1.7
P-3	Q-8	55.2	0.5	43.7		6.1	3.6
PSB 4	Q-8	62.7	0.5	50.5		7.5	2.8
T 2	Q-8	63.0	0.4	46.8		3.7	1.2
<i>C. testosteroni</i> DSM 1622	Q-8	70.8	0.2	50.1		5.2	1.5
B 1	Q-8	64.1	0.5	61.5		7.4	4.2
O-2	Q-8	72.2		50.4		5.8	3.4
PSB 1	Q-8	41.7	tr	37.9		1.9	0.8
NRRL B-12227	Q-8	66.9	1.3	50.8		13.3	3.4
<i>C. acidovorans</i> DSM 1621	Q-8	61.3	0.9	48.5		1.5	0.4
NRRL B-12228	Q-8	74.8	0.7	50.8	0.9	17.0	6.0

aligned relative to previously published sequences. The 16S rRNA fragment sequences have been deposited at the European Molecular Biology Laboratory, Heidelberg, Germany under the following accession numbers: X59160 (strain BN9), X59161 (strain PSB 1), X59162 (strain A 3-C), X59163 (*Alcaligenes xylosoxidans* subsp. *xylosoxidans*), X59164 (strain 26-8/1), X59165 (*Pseudomonas* sp. strain D7-3), X59166 (strain A 3), X59167 ("*Alcaligenes eutrophus*"), X59168 (*Comamonas acidovorans*), X59169 (*Acidovorax facilis*), X59170 (strain NRRL B-12228), and X59171 (strain KTW11).

RESULTS

Phenotypic characterization. The results of the initial identification tests obtained with the commercial API 20NE test system are shown in Table 1. Identification of the isolates by the API 20NE test system gave miscellaneous results. In most cases more than one identity for each isolate was proposed. Except for strain DSM 1621, which was identified as *Pseudomonas acidovorans* (now *C. acidovorans*), and strain NRRL B-12228, which was identified as "*Pseudomonas cepacia*," none of the xenobiotic compound-degrading isolates could be assigned to a previously described species with a probability greater than 90%. No species was proposed for isolates NRRL B-12227 and A 3 by the API 20NE analytical profile index. Isolate BN9 did not grow in this test system, not even after 4 days of incubation; therefore, no results were available for isolate BN9.

Chemotaxonomic investigations. The failure of the API 20NE system prompted us to analyze the isoprenoid quinones, which have been shown to be of great value for identification of higher taxa (10). The xenobiotic compound-degrading isolates which we studied were characterized by the presence of ubiquinone Q-8 (Table 2). This characteristic is common to all members of the beta subclass of the *Proteobacteria* investigated to date (10, 22, 29, 35, 47, 65). Nevertheless, the presence of ubiquinone Q-8 does not permit unambiguous allocation to the beta subclass, because this type of ubiquinone is also present in some members of

the alpha and gamma subclasses of the *Proteobacteria* (10, 30, 42).

This uncertainty was resolved by polyamine analysis as the presence of the unusual diamine 2-hydroxyputrescine is a beta subclass-specific marker (2, 7-9, 65) that is not present in other gram-negative bacteria (26-28). All of the isolates had a characteristic polyamine pattern (Table 2) with putrescine and 2-hydroxyputrescine as the major components. The triamine spermidine and the tetraamine spermine were minor components. Small amounts of 1,3-diaminopropane were detected in a few isolates, and significant amounts of cadaverine were found in isolate BN9. The presence of 2-hydroxyputrescine in all of the isolates which we investigated clearly demonstrated that these organisms belong to the beta subclass of the *Proteobacteria*. Our chemotaxonomic results excluded alternative identification of isolates P-3, PSB 4, T 2, and A 3, and *Comamonas testosteroni* DSM 1622 as *Pseudomonas alcaligenes* strains, as suggested by the results of the multiple-test system (Table 1). Insufficient differentiation of the latter two species not only is a failure of the API 20NE multiple-test system, but also is a general problem of the present phenotypic approaches which has attracted much attention (23, 49, 50). However, as we demonstrated, analysis of either quinones or polyamines can easily solve this problem because *P. alcaligenes* contains ubiquinone Q-9 (68) and putrescine and spermidine as major polyamine compounds (9).

Identification by protein pattern and DNA studies. Determination of the G+C contents of DNAs is recommended for minimum descriptions of species (54). The values which we obtained were in a rather narrow range, between 61 and 68 mol% G+C (Table 1). On the basis of the results of phenotypic investigations, single strains were allocated with high levels of probability to *C. acidovorans*, *C. testosteroni*, "*P. cepacia*," and species of the genus *Alcaligenes* sensu stricto (7, 16, 36). The isolates were further characterized by DNA-DNA hybridization as the method of choice for refined identification at the species level (33, 62). However, DNA homology studies were carried out (Table 3) only if the results of the other methods indicated a close relationship, as

TABLE 3. Levels of DNA homology between xenobiotic compound-degrading isolates and reference strains

Organism	% of binding with strain ^a :					KTW11
	DSM 39 ^T	DSM 38	DSM 30026 ^T	DSM 649 ^T	<i>Pseudomonas</i> sp. strain D7-3	
<i>C. acidovorans</i> DSM 39 ^T	(100)					
<i>C. acidovorans</i> DSM 1621	81					
<i>C. testosteroni</i> DSM 38		(100)				
<i>C. testosteroni</i> DSM 1622		82				
A 3		84				
P-3		72				
PSB 4		68				
T 2		67				
<i>Alcaligenes xylosoxidans</i> subsp. <i>denitrificans</i> DSM 30026 ^T			(100)			
A 3-C			73			
B 1			52			
O-2			45			
<i>Acidovorax facilis</i> DSM 649 ^T				(100)		
NRRL B-12227				61		
<i>Pseudomonas</i> sp. strain D7-3					(100)	
A7-2					94	
<i>Pseudomonas</i> sp. strain D7-4					64	
26-8/1					53	
KTW11						(100)
KTW13						69

^a Percentages of binding were calculated from the initial renaturation rates (15). The values are the averages of four measurements.

follows: (i) an obvious similarity in the protein patterns or (ii) a significant sequence homology in the 16S rRNA fragment (see below). For example, the high level of similarity between isolate A 3 and *C. testosteroni* DSM 38 recognized on the basis of nearly identical protein patterns (Fig. 1) was confirmed by a high level of DNA homology. The identical 16S rRNA fragment sequences of another pair of strains, isolate NRRL B-12227 and *Acidovorax facilis* DSM 649, corresponded to the relatively high level of DNA homology which we determined.

On the basis of their high levels of DNA homology with a single reference strain, strains DSM 1622, A 3, P-3, PSB 4, and T 2 were classified as *C. testosteroni* according to the definition of Johnson (33). By using the same argument strain DSM 1621 was classified as *C. acidovorans*, isolate A 3-C was classified as *Alcaligenes xylosoxidans* subsp. *denitrificans*, and strain NRRL B-12227 was classified as *Acidovorax facilis*. On the other hand, the results of DNA-DNA hybridizations allowed allocation of isolates B 1 and O-2 only to the genus *Alcaligenes*. Rather high levels of internal DNA homology were observed among *Pseudomonas* sp. strain D7-3, *Pseudomonas* sp. strain D7-4, isolate A7-2, and isolate 26-8/1 and between strains KTW11 and KTW13 (Table 3).

Sequence analysis of a 16S rRNA fragment. In order to identify the 11 isolates that were not identified by the methods described above, we analyzed a fragment of 16S rRNA. The sequence which we analyzed (positions 1220 to 1377, *Escherichia coli* nomenclature) (5) is characterized by a high number of variable nucleotides in different species belonging to the alpha, beta, and gamma subclasses of the *Proteobacteria* (5, 21, 41, 60, 63, 64, 69). A comparison of the sequences obtained for this 16S rRNA fragment (Fig. 2) revealed that of the 158 nucleotides determined, at least 36 were variable. Nevertheless, the maximum number of sequence differences that were detected between isolates and selected reference strains belonging to the beta subclass of the *Proteobacteria* was only 23 (Fig. 2).

Identical fragment sequences were found in strain NRRL B-12227 and *Acidovorax facilis*, in isolate A 3 and *C. testosteroni* (69), in *Pseudomonas* sp. strain D7-3, *Pseudomonas* sp. strain D7-4, and isolate A7-2, and in isolates KTW11 and KTW13. The fragment sequence of isolate A 3-C differed by one nucleotide from that of *Alcaligenes*

FIG. 1. Patterns of soluble proteins. Lane a, *C. testosteroni*; lane b, strain A 3; lane c, *Acidovorax facilis*; lane d, strain NRRL B-12227.

	1220	1240	1260	1280
Ac.fa.	GGGCUACACAGCUAUACAA	UGGUGUACAGAGGGUUGC	CAACCCGCGAGGGGAGCCA	AUCCCAUAAAGCCAGUCGUA
C.ac.	GGGCUACACAGCUAUACAA	UGGUGUACAGAGGGUUGC	CAACCCGCGAGGGGAGCUA	AUCCCAUAAAACGAGUCGUA
A 3	GGGCUACACAGCUAUACAA	UGGUGUACAAAGGGUUGC	CAACCCGCGAGGGGAGCUA	AUCCCAUAAAGCCAGUCGUA
KTW11	GGGCUACACAGCUAUACAA	UGGUGUACAGAGGGUUGC	CAAGCccCGAGGUGGAGCUA	AUCCAGAAAAUGCAUCGUA
Al.eu.	GGGCUACACAGCUAUACAA	UGGUGCGUACAGAGGGUUGC	GACCGCGGAGGGGAGCUA	AUCCAGAAAAACGCAUCGUA
D7-3	GGGCUACACAGCUAUACAA	UGGUGCGUACAGAGGGUUGC	CAACCCGCGAGGGGAGCUA	AUCCAGAAAAACGCAUCGUA
26-8/1	GGGCUACACAGCUAUACAA	UGGUGCGUACAGAGGGUUGC	CAACCCGCGAGGGGAGCCA	AUCCAGAAAAACGCAUCGUA
Al.xy1.	GGGCUACACAGCUAUACAA	UGGUGCGGACAGAGGGUUGC	CAACCCGCGAGGGGAGCCA	AUCCAGAAAAACGCAUCGUA
A 3-C	GGGCUACACAGCUAUACAA	UGGUGCGGACAGAGGGUUGC	CAACCCGCGAGGGGAGCUA	AUCCAGAAAAACGCAUCGUA
BN9	GGGCUACACAGCUAUACAA	UGGUGCGGACAGAGGGUUGC	CAACCCGCGAGGGGAGCCA	AUCCAGAAAAACGCAUCGUA
PSB-1	GGGCUACACAGCUAUACAA	UGGUGCGGACAGAGGGUUGC	CAACCCGCGAGGGGAGCCA	AUCCAGAAAAACGCAUCGUA
NRRL B 12228	GGGCUACACAGCUAUACAA	UGGUGUACAGAGGGGCCG	CAACCCGCGAGGGGAGCUA	AUCCAGAAAAUGUGUAUCGUA
	1300	1320	1340	1360
Ac.fa.	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGAAUGUCGCGGUGAAUA
C.ac.	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
A 3	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGUGGAUC	AGAAUGUCACGGUGAAUA
KTW11	GUCCGGAUCGAGUCUGCAA	CUCGACUACGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
Al.eu.	GUCCGGAUCGAGUCUGCAA	CUCGACUACGUGAAGUCGGA	AUCCGUAGUAAUCGUGGAUC	AGCAUGCCACGGUGAAUA
D7-3	GUCCGGAUCGAGUCUGCAA	CUCGACUACGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
26-8/1	GUCCGGAUCGAGUCUGCAA	CUCGACUACGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
Al.xy1.	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
A 3-C	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
BN9	GUCCGGAUCGAGUCUGCAA	CUCGACUGCGUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
PSB-1	GUCCGGAUCGAGUCUGCAA	CUCGACUGCAUGAAGUCGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA
NRRL B 12228	GUCCGGAUCGAGUCUGCAA	CUCGACUCCAUAGAUGUGGA	AUCCGUAGUAAUCGCGGAUC	AGCAUGCCGCGGUGAAUA

FIG. 2. Sequences of 16S rRNA fragments from position 1220 to position 1377. Ac.fa., *Acidovorax facilis*; C. ac., *Comamonas acidovorans*; Al. eu., "*Alcaligenes eutrophus*"; Al. xy1., *Alcaligenes xylosoxidans*.

xylosoxidans subsp. *xylosoxidans*. The same difference was found between isolate 26-8/1 and group III bacteria.

With one exception, the fragment sequences of the other isolates exhibited intermediate levels of similarity to a previously described reference strain, thus forming three groups (Fig. 2). Isolates A 3-C, BN9, and PSB 1 clustered with *Alcaligenes xylosoxidans* subsp. *xylosoxidans* with a maximum sequence difference of eight nucleotides. Strain NRRL B-12227 and isolate A 3 grouped with *C. acidovorans* and *Acidovorax facilis* with a maximum sequence difference of six nucleotides. *Pseudomonas* sp. strain D7-3, *Pseudomonas* sp. strain D7-4, and isolates A7-2, 26-8/1, KTW11, and KTW13 clustered with "*Alcaligenes eutrophus*" with a maximum sequence difference of 10 nucleotides. Strain NRRL B-12228 differed by at least 13 nucleotides from the next phylogenetically described reference strain, an "*Alcaligenes eutrophus*" strain (Fig. 2).

DISCUSSION

According to the definition of Johnson (33), only 8 of the 19 isolates which we investigated could be allocated to a recognized species because of the high DNA homology values obtained with reference DNAs (Table 3). When the stricter definition of Wayne et al. (62) was used, three of the isolates could be allocated only to the genus level (i.e., strains TS 2 and PSB 4 were allocated to the genus *Comamonas*, and strain NRRL B-12227 was allocated to the genus *Acidovorax*). However, we think that strains TS 2 and PSB 4 are true members of *C. testosteroni* because of the phenotypic similarity which we observed (Table 1). Following Willems et al. (66), who included another strain in *Acidovorax facilis* on the basis of a DNA homology level of 60%, we allocated strain NRRL B-12227 to *Acidovorax facilis*. The results of our DNA studies clearly contradict the alternative identifications by API 20NE tests of isolates P-3 and T 2 as

Alcaligenes xylosoxidans subsp. *denitrificans* and of isolate A 3-C as *C. acidovorans*.

On the basis of their intermediate levels of DNA homology with *Alcaligenes xylosoxidans* subsp. *denitrificans* (Table 3), isolates B 1 and O-2 cannot be assigned to this taxon, not even when the definition of Johnson is used (33). Although a phylogenetic relationship to the genus *Alcaligenes* is obvious, these isolates clearly deviate from the type species of this genus, *Alcaligenes faecalis*, in their higher G+C contents (i.e., 66 or 65 mol%, compared with the values of 55 to 60 mol% reported for *Alcaligenes faecalis*) (36). Thus, these organisms may be placed in future studies in either *Alcaligenes xylosoxidans* subsp. *xylosoxidans*, *Alcaligenes piechaudii* (37), or a new species of the heterogeneous genus *Alcaligenes* (7).

The results of the API 20NE tests (Table 1) were not useful for classification of the remaining isolates. For example, *Pseudomonas* sp. strain D7-3, *Pseudomonas* sp. strain D7-4, and isolates A7-2 and PSB 1 were identified by the API 20NE tests as members of CDC (Centers for Disease Control, Atlanta, Ga.) group IVC-2. CDC group IVC-2 consists of bacteria that were obtained from clinical specimens and are biochemically almost identical to *Bordetella bronchiseptica* (53). Phylogenetically, CDC group IVC-2 belongs to the beta subclass of the *Proteobacteria*, with "*Alcaligenes eutrophus*" and "*Pseudomonas solanacearum*" as the nearest neighbors (51). However, there is no indication that members of CDC group IVC-2 should be included in the species "*Alcaligenes eutrophus*" or "*P. solanacearum*."

An example of the fallibility of the multiple-test system which we examined is the identification of isolates KTW11 and KTW13 as either "*Pseudomonas diminuta*" or *Moraxella* spp. (Table 1), which contradicts the chemotaxonomic results. Identification of these isolates as "*P. diminuta*," which belongs to the alpha subclass of the *Proteobacteria* (67), would have required the presence of ubiquinone Q-10

(68) and the presence of high concentrations of *sym*-homospermidine in the polyamine pattern (9) and not the presence of ubiquinone Q-8 and 2-hydroxyputrescine, which were detected (Table 2). Identification as *Moraxella* spp. must also be rejected because this genus belongs to the gamma subclass of the *Proteobacteria* and is related to the genus *Acinetobacter* (52). 1,3-Diaminopropane is the main polyamine component of the members of the genus *Moraxella* investigated so far (7a).

The isolates which gave miscellaneous results required an alternative approach for classification. The 16S rRNA fragment that was sequenced in this work did not have any of the signature nucleotides known from previous oligonucleotide cataloging experiments (67). Two successive bases, cytosine-adenine at positions 1233 and 1234, were found to be characteristic of the beta subclass of the *Proteobacteria* and to be common to the reference strains and the xenobiotic compound-degrading isolates (Fig. 2) (21, 69). For rapid identification of gram-negative bacteria it is important that this marker is absent in the alpha, gamma, and delta subclasses of the *Proteobacteria*, in flavobacteria, and in members of the genus *Bacteroides* (5, 41, 60, 61, 63, 64).

A close relationship between strain NRRL B-12227 and *Acidovorax facilis* was deduced from the identical fragment sequences and was confirmed by a high level of DNA-DNA homology (61%) but could not be detected by comparing between the protein patterns (Fig. 1). The close relationship between isolate A 3 and *C. testosteroni*, which was first detected by identifying identical 16S rRNA fragment sequences, was confirmed by the similarity of the protein patterns (Fig. 1) and a high level of DNA homology (Table 3). We suggest that the description of *C. testosteroni* (48, 57) should be amended to include the presence of urease in single strains. As shown here, the detection of urease activity prevented identification of isolate A 3 as *C. testosteroni* when the API 20NE tests were used.

The fragment sequences of isolates A 3-C, BN9, and PSB 1 exhibited the smallest differences with the *Alcaligenes xylosoxidans* subsp. *xylosoxidans* sequence. The results of both DNA homology and sequence similarity studies clearly placed isolate A 3-C in the species *Alcaligenes xylosoxidans* (Table 3 and Fig. 2). The question of whether isolates BN9 and PSB 1 are members of the genus *Alcaligenes* could not be resolved by comparing sequences because only an incomplete sequence for *Alcaligenes faecalis* (the type species of the genus) is available (21). Nevertheless, we believe that these biodegradative isolates are members of the family *Alcaligenaceae* (16); further information will be required for final allocation to the genus *Alcaligenes* (7, 36, 37).

The six isolates which clustered together with "*Alcaligenes eutrophus*" with a maximum difference of 10 nucleotides (Fig. 2) may form the nucleus of a new genus if further common properties can be found. On the basis of our DNA homology and 16S rRNA fragment sequencing data, the emerging genus, which is centered around "*Alcaligenes eutrophus*" as the type species, could contain at least three other species; one of these is represented by isolates KTW11 and KTW13, a second is represented by the misnamed organisms *Pseudomonas* sp. strain D7-3 and *Pseudomonas* sp. strain D7-4 and isolate A7-2, and the third is represented by isolate 26-8/1. However, a formal proposal of a new genus will require a broader study of these isolates, including strains belonging to CDC group IVc-2 which have been found to be phylogenetically related to "*Alcaligenes eutrophus*" (51). It would be prudent to include in such a study additional strains which are clearly different from "*Alcali-*

genes eutrophus" but are related to it at the genus level (32, 53a).

For further studies dealing with identification of isolates belonging to the beta subclass of the *Proteobacteria* we recommend a chemotaxonomic approach as the first step (i.e., the detection of the unique polyamine 2-hydroxyputrescine as the beta subclass-specific marker). An identification can be verified in a second step if the 16S rRNA fragment (positions 1220 to 1377) sequenced in this study has the characteristic nucleotides cytosine-adenine at positions 1233 and 1234 and differs by less than 23 nucleotides from the fragment of a reference strain belonging to the beta subclass. Sequence identity or a difference of only one nucleotide indicates relatedness at the species level. This may be confirmed either by the similarity of the of soluble protein patterns or quantitatively by a high level of DNA-DNA homology.

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