APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3818–3825 Vol. 62, No. 10
0099-2240/96/$04.00+0

Phenotypic and Genotypic Characterization of Phenanthrene-Degrading Fluorescent *Pseudomonas* Biovars

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Received 1 February 1996/Accepted 24 July 1996

A total of 41 phenanthrene degraders were isolated from a former coal gasification site by using *Pseudomonas*-selective Gould's S1 medium. All isolates were found to belong to the fluorescent *Pseudomonas* group and were subjected to characterization by phenotypic methods, including classical taxonomic tests, API 20NE, and Biolog GN, and the strains were further characterized by the genotypic method repetitive extragenic palindromic PCR (REP-PCR). By using classical tests, the population was found to consist of 38 strains belonging to *P. fluorescens*, 2 *P. putida* strains, and 1 *Pseudomonas* sp. Bacteria in phenograms from Biolog GN and REP-PCR data were divided into groups, which were in good agreement with classical test and API 20NE results. We found a nonfluorescent group of 22 bacteria inconsistent with any *Pseudomonas* sp. in *Bergey's Manual of Systematic Bacteriology*. The group showed small differences in the genotypic test, indicating that all 22 isolates were not recent clones of the same isolate. Analyses of the nonfluorescent group indicated that it belonged to *Pseudomonas*, but the group could not be affiliated with *P. fluorescens* because of differences in DNA-DNA hybridization. Identifications using classical tests and API 20NE were found to correlate, but Biolog GN identifications after 24-h incubation resulted very often in the distantly related *P. corrugata*. The reproducibilities of individual tests of each phenotypic method were assessed, and low reproducibilities were mainly found to be associated with specific Biolog GN test wells. Classical tests and API 20NE proved to be the best for identification of isolates, whereas Biolog GN and REP-PCR were found to be the best tests for high resolution among these closely related isolates.

The genus *Pseudomonas* is a group of gram-negative motile rods known for its large metabolic versatility as well as pathogenicity to plants, animals, and humans (27). A large number of bacteria from this group capable of degrading polycyclic aromatic hydrocarbons have been isolated in soils and aquifers (4, 8, 18, 29, 30). This is striking, since the isolation of bacteria degrading polycyclic aromatic hydrocarbons frequently selects for specific properties such as high degradation rates rather than selecting for *Pseudomonas* spp. The identification of strains is often conducted only to the *Pseudomonas* sp. level in this very diverse genus, not allowing full characterization.

The phylogenetic center of *Pseudomonas* spp. consists of RNA homology group I, known as the "true" *Pseudomonas* spp. (26). Among these, *P. aeruginosa*, *P. fluorescens*, *P. putida*, and *P. chlororaphis* constitute an oxidase-positive group of saprophytic or opportunistic fluorescent bacteria; *P. fluorescens* and *P. putida* are further divided into five and two biovars, respectively (25).

Traditionally, the characterization of species has been conducted by using classical phenotypic tests, such as growth on specific media, enzymatic reactions, and microscopy. However, these approaches are rather time-consuming and often inappropriate when handling large populations of closely related bacteria. Bacterial identification methods aiming to simplify identification and characterization are desired (5, 33); among the most commonly used commercially available systems are the API and Biolog systems.

The API 20NE kit is a test kit consisting of 20 enzymatic and carbon compound assimilation tests, whereas the Biolog GN system contains 95 wells with different carbon compounds. Bacterial respiration in Biolog GN wells is indicated by the resulting reduction of the redox dye tetrazolium violet to produce a purple coloration.

The characterization of bacteria by using phenotypic tests has been subject to debate as it is dependent on the ability of strains to grow under certain environmental conditions, and characterization may be obscured by the involvement of plasmids (25). The *Pseudomonas* RNA homology group I shares so many characteristics with other pseudomonads that strict differentiation is not possible with ordinary phenotypic tests (27). Therefore, methods based on analysis of the genome have been proposed; they take advantage of differences in the bacterial genotype. Repetitive extragenic palindromic PCR (REP-PCR) is one of these methods and uses the REP sequences originally found in *Escherichia coli* as primers for PCRs (36), producing specific band patterns and thereby allowing the discrimination of even closely related isolates (7).

Our aim with this study was to characterize a group of bacteria from the fluorescent *Pseudomonas* group degrading the polycyclic aromatic hydrocarbon compound phenanthrene by four different methods to assess the bacterial diversity of this closely related group. Using environmental isolates, we could evaluate the ability to identify bacteria not included in the database of each identification system.

MATERIALS AND METHODS

Isolation of bacteria. Bacteria were isolated from a sandy soil from a former coal gasification site in Copenhagen, Denmark. Three soil samples were taken, pooled, thoroughly mixed, and passed through a sieve (mesh size, 2 mm). Sixty-five grams (wet weight) of soil was suspended in 200 ml of 0.015 M phosphate buffer (pH 7.4) and blended three times for 1 min with 1-min intervals in a Waring blender at low speed (2). Aliquots (100 μl) in direct and 10^{-1} dilutions were spread on Gould's S1 medium selective for fluorescent pseudomonads (9,

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19). The spray plates were incubated at 20°C for 4 days. Colonies were transferred with sterile toothpicks to phenanthrene spray plates (17). The plates were slightly modified in that we applied the phenanthrene (Sigma, St. Louis, Mo.) in a 750-μl acetone solution (0.5% [wt/vol]) with a pipette on top of the agar in each plate, allowing the acetone to evaporate before transferring the bacteria. The following two sets of agar plates were used: one with only the hydrocarbon minimal medium (HCMM2) described by Ridgway et al. (29) and one with HCMM2 supplemented with 0.03% (wt/vol) Casamino Acids (Difco, Detroit, Mich.), allowing the growth of dissimilative bacteria. Both were solidified by 1.5% (wt/vol) Noble agar (Difco). The plates were incubated at 30°C and screened for bacteria producing clearing zones in the opaque phenanthrene layer several times during a period of approximately 10 days. Bacteria producing clearing zones, subsequently designated phenanthrene degraders, were transferred to aqueous phenanthrene solutions in HCMM2 or HCMM2 supplemented with Casamino Acids, depending on the type on which the isolates originally were isolated, and incubated at 30°C on a rotary shaker (200 rpm) for approximately 14 days. All bacterial suspensions were stored in 50% (vol/vol) glycerol at −80°C.

All bacteria in this work, unless stated otherwise, were grown on rich Luria-Bertani medium (22) supplemented with 0.10% (wt/vol) glucose and 1.5% (wt/vol) BiTek Agar (Difco).

Identification by classical tests. Forty-one isolates were Gram stained by the classical staining method (Hucker) (23) and the KOH test (10). Furthermore, an oxidase test (*Dry*slide Oxidase; Difco) and microscopy for shape and size were conducted.

To assign bacteria to biovars the following classical tests according to *Bergey's Manual of Systematic Bacteriology* (24) were performed: denitrification using Durham tubes in tryptic soy broth (Difco) supplemented with 10 mM KNO_3 (production of N_2 and/or N_2O and growth) (32); fluorescence in UV light on Gould's S1 (9); growth at 42°C; growth on a minimal medium supplemented with *meso*-inositol, sorbitol, or L-tryptophan (0.1% NH_4Cl, 0.2% KH_2PO_4, 0.2% $K_2HPO_4 \cdot 3H_2O$, 0.01% $MgSO_4$, 10 ppm of $Fe_2(SO_4)_3$, 1 μM [each] $CuCl_2$, $ZnSO_4$, $MnSO_4$, $CoCl_2$, $NiCl_2 \cdot 6H_2O$, and $NaMoO_4$, and 0.4% carbon compound); production of levan from sucrose-rich medium (5.0% [wt/vol] sucrose and 2.3% [wt/vol] Bacto Nutrient Agar [Difco]); and pigment production on potato dextrose agar (12). Additionally, fluorescence in UV light on King's B medium (16) was examined.

Identification by API 20NE. The API 20NE test kit (bioMérieux, Marcy-l'Etoile, France) was used, and the manufacturer's instructions were followed by incubating bacteria at 30°C. Examinations of the strips were conducted after 24 and 48 h, and the results from 48 h were used. However, in the denitrification test, the results from 24 h were used, because the denitrification well was sacrificed at 24 h. The identification of strains was carried out according to the API 20NE identification manual (1).

Identification and characterization by Biolog GN. One or two days before the inoculation of Biolog GN plates (Biolog, Inc., Hayward, Calif.), the isolates were streaked on tryptic soy agar (Difco) plates from freeze cultures. On the day of inoculation, a prewetted sterile cotton swab was used to transfer bacteria to a 30-ml muffled (600°C) glass tube with 19 ml of 0.85% NaCl solution and adjusted to a bacterial concentration of optical density at 600 nm (OD_{600}) of 0.12 ($\sim 3 \times 10^8$ cells per ml). Within 10 min, the Biolog GN plates were inoculated with 150 μl of the adjusted bacterial suspension in each well and incubated at 30°C for 24 h, leaving out the 4-h measurement as recommended by Holmes et al. (13). The development of color was read the following day by using a 590-nm wavelength filter in a microplate reader (Microplate Bio-Kinetics Reader EL 312e; Bio-tek Instruments, Inc., Winooski, Vt.), as well as read manually. Reactions were recorded as positive (+), negative (−), or borderline (\), when it was impossible to distinguish positive from negative. Manual readings from the first and second runs were entered in the Biolog GN database, release 3.50, to provide identification. An identification was acknowledged when the similarity index used by Biolog was 0.5 or more.

Culture collection strains from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM) were also tested to evaluate identification and reproducibility performance compared with that of our isolates. The strains used were *P. aeruginosa* DSM50071, *P. fluorescens* biovar I DSM50090, *P. fluorescens* biovar III DSM50124, *P. fluorescens* biovar IV DSM50415, *P. fluorescens* biovar V DSM50148, *P. chlororaphis* DSM50083, *P. putida* biovar A DSM50208, and *P. putida* biovar B DSM50222.

Characterization by REP-PCR. Template DNA was prepared by boiling 200 μl of bacterial suspension in MilliQ H_2O (OD_{600} = 0.6) in safe-lock Eppendorf tubes for 10 min. The tubes were immediately cooled on ice and centrifuged (20,000 × *g*, 10 min, 5°C), and the supernatants were subsequently kept on ice or at −20°C. One or two microliters of template DNA suspension was used per reaction. After optimization of the $MgCl_2$ concentration to 4.0 mM, PCR was carried out in PCR tubes with 13.3 μl of MilliQ H_2O, 2.5 μl of 10× Stoffel PCR buffer (100 mM Tris-HCl, pH 8.3, 100 mM KCl) (Perkin-Elmer, Vatterstetten, Germany), 2.0 μl of deoxynucleoside triphosphate (dNTP) mix (0.2 mM [each] dNTP [Boehringer Mannheim, Munich, Germany]), 1.5 μl (16.7 μM) of both REP1R-I (5′ IIIICGICGICATCIGGC 3′) (36) and REP2-I (5′ ICGICTTATC IGGCCTAC 3′) (35) primers (Oswel DNA Service, Southampton, England), 0.2 μl (5 U/μl) of Stoffel fragment DNA polymerase (Perkin-Elmer), and 4.0 μl (25 mM) $MgCl_2$. In each PCR run, a negative control with no template DNA was included. The PCR tubes were overlaid with 2 drops of mineral oil. The reactions were performed in a DNA thermal cycler (Perkin-Elmer) (1 cycle of 94°C for 6 min followed by 30 cycles of 94°C for 1 min, 40°C for 1 min, and 65°C for 6 min and a final extension of 65°C for 16 min prior to cold storage at 5°C until loaded on a gel or frozen at −20°C).

The amplified PCR fragments were separated on 3.0% (wt/vol) NuSieve agarose gels (20 by 20 cm) (FMC Bioproducts, Solrød, Denmark) in 1× Tris-acetate-EDTA buffer at 29 V by using 10 μl of PCR product and 3 μl of dye (Promega, Madison, Wis.) per slot. Postelectrophoresis staining of gels was done in 0.5 μg of ethidium bromide per ml, and after destaining, the gels were photographed.

After the first run, isolates were manually grouped according to similarity to facilitate comparison. The Polaroid pictures of gels were evaluated by using CREAM 1-D for Windows 1.0, revision A (Kem-En-Tec A/S, Copenhagen, Denmark).

Data and cluster analyses. All tests were performed independently twice. NTSYS-pc 1.80 (Applied Biostatistics, Inc., Setauket, N.Y.) was used to calculate simple matching coefficients for the 49 operational taxonomic units (strains) for API 20NE, Biolog GN, and REP-PCR results as binary matrices. In cases of opposite results for the first and second Biolog GN runs of the same test with the same isolate, the test results for that well and isolate were treated as neither positive nor negative and excluded in calculating simple matching coefficients. For Biolog GN manual examinations, a borderline and a positive were treated as positive, a borderline and a negative were treated as negative, and two borderlines were treated as neither positive nor negative. For REP-PCR results, only bands of between 100 and 1,000 bp were included in the analysis and bands of similar sizes were regarded as the same, since the system had an accuracy of approximately ±2% in measuring fragment lengths. Phenograms were produced by the unweighted-pair-group method with arithmetic average (UPGMA) facility of NTSYS-pc. In case of ties, 25 trees were examined for agreement with the tree produced. Finally, trees were produced with the complete-linkage and single-linkage utilities of the same computer program to evaluate dissimilarities between cluster methods.

RESULTS

Isolation of bacteria. A total of 522 colonies were isolated from the Gould's S1 plates. Of these, 53 (10%) were found to degrade phenanthrene. Of these, 7 (13%) produced clearing zones on phenanthrene spray plates without Casamino Acids, 38 (72%) produced clearing zones on plates with Casamino Acids, and 8 (15%) produced clearing zones on plates both with and without Casamino Acids. The isolates growing on spray plates with Casamino Acids generally produced clearing zones at a higher rate than did isolates on spray plates with pure minimal medium. A subsample of 41 randomly chosen isolates of the total group of 53 phenanthrene degraders was used in the subsequent studies.

Identification by classical tests, API 20NE, and Biolog GN. All 41 isolates were shown to be gram-negative, oxidase-positive rods of a size of approximately 1 by 3 μm. Twenty-four strains were not UV fluorescent on Gould's S1 or King's B medium. The tentative biovar allocations found by using the classical tests are listed in Table 1. Twenty-six strains met the criteria of classical tests for *P. fluorescens* biovar III without regard to fluorescence, but 22 of the isolates not producing fluorescence constituted a group with identical results from API 20NE and classical tests. The test results of this group were inconsistent with any *Pseudomonas* sp. according to *Bergey's Manual of Systematic Bacteriology* (24), and the group is hereafter designated *P. fluorescens* biovar IIIc. Likewise, three bacteria constituted a group different from the classical *P. fluorescens* biovar III JAJ19 in *meso*-inositol, *N*-acetyl-D-glucosamine of API 20NE, and several Biolog GN tests, so these groups are designated biovars IIIb and IIIa, respectively.

There was good correspondence between identification results from the classical tests and API 20NE. Of the 38 strains identified as *P. fluorescens* by classical tests, 36 were also identified as *P. fluorescens* by API 20NE. The two strains left gave results of *P. aureofaciens* and no match by API 20NE. JAJ18, identified as a *Pseudomonas* sp. in classical tests as a consequence of results incompatible with any biovar, was found to

TABLE 1. Identifications and characteristics of 41 phenanthrene-degrading isolates

Test	% Strains positive[a]							
	P. fluorescens						*P. putida* B (2)	*Pseudomonas* sp. (1)
	II (3)	IIIa (1)	IIIb (3)	IIIc (22)	IV (8)	V (1)		
Classical								
Fluorescence on Gould's S1	100	100	33	0	100	100	100	100
Denitrification	100	100	100	100	100	0	0	100
Levan production	100	0	0	0	100	0	0	100
Blue pigment on potato dextrose agar	0	0	0	0	100	0	0	100
meso-Inositol	100	100	0	100	100	100	0	100
Sorbitol	100	100	100	0	100	100	0	0
L-Tryptophan	0	0	0	0	0	0	100	0
API 20NE								
Indol production on tryptophan	0	0	0	0	0	0	0	0
Glucose acidification	0	0	0	0	0	0	0	0
Arginine dihydrolase	100	100	33[b]	0	100[b]	0	100	100[b]
Urease	0	0	0	0	0	0	0	0
Esculin hydrolysis	0	0	0	0	0	0	0	0
Gelatin hydrolysis	100[b]	100[b]	33	0	63	0	0	100[b]
β-Galactosidase	0	0	0	0	0	0	0	0
D-Glucose	100	100	100	100	100	100	100	100
L-Arabinose	100	100	100	100	100	100	100	100
D-Mannose	100	100	100	100	100	100	100	100
D-Mannitol	100	100	100	100	100	100	0	100
N-Acetyl-D-glucosamine	66	100	0	100	75[b]	0	50[b]	100
Maltose	0	0	0	0	0	0	0	0
Gluconate	100	100	100	100	100	100	100	100
Caprate	100	100	100	100	100	100	100	100
Adipate	33	0	0	0	0	0	0	0
L-Malate	100	100	100	100	100	100	100	100
Citrate	100	100	100	100	100	100	100	100
Phenylacetate	0	0	0	0	13[b]	100	100	0
Biolog[c]								
N-Acetyl-D-glucosamine	66	100	0	100	0	0	0	100
D-Arabitol	100	100	100	100	100	100	0	100
meso-Inositol	100	100	0	100	100	100	0	100
D-Mannitol	100	100	100	100	100	100	0	100
D-Sorbitol	100[b]	100	100	5[b]	100	100	0	100[b]
Sucrose	100	0	100	100	100	100	0	100
D-Trehalose	100	100	100	100	63[d]	100	0	100
D-Galacturonic acid	100	100	100	100	100	100	0	100
D-Glucuronic acid	100	100	100	100	100	100	0	100
τ-Hydroxybutyric acid	33	100	33	0	0	0	50	0
Quinic acid	100	100	100	100	63	100	100	100
Glucuronamide	100	100	100	100	100	100	0	100
L-Ornithine	100[b]	0	100	100	13[b]	100	100	100
L-Phenylalanine	0	0	33	82[e]	0	0	100	0
Urocanic acid	100	100	100	100	100	0	100	100
Phenylethylamine	0	0	0	0	0	0	100	0
2,3-Butanediol	66	0	0	59[b]	38[b]	0	0	0

[a] The total number of strains tested per category is given parenthetically.
[b] One isolate gave varying results and was scored as positive.
[c] Data from manual readings for selected characters are shown.
[d] Two isolates gave varying results and were scored as positive.
[e] Ten isolates gave varying results and were scored as positive.

be *P. fluorescens*, and both *P. putida* isolates were confirmed as such by API 20NE. In a few cases, API 20NE gave an identification of *P. aureofaciens* or *P. chlororaphis* in one of the runs and an identification of *P. fluorescens* in the other.

All Biolog GN identifications by manual registrations resulted in the right identification of the genus, but for 31 of 38 *P. fluorescens* strains, the RNA homology group V member *P. corrugata* rather than *P. fluorescens* was found in at least one of the two replicate runs. This identification was not specific for any of our biovar groupings. Furthermore, most strains gave different identifications in the two runs.

Reproducibility of methods. All classical tests had good reproducibility, since all produced fewer than four strains with opposite results in the two runs among the 41 isolates tested. API 20NE wells were often considerably easier to examine after 48 h rather than 24 h; consequently, the 48-h results were used, except for denitrification. The following two API 20NE test wells had low-level reproducibility: denitrification (46% of

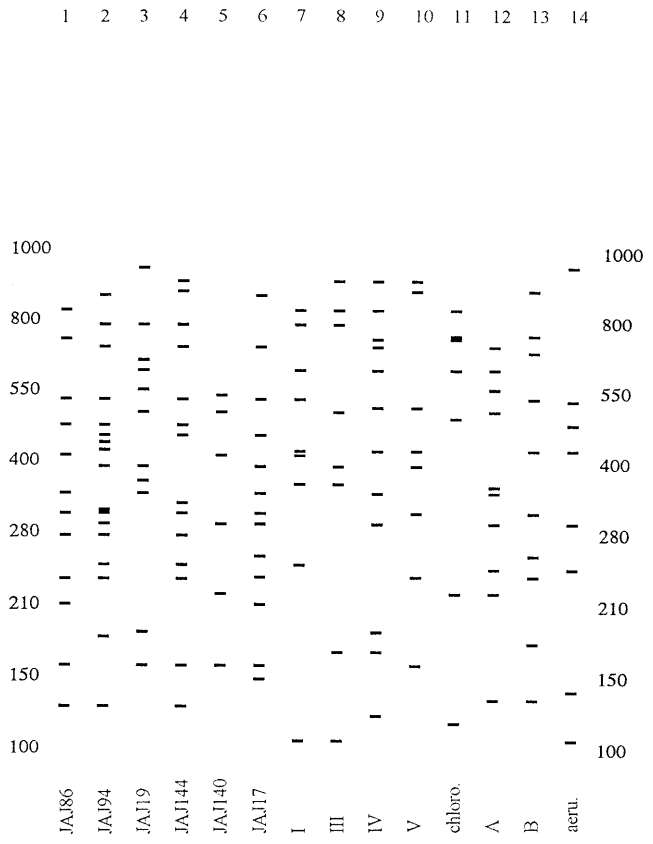

FIG. 1. REP-PCR patterns typical of the different groups and culture collection strains. The numbers above the bands indicate lanes, and the numbers on the sides indicate REP-PCR product sizes (in base pairs). Below the bands example strain names are listed. I, III, IV, V, chloro., A, B, and aeru., the corresponding DSM strains.

the strains gave different results) and arginine dehydrolase (10%).

Biolog GN results represent the results from manual examinations, since the automatic readings yielded a greater number of +− cases, in which one run gave a positive result and the other was negative. In automatically examined tests, the development in coloration used as the limit between positive and negative is crucial for reproducibility. By using Biolog's recommended limit (3) of a 40% higher OD_{590} in wells compared with that of the negative control well without a carbon source, 11.8% of all wells gave opposite results in the two runs. When we changed the limit to 86%, which is the percentage giving the lowest number of contradictory results for our isolates, 7.2% were +− cases. However, for each isolate, this limit is different; it is possible to reduce the +− case ratio to less than 5% by using separate limits for each isolate. Wells giving general growth near the limit were especially prone to +− cases. Manual readings gave 4.8% +− cases, but for 12.4% of the wells, at least one run gave borderline growth. Low-level reproducibility was associated with the reproducibility of specific tests rather than with specific isolates. In wells for dextrin and α-hydroxybutyric acid tests, fewer than 10 isolates gave two positive or two negative results in two runs.

The REP-PCRs gave 58 different visible bands in the population, with individual isolates varying between 6 and 17 bands. The different REP-PCR band patterns typical of each group are shown in Fig. 1. In the case of *P. fluorescens* biovar IIIc isolates, the strain banding patterns resemble each other but are not exactly identical. The REP-PCR results for duplicate runs were identical. In some cases, weaker bands shown in one of the runs could not be seen in the other run. However, this was the case only when the general band intensity of the isolate was reduced. In such cases, the bands present in only one run were included in analyses. The mixture giving the highest band intensity for each isolate was used for the final separation on gels.

Cluster analyses of Biolog GN and REP-PCR data. The phenogram produced on the basis of manual Biolog GN results is shown in Fig. 2. Alternate trees produced from the same data by UPGMA (tied trees) showed good agreement with the tree shown. The groups (marked by lowercase letters) were internally regrouped in some trees but were distinct clusters compared with each other. The only exceptions were DSM50090 and DSM50148, whose placements varied. This was considered to be consistent, since the results were the same when single- and complete-linkage clustering methods were used. The REP-PCR phenogram (Fig. 3) divided the strains into groups similar to the Biolog GN groups. The cluster grouping was consistent in tied UPGMA trees as well as in single- and complete-linkage trees, but internally these clusters varied.

As can be seen from Fig. 2 and 3, there was homology between these phenograms. The largest differences are the division of cluster b and the varying placement of most culture collection strains, JAJ19, and JAJ102. The *P. fluorescens* biovar IIIc isolates shared a cluster; so did the three *P. fluorescens* biovar IIIb isolates, a *P. fluorescens* biovar V isolate, and two *P. putida* B isolates. The *P. fluorescens* biovar II and IV isolates were to some extent interspersed. *P. fluorescens* culture collection strains in the Biolog GN-generated phenogram were distributed among *P. fluorescens* isolates as *P. putida* and *P. aeruginosa* clustered outside these. However, in the REP-PCR phenogram, culture collection strains clustered more randomly.

DISCUSSION

Isolation. The percentage of phenanthrene-degrading *Pseudomonas* isolates relative to all *Pseudomonas* isolates was 13% for this soil, which is lower than the 41% found by using King's B and phenanthrene spray plates by Campbell et al. (6) at the same coal gasification site with a different soil. However, their selection procedures were different from ours, since the degraders were isolated for UV fluorescence on King's B plates after 2 to 3 days and spray plates were incubated for 3 to 4 weeks. The Casamino Acids used in our experiments can support minor growth, allowing constitutive unspecific genes transforming phenanthrene to be expressed, even though bacteria do not benefit from the degradation.

Identification. Classical tests gave us tentative species and biovar distributions. The use of very specifically chosen tests from *Bergey's Manual of Systematic Bacteriology* has the advantage of making it possible to use a minimum number of tests. However, if only one test separates two species or biovars, an isolate can be misplaced because of minor changes in metabolic profiles compared with the general pattern. For instance, pigment formation on potato dextrose agar is the only character separating *P. fluorescens* biovars II and IV. Furthermore, the results of the large *P. fluorescens* group denominated IIIc conflict with the taxonomy in *Bergey's Manual of Systematic Bacteriology* (24) since no groups in this review are consistent with *P. fluorescens* biovar IIIc. However, the identifications by API 20NE were in good agreement with the classical test

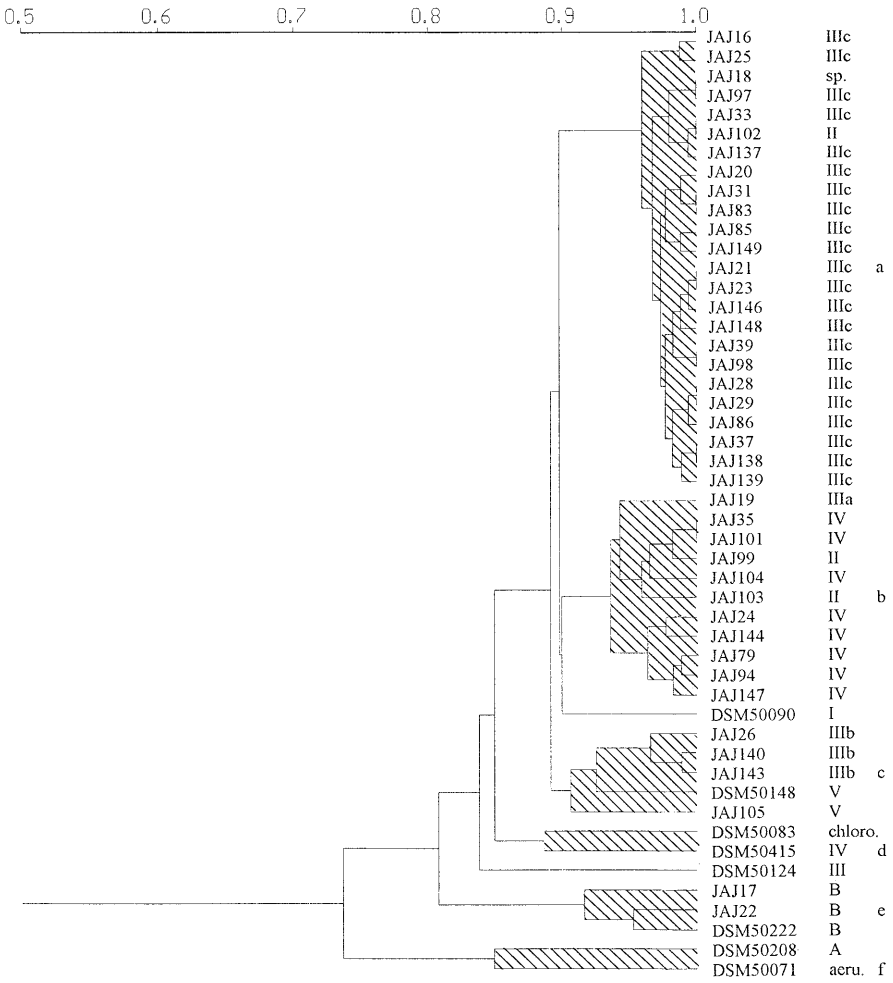

FIG. 2. UPGMA tree formed on the basis of simple matching coefficients from Biolog GN results (manual examinations). Wells which gave opposite results, i.e., positive and negative (+− cases) and wells for which both results were borderline were treated as neither positive nor negative. Wells with positive and borderline results and negative and borderline results were treated as positive and negative, respectively. II, IIIa, IIIb, IIIc, IV, and V, *P. fluorescens* biovars II, IIIa, IIIb, IIIc, IV, and V, respectively; A and B, *P. putida* A and *P. putida* B, respectively; chloro., *P. chlororaphis*; aeru., *P. aeruginosa*; sp., a *Pseudomonas* sp., as determined by classical tests. Lowercase letters indicate consistent groups (see text). The upper axis indicates distance value.

results that identify these strains as *P. fluorescens*. API 20NE and classical test results were different for only 3 of 39 *P. fluorescens* strains (3 others were different in one API 20NE run) and were consistent for both *P. putida* strains. Wauters et al. (37) tested 11 *P. putida* isolates and 13 *P. fluorescens* isolates of clinical origin and identified 6 and 10 strains correctly, respectively, compared with conventional test results. Our results thus show a similar identification rate.

The Biolog GN identifications were quite different, often giving the result *P. corrugata*. This identification was not specific to any of our biovar groupings, including the *P. fluorescens* IIIc group. Considering that *P. corrugata* is positive in gelatin hydrolysis as opposed to our group IIIc results (31) and the results from our API 20NE identifications, we find it unlikely that the *P. fluorescens* group IIIc strains are *P. corrugata* isolates. This is supported by the results of Kragelund et al. (19), which indicate that Gould's S1 medium allows the growth of only *Pseudomonas* RNA homology I members.

Cluster analyses. The two phenograms presented (Fig. 2 and 3) show generally good agreement between one another and with classifications from classical tests and API 20NE. Differences are mainly represented by culture collection strains, JAJ19, and JAJ102. In the REP-PCR diagram, the location of JAJ19 is different from what would be expected from Biolog GN, API 20NE, or classical test results. However, in both diagrams (Fig. 2 and 3), JAJ19 is separated from both IIIb and IIIc group members, supporting this distinction. JAJ102 produced +− cases in several Biolog GN tests, making it difficult to discern it from members in the *P. fluorescens* biovar IIIc cluster and the *P. fluorescens* biovar II-IV cluster. If the reproducibility of the Biolog GN results can be increased, this problem may be reduced. The Biolog GN phenogram presented indicates that the nonfluorescent cluster is more closely related to *P. fluorescens* than to *P. putida* or *P. aeruginosa*. The culture collection strains in the REP-PCR tree are not as well ordered as they are in the Biolog GN phenogram. This is most likely due to the nature of REP-PCR, in which a large number of characters in diverse strains randomly give identical bands in strains with no special relation and fewer true bands in common, emphasizing the strength of REP-PCR in discerning closely related strains.

Reports on nonfluorescent strains in the literature. The number of nonfluorescent strains isolated in this study was somewhat surprising to us, since we had used a very selective

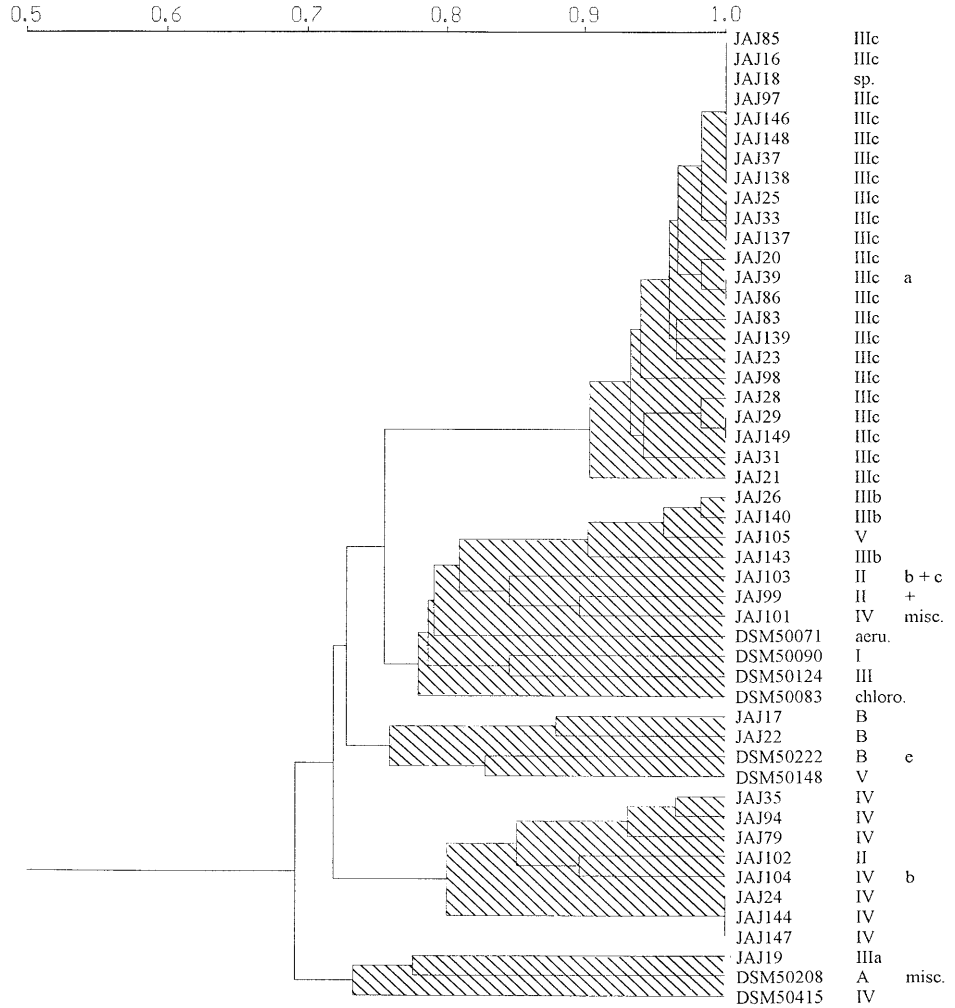

FIG. 3. UPGMA tree formed on the basis of simple matching coefficients from REP-PCR results by using the PCR suspension of the isolate giving the most intense bands. II, IIIa, IIIb, IIIc, IV, and V, *P. fluorescens* biovars II, IIIa, IIIb, IIIc, IV, and V, respectively; A and B, *P. putida* A and *P. putida* B, respectively; chloro., *P. chlororaphis*; aeru., *P. aeruginosa*; sp., a *Pseudomonas* sp., as determined by classical tests. Lowercase letters indicate the placements of Biolog GN groups (see text); misc., miscellaneous strains. The upper axis indicates distance value.

isolation medium. In *Bergey's Manual of Systematic Bacteriology* (24), it is stated that the members of the oxidase-positive group of saprophytic or opportunistic fluorescent *Pseudomonas* spp. produce fluorescence in UV light, which in the terminology means that more than 90% of the isolates contained in the samples used are fluorescent. These results, mainly based on large studies by Jessen (15) and Stanier et al. (34), could overemphasize the share of fluorescent strains caused by the origin of these isolates. These have often been isolated on account of fluorescence on media such as King's B that allow relatively unspecific growth but enable bacteria to exhibit fluorescence. To our knowledge, two descriptions of a considerable number of nonfluorescent members of this group have been made. In the first case, Holmes et al. (14) found this in an examination of the fluorescence of 34 strains on King's B. Nevertheless, they do not comment on this observation in the text. Lambert et al. (20) isolated several *P. fluorescens* strains from maize root and rhizosphere and noted that among these strains, there were fluorescent bacteria as well as nonfluorescent bacteria.

As a consequence of classical test and API 20NE identifications, the use of Gould's S1 as the selection medium, and the relation indicated by both phenograms, we consider the strains designated *P. fluorescens* IIIc to be members of *Pseudomonas* RNA homology group I. This was confirmed by partial 16S sequencing and cellular fatty acid profiles performed at the DSM (34a). However, DNA-DNA hybridization tests showed 44.5% homology between JAJ28 and the type strain of *P. fluorescens*. Therefore, the group hitherto designated *P. fluorescens* IIIc may be a novel species within the *Pseudomonas* RNA homology group I. Additionally, the differences in REP-PCR-generated fingerprints indicate that these bacteria are not recent multiplications of a single bacterium.

The less evident differences of *P. fluorescens* IIIb do not warrant a similar proposal of a new group.

Reproducibility of methods. All classical tests had good reproducibility. In general, API 20NE had good reproducibility, but there were minor difficulties with the arginine dihydrolase and major difficulties with the denitrification wells. In light of this result, the classical denitrification test was incorporated in this study; this denitrification assay proved to have good reproducibility. The performance of the API 20NE denitrification test may improve upon incubation for 48 h. Parekh et al. (28) used three different bacterial suspensions to inoculate

duplicate API 20NE strips and found 100% similarity between replicates. They do not indicate the identities of bacteria, except for their gram-negative status.

In general, the Biolog GN well results were less reproducible than those of tests for other phenotypic methods. The wells especially prone to +− cases were those producing the smallest general increase in OD_{590}. Our findings are supported by Haack et al. (11), who performed seven replicate Biolog GN runs of a *Pseudomonas* sp. isolated from soil. The incubation times were 24 and 72 h, and within the 95 carbon source wells, 16 and 6 wells gave varying results, respectively. The study by Parekh et al. (28) mentioned above found Biolog GN plates to give 95.8 to 98.9% reproducibility in an experiment similar to that with API 20NE. The incubation time recommended by Biolog is 4 or 24 h. We used the 24-h incubation time recommended by others in identification studies (13). However, in comparing the performances of two systems such as API 20NE and Biolog GN, we must take into account that the incubation temperatures are equal but the times are quite different. In studies like this, a short incubation time is not as vital as it can be in clinical identifications, and with a performance per well similar to that of API 20NE, Biolog GN will presumably provide more information because of the larger number of wells. REP-PCR has been shown by others to correlate well with characterization by a large number of phenotypic tests (21). The REP-PCRs in our study produced bands of varying intensity, sometimes leaving the weakest bands out in one of the runs. Therefore, isolates giving band profiles with single differences can be the result of identical bacteria. However, the large nonfluorescent group consisted of strains which had minor but distinct differences and this genetic tool proved suitable for assessing the diversity of our isolates.

Concluding remarks. In studying this closely related group, classical tests and API 20NE appeared to give the best performance in identifying the strains to biovar and species levels, respectively. However, Biolog GN and REP-PCR gave the best diversity assessments, especially because of their high resolution. Increasing the incubation time may prove to improve Biolog GN test reproducibility.

ACKNOWLEDGMENTS

This work was supported by a grant from the Danish Centre for Ecotoxicological Research under the Danish Environmental Research Programme.

Søren Andersen and Kaare Johnsen contributed equally to this work.

We thank Mette Neiendam Nielsen and Jan Sørensen for critically reading the manuscript and inspiring discussions of the manuscript and Anita Jørgensen for valuable suggestions in the laboratory. Finally, Søren J. Sørensen from the University of Copenhagen and Jens Efsen Johansen from Novo Nordisk are thanked for help during this study.

REFERENCES

1. **Analytab Products.** 1992. API 20NE analytical profile index, 5th ed. Analytab Products, Marcy-l'Etoile, France.
2. **Bakken, L. R.** 1985. Separation and purification of bacteria from soil. Appl. Environ. Microbiol. **49:**1482–1487.
3. **Biolog.** 1993. GN MicroPlate: instructions for use. Biolog, Hayward, Calif.
4. **Bouchez, M., D. Blanchet, and J.-P. Vandecasteele.** 1995. Degradation of polycyclic aromatic hydrocarbons by pure strains and by defined strain associations: inhibition phenomena and cometabolism. Appl. Microbiol. Biotechnol. **43:**156–164.
5. **Busse, H.-J., T. El-Banna, and G. Auling.** 1989. Evaluation of different approaches for identification of xenobiotic-degrading pseudomonads. Appl. Environ. Microbiol. **55:**1578–1583.
6. **Campbell, J. I. A., C. S. Jacobsen, and J. Sørensen.** 1995. Species variation and plasmid incidence among fluorescent *Pseudomonas* strains isolated from agricultural and industrial soils. FEMS Microbiol. Ecol. **18:**51–62.
7. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58:**2180–2187.
8. **Evans, W. C., H. N. Fernley, and E. Griffiths.** 1965. Oxidative metabolism of phenanthrene and anthracene by soil pseudomonads, the ring-fission mechanism. Biochem. J. **95:**819–831.
9. **Gould, W. D., C. Hagedorn, T. R. Bardinelli, and R. M. Zablotowicz.** 1985. New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats. Appl. Environ. Microbiol. **49:**28–32.
10. **Gregersen, T.** 1978. Rapid method for distinction of gram-negative from gram-positive bacteria. Eur. J. Appl. Microbiol. **5:**123–127.
11. **Haack, S. K., H. Garchow, M. J. Klug, and L. J. Forney.** 1995. Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. Appl. Environ. Microbiol. **61:**1458–1468.
12. **Harrigan, W. F., and M. E. McCane.** 1976. Laboratory methods in food and dairy microbiology. Academic Press, London.
13. **Holmes, B., M. Costas, M. Ganner, S. L. W. On, and M. Stevens.** 1994. Evaluation of biolog system for identification of some gram-negative bacteria of clinical importance. J. Clin. Microbiol. **32:**1970–1975.
14. **Holmes, B., C. A. Pinning, and C. A. Dawson.** 1986. A probability matrix for the identification of gram-negative, aerobic, non-fermentative bacteria that grow on nutrient agar. J. Gen. Microbiol. **132:**1827–1842.
15. **Jessen, O.** 1965. *Pseudomonas aeruginosa* and other green fluorescent pseudomonads. Munksgaard, Copenhagen.
16. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44:**301–307.
17. **Kiyohara, H., K. Nagao, and K. Yana.** 1982. Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. **43:**454–457.
18. **Kiyohara, H., N. Takizawa, and K. Nagao.** 1992. Natural distribution of bacteria metabolizing many kinds of polycyclic aromatic hydrocarbons. J. Ferment. Bioeng. **74:**49–51.
19. **Kragelund, L., K. Leopold, and O. Nybroe.** 1996. Outer membrane protein heterogeneity within *Pseudomonas fluorescens* and *P. putida* and use of an OprF antibody as a probe for rRNA homology group I pseudomonads. Appl. Environ. Microbiol. **62:**480–485.
20. **Lambert, B., F. Leyns, L. van Rooyen, F. Gossele, Y. Papon, and J. Swings.** 1987. Rhizobacteria of maize and their antifungal activities. Appl. Environ. Microbiol. **53:**1866–1871.
21. **Lemanceau, P., T. Corberand, L. Gardan, X. Latour, G. Laguerre, J.-M. Boeufgras, and C. Alabouvette.** 1995. Effect of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.), on the diversity of soilborne populations of fluorescent pseudomonads. Appl. Environ. Microbiol. **61:**1004–1012.
22. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Murray, R. G. E., R. N. Doetsch, and C. F. Robinow.** 1994. Determinative and cytological light microscopy, p. 21–41. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
24. **Palleroni, N. J.** 1984. Pseudomonas, p. 141–199. *In* B. Tansill (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore.
25. **Palleroni, N. J.** 1986. Taxonomy of the pseudomonads, p. 3–25. *In* J. R. Sokatch (ed.), The bacteria (the biology of pseudomonads). Academic Press Inc., Orlando, Fla.
26. **Palleroni, N. J.** 1992. Present situation of the taxonomy of aerobic pseudomonads, p. 105–115. *In* E. Galli, S. Silver, and B. Winholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
27. **Palleroni, N. J.** 1993. *Pseudomonas* classification. Antonie Leeuwenhoek **64:**231–251.
28. **Parekh, N. R., A. Hartmann, M.-P. Charnay, and J.-C. Fournier.** 1995. Diversity of carbofuran-degrading soil bacteria and detection of plasmid-encoded sequences homologous to the *mcd* gene. FEMS Microbiol. Ecol. **17:**149–160.
29. **Ridgway, H. F., J. Safarik, D. Phipps, P. Carl, and D. Clark.** 1990. Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. Appl. Environ. Microbiol. **56:**3565–3575.
30. **Sanseverino, J., B. M. Applegate, J. M. H. King, and G. S. Sayler.** 1993. Plasmid-mediated mineralization of naphthalene, phenanthrene, and anthracene. Appl. Environ. Microbiol. **59:**1931–1937.
31. **Scarlett, C. M., J. T. Fletcher, P. Roberts, and R. A. Lelliott.** 1978. Tomato pith necrosis caused by *Pseudomonas corrugata* n. sp. Ann. Appl. Biol. **88:** 105–114.
32. **Smibert, R. M., and N. R. Krieg.** 1994. Phenotypic characterization, p. 607–654. *In* P. Gerhardt, R. G. E. Murray, W. A. Wood, and N. R. Krieg (ed.), Methods for general and molecular bacteriology. American Society for Microbiology, Washington, D.C.
33. **Sørensen, J., J. Skouv, A. Jørgensen, and O. Nybroe.** 1992. Rapid identifi-

cation of environmental isolates of *Pseudomonas aeruginosa*, *P. fluorescens* and *P. putida* by SDS-PAGE analysis of whole-cell protein patterns. FEMS Microbiol. Ecol. **101:**41–50.

34. **Stanier, R. Y., N. J. Palleroni, and M. Doudoroff.** 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:**159–271.

34a. **Verbarg, S.** Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. Personal communication.

35. **Versalovic, J., V. Kapur, E. O. Mason, U. Shah, T. Koeuth, J. R. Lupski, and J. M. Musser.** 1993. Penicillin resistant *Streptococcus pneumoniae* strains recovered in Houston, Texas: identification and molecular characterization of multiple clones. J. Infect. Dis. **167:**850–856.

36. **Versalovic, J., T. Koeuth, and J. R. Lupski.** 1991. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. Nucleic Acids Res. **19:**6823–6831.

37. **Wauters, G., A. Boel, G. P. Voorn, J. Verhaegen, F. Meunier, M. Janssens, and L. Verbist.** 1995. Evaluation of a new identification system, crystal enteric/non-fermenter, for gram-negative bacilli. J. Clin. Microbiol. **33:**845–849.