International Journal of Systematic and Evolutionary Microbiology (2002), **52**, 131–139 Printed in Great Britain

PAH-degradation by *Paenibacillus* spp. and description of *Paenibacillus naphthalenovorans* sp. nov., a naphthalene-degrading bacterium from the rhizosphere of salt marsh plants

L. L. Daane,[1]† I. Harjono,[1] S. M. Barns,[2] L. A. Launen,[1,3] N. J. Palleroni[1,3] and M. M. Häggblom[1,3]

[1] Biotechnology Center for Agriculture and the Environment, Foran Hall, Cook College, Rutgers University, 59 Dudley Road, New Brunswick, NJ 08901-8520, USA

[2] Bioscience Division, Environmental Molecular Biology Group, M888, Los Alamos National Laboratory, Los Alamos, NM 87545, USA

[3] Department of Biochemistry and Microbiology, Lipman Hall, Cook College, Rutgers University, 76 Lipman Drive, New Brunswick, NJ 08901-8525, USA

Author for correspondence: Max Häggblom. Tel: +1 732 932 9763 ext. 326. Fax: +1 732 932 8965. e-mail: haggblom@aesop.rutgers.edu

Bacteria belonging to the genus *Paenibacillus* were isolated by enrichment from petroleum-hydrocarbon-contaminated sediment and salt marsh rhizosphere using either naphthalene or phenanthrene as the sole carbon source, and were characterized using phenotypic, morphological and molecular techniques. The isolates were grouped by their colony morphologies and polyaromatic hydrocarbon-degradation patterns. Phenanthrene-degrading isolates produced mottled colonies on solid media and were identified as *P. validus* by fatty acid methyl ester and 16S rRNA gene sequence analyses. In contrast, the naphthalene-degrading isolates with mucoid colony morphology were distantly related to *Paenibacillus validus*, according to fatty acid methyl ester and 16S rRNA gene sequence analyses. The predominant fatty acids of the mucoid isolates were 15:0 anteiso, 16:1ω11c, 16:0 and 17:0 anteiso, constituting, on average, 50·5, 12·0, 11·2 and 6·5% of the total, respectively. The G+C contents of their DNA ranged from 47 to 52 mol%. The 16S rDNA sequence analysis revealed the highest (≤94%) similarity to *P. validus*. In addition, phylogenetic analyses based on 16S rDNA sequences showed that the mucoid isolates formed a distinct cluster within *Paenibacillus*. DNA–DNA hybridization experiments showed only a 6% DNA similarity between the type strain of *P. validus* and mucoid strain PR-N1. On the basis of the morphological, phenotypic and molecular data, the naphthalene-degrading isolates merit classification as a new *Paenibacillus* species, for which the name *Paenibacillus naphthalenovorans* sp. nov. is proposed, with PR-N1^T (= ATCC BAA-206^T = DSM 14203^T) as the type strain.

Keywords: *Paenibacillus*, rhizosphere, polycyclic aromatic hydrocarbons

INTRODUCTION

In 1991, Ash *et al*., determined the phylogenetic structure of the genus *Bacillus* by using the small-subunit rRNA sequences of 51 species. The study revealed five phylogenetically distinct clusters, group 3 bacilli being separated as the new genus *Paenibacillus* (Ash *et al*., 1993). Since 1993, the genus *Paenibacillus* has grown from 11 species to over 26 species. Sporogenesis enables *Bacillus*, *Paenibacillus* and related organisms to withstand environmentally stressful conditions, allowing long-term survival and making them virtually ubiquitous (Claus & Berkeley, 1986). *Paenibacillus* species have been isolated from a wide variety of sources including soil, water, the plant rhizosphere, plant materials, food, fodder, faeces and diseased insect larvae (Alexander & Priest, 1989; Claus & Berkeley, 1986; Kanzawa *et al*., 1995; Montefusco *et al*., 1993; Nakamura, 1984, 1987; Seldin *et al*., 1984; Shida *et al*., 1997b; Yoon *et al*., 1998).

We have isolated a variety of Gram-positive and

†**Present address:** Avon Products, Inc., Avon Place, Suffern, NY 10901-5605, USA.

Abbreviations: ED, Euclidean distance; FAME, fatty acid methyl ester; PAH, polyaromatic hydrocarbon.

Gram-negative polyaromatic hydrocarbon (PAH)-degrading bacteria from estuarine sediment and the rhizospheres of salt marsh plants (*Distichlis spicata*, *Juncus gerardi*, *Spartina alterniflora* and *Sporobolus airoides*). In the process, we have isolated endospore-forming bacteria by using a pasteurization enrichment technique (Daane *et al*., 2001). Initial assignment of the isolates to the genus *Paenibacillus* was based on their fatty acid methyl ester (FAME) profiles. These isolates were further grouped by their colony morphologies and PAH-degradation profiles. Isolates exhibiting non-mucoid, mottled colonies were tentatively identified as *Paenibacillus validus*, while isolates forming mucoid colonies were judged to be distant relatives of *P. validus*, *Paenibacillus alvei* and *Paenibacillus pabuli*. In the present investigation, we used morphological, phenotypic and molecular techniques to assess the taxonomic position of the mucoid *Paenibacillus* isolates. We have determined that the mucoid group is distinct from previously identified species of the genus *Paenibacillus*, and we propose the new species *Paenibacillus naphthalenovorans*.

METHODS

Bacterial isolates and growth conditions. Spore-forming PAH-degrading bacteria were obtained from pasteurized (80 °C, 10 min), petroleum-hydrocarbon-contaminated estuarine sediment and salt marsh plant rhizospheres by aerobic enrichment using either naphthalene, phenanthrene or biphenyl as the sole carbon source (Daane *et al*., 2001). The tentatively identified *Paenibacillus* isolates separated into two distinct morphological groups: one formed mucoidal colonies, and the other did not (Table 1). The colony morphologies were maintained over repeated subculturing as well as reculturing from frozen stocks. The type strains of the following species were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and used as controls in the phenotypic and whole-cell fatty acid analyses: *P. alvei* ATCC 6344, *P. pabuli* ATCC 43899, *Paenibacillus polymyxa* ATCC 842 and *Paenibacillus validus* ATCC 43897^T.

Bacterial strains were grown on trypticase soy (Beckton Dickinson) agar (TSA) plates for phenotypic, phylogenetic and FAME analyses. Mineral salts basal (MSB) medium (Stanier *et al*., 1966), containing 2% (w/v) Noble agar (Difco) and naphthalene, biphenyl or phenanthrene as the carbon source was used routinely for maintaining the PAH-degrading phenotype. The bacteria were routinely grown aerobically at 30 °C except where indicated otherwise. Isolates were maintained at 4 °C, and were kept in 50% (v/v) glycerol at −80 °C for long-term storage.

FAME analysis. Whole-cell fatty acid (FAME) analyses were performed on all of the ATCC type strains and PAH-degrading isolates by growing the cells at 28 °C for 24 h on TSA plates. Cellular fatty acids were saponified, methylated and extracted and then analysed by GC by following the procedures given for the Sherlock Microbial Identification System (MIDI). Identification and comparison were made using the Aerobe (TSBA version 3.9) database of the Sherlock Microbial Identification System. The DENDROGRAM program resident in the MIDI software package was used to construct unweighted pair matching based on fatty acid data.

Table 1. Polyaromatic hydrocarbon (PAH)-degrading spore-forming bacteria isolated by pasteurized enrichment from contaminated estuarine sediment and plant rhizospheres

Isolate*	Source†	Enrichment substrate
Mucoid		
PR-N1^T	*Spartina alterniflora*	Naphthalene
PR-N2	*Spartina alterniflora*	Naphthalene
PR-N5	*Spartina alterniflora*	Naphthalene
PR-N11	*Spartina alterniflora*	Naphthalene
PR-N12	*Spartina alterniflora*	Naphthalene
PR-N16	*Spartina alterniflora*	Naphthalene
PS-N1	Estuarine sediment	Naphthalene
PS-N6	Estuarine sediment	Naphthalene
SA-N1	*Sporobolus airoides*	Naphthalene
Non-mucoid		
PR-N3	*Spartina alterniflora*	Naphthalene
PR-N4	*Spartina alterniflora*	Naphthalene
PR-N13	*Spartina alterniflora*	Naphthalene
PR-N19	*Spartina alterniflora*	Naphthalene
PR-N20	*Spartina alterniflora*	Naphthalene
PR-N21	*Spartina alterniflora*	Naphthalene
PR-N22	*Spartina alterniflora*	Naphthalene
DS-N1	*Distichlis spicata*	Naphthalene
DS-N2	*Distichlis spicata*	Naphthalene
DS-N3	*Distichlis spicata*	Naphthalene
JG-N1	*Juncus gerardi*	Naphthalene
JG-N2	*Juncus gerardi*	Naphthalene
JG-N3	*Juncus gerardi*	Naphthalene
PR-B1	*Spartina alterniflora*	Biphenyl
PR-B2	*Spartina alterniflora*	Biphenyl
PR-P1	*Spartina alterniflora*	Phenanthrene
PR-P2	*Spartina alterniflora*	Phenanthrene
PR-P6	*Spartina alterniflora*	Phenanthrene
PR-P9	*Spartina alterniflora*	Phenanthrene
PR-P10	*Spartina alterniflora*	Phenanthrene
PR-P11	*Spartina alterniflora*	Phenanthrene
PR-P13	*Spartina alterniflora*	Phenanthrene

* Mucoid and non-mucoid refer to colony morphologies when grown on solid media.

† Estuarine sediment and *Spartina alterniflora* were obtained from Piles Creek, Linden, NJ, USA. *Distichlis spicata*, *Juncus gerardi* and *Sporobolus airoides* were obtained from The University of Delaware Marine Station, Lewes, DE, USA.

Morphological and phenotypic characterizations. The morphology of the ATCC type strains and PAH-degrading isolates was observed using a phase-contrast microscope. Trypticase soy broth (2 ml) was inoculated with the bacteria being tested and was incubated at 37 °C on a rotary wheel. Motility and morphology were observed at 24, 48 and 72 h in drops of culture placed onto 1% (w/v) agarose-coated and -uncoated glass slides. The agarose coating enabled more detailed examination of motile cells. Flagella were stained according to the method of Leifson (1951). Photomicrographs were taken using a Leitz Planfluotar oil immersion lens (NA 1.32).

The bacterial isolates and type strains were phenotypically

characterized by using the API 20E and 50CH system tests (bioMérieux Vitek) as described by Logan & Berkeley (1984). Other phenotypic tests included catalase activity, starch and casein hydrolyses, and the effects of temperature, anaerobiosis and salinity on growth (Smibert & Krieg, 1994). For testing individual aromatic growth substrates, MSB agar medium, containing one of the following aromatic compounds, was used: naphthalene, phenanthrene, biphenyl, phthalate, gentisate, *o*-, *m*- and *p*-hydroxybenzoate or benzoate (minimum 98% pure; Aldrich). Naphthalene and biphenyl were added in the vapour phase as crystals in the Petri-dish lid. Phenanthrene was added as a 2% (w/v) Noble agar overlayer onto MSB agar plates at a final concentration of 1 mg ml^{-1} ($\sim$ 5·6 mM). The remaining organic acids were added from filter-sterilized stock solutions to autoclaved molten MSB agar medium at final concentrations ranging from 5 to 10 mM.

DNA preparation. Total genomic DNA for phylogenetic and G+C-content analyses was isolated from bacterial cells grown on TSA plates for 48 h using a modification of a method described by Wilson (1994). The collected bacterial pellets were pretreated with lysozyme (2·5 mg ml^{-1}) for 1 h at 37 °C. The preparation and labelling of reference strain DNA for hybridization experiments were carried out as described by Song *et al.* (1998). DNA was diluted in 0·1 × standard saline citrate (1 × standard saline citrate is 0·15 M sodium citrate, 0·15 M NaCl, pH 7·0) and examined for purity spectrophotometrically at 260 and 280 nm. The reading at 260 nm was used for quantitative estimation, by assuming an absorption coefficient of 20.

Phylogenetic analysis of 16S rDNA sequences. The 16S rDNA was PCR-amplified as described by Daane *et al.* (2001), using the eubacterial primers 27f and 1522r (Johnson, 1994b). Five microlitres of each reaction was run on a 1% agarose gel, and the DNA was visualized by UV illumination following staining with ethidium bromide. The amplified PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. DNA sequences were determined directly from the purified PCR products with automated fluorescent *Taq* cycle sequencing using an ABI 373A sequencer (Applied Biosystems). Approximately 100 ng purified DNA was used for one automated fluorescent sequencing reaction. The primers for sequencing used in this study were 27f, 704f, 926f, 1242f, 321r, 685r, 907r, 1220r and 1522r (Johnson, 1994b).

Small-subunit rRNA sequences of *Bacillus* and *Paenibacillus* reference strains were obtained from GenBank and the Ribosomal Database Project (Maidak *et al.*, 1999). Sequences were aligned initially using the CLUSTAL X editor (Thompson *et al.*, 1999). This alignment was refined manually, using the Genetic Data Environment Editor (Maidak *et al.*, 1999). Because sequence positions corresponding to *Escherichia coli* 16S rRNA (Gutell, 1993) nucleotides 69–94 were highly heterogeneous and unalignable, they were excluded from the analyses.

Maximum-likelihood analysis was performed using fastDNAml (Olsen *et al.*, 1994) with empirical base frequencies and an optimized transition/transversion ratio of 1·3. The tree of highest likelihood was found by repeated tree building (100 ×) using random sequence input order, with global branch rearrangement of the final tree. Unweighted least squares distance matrix (Jukes–Cantor correction) and unweighted maximum-parsimony trees were inferred with PAUP 4.0b3a (D. Swofford, distributed by Sinauer Associates), using 100 rounds of random sequence addition order and optimized by tree-bisection-reconstruction branch swapping. Bootstrap analyses by maximum-likelihood and maximum-parismony methods were performed on 100 resampled datasets each. A dissimilarity matrix was calculated from the mean character differences for each pair of sequences by using PAUP 4.0b3a.

DNA base composition. The G+C content of the genomic DNA was estimated using the method described by Mesbah *et al.* (1989), with some modifications. The samples were analysed by HPLC (model SCL-10A; Shimadzu) equipped with a Supelcosil LC-18 column (250 × 4·6 mm; particle size, 5 μm; Supelco) with KH_2PO_4 (49 mM, adjusted to pH 4·0 with H_3PO_4)-methanol (90:10, 1·5 ml min^{-1}) as the eluant, with detection at 280 nm using a Shimadzu SPD-10A UV-visible variable-wavelength detector.

DNA–DNA hybridization. The DNA preparations were sheared by two passages through a French press. DNA–DNA hybridization was done using the S1 nuclease method (Johnson, 1994a), with the modifications described by Song *et al.* (1998). The hybridization was performed at a reannealing temperature of 57 °C based on the G+C content of the DNA.

RESULTS AND DISCUSSION

Morphological features of the PAH-degrading, spore-forming *Paenibacillus* isolates

Two morphologically distinct groups of spore-formers were isolated from petroleum-hydrocarbon-contami-

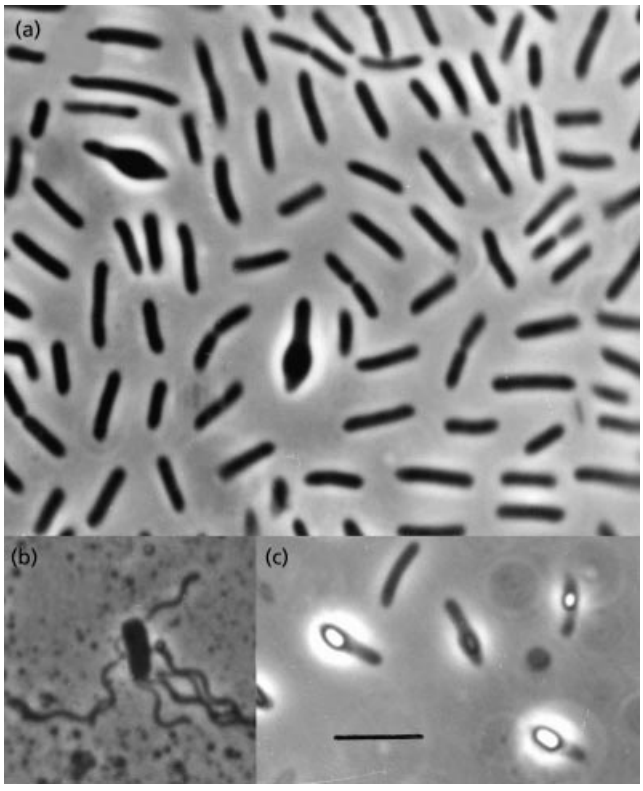

Fig. 1. Strain PR-N1^T. (a) A crowded area of a wet mount showing that the cells are separated from one another because of the capsular material. (b) Peritrichous flagella stained by Leifson's method. (c) Spores in swollen sporangia. Bar, 5 μm.

Table 2. Whole-cell fatty acid compositions (%, w/w of total) of the PAH-degrading *Paenibacillus* isolates and several type species of the genera *Paenibacillus*, as determined by GC analysis of their methyl esters

Species or isolate*	14:0 iso	14:0	15:0 iso	15:0 anteiso	15:0	16:1 ω7*c* alcohol	16:0 iso	16:1 ω11*c*	16:0	iso 17:1 ω10*c*	17:0 iso	17:0 anteiso
Mucoid												
PR-N1^T	ND	2·5	5·3	51·8	ND	ND	5·6	9·3	14·4	ND	3·2	7·9
PR-N5	ND	3·7	4·7	47·9	ND	ND	4·3	16·7	13·4	ND	2·8	6·5
PS-N1	2·3	2·8	6·0	55·0	1·5	ND	5·5	9·8	9·0	ND	1·9	6·3
PS-N6	1·8	2·8	5·3	52·9	1·5	1·9	5·9	12·0	8·5	ND	1·7	5·8
SA-N1	1·9	2·8	5·6	49·8	2·1	1·5	5·2	12·8	8·9	ND	2·9	6·5
PR-N16	1·3	2·7	6·8	45·4	3·5	1·0	4·7	11·6	12·7	ND	4·3	6·1
Non-mucoid												
DS-N2	3·7	1·7	13·7	50·7	0·9	2·8	7·0	5·7	4·7	1·0	3·3	4·3
JG-N2	3·9	1·8	14·4	49·4	0·7	3·1	8·1	5·4	4·5	1·0	3·4	3·8
PR-N13	2·6	1·4	10·1	52·3	1·0	1·7	7·9	4·5	7·0	0·9	4·3	5·7
PR-N19	2·9	1·6	11·0	50·7	1·0	2·3	8·3	5·3	6·7	0·9	3·4	5·1
PR-N21	3·0	1·3	13·8	53·9	ND	2·7	7·3	4·3	4·3	1·1	3·5	4·7
PR-P1	3·4	1·7	11·7	54·0	0·8	3·2	6·6	6·1	4·2	ND	3·0	4·4
PR-P9	2·9	1·7	12·7	46·3	1·0	3·1	7·5	7·1	5·6	1·4	4·2	5·1
P. validus ATCC 43897^T	2·7	1·8	13·2	48·0	0·9	2·2	6·8	6·9	6·4	1·7	4·3	5·3
P. polymyxa ATCC 842^T	2·9	3·4	6·8	48·6	ND	ND	11·0	1·8	15·9	ND	3·7	5·8
P. pabuli ATCC 43899^T	3·5	3·7	10·9	48·6	ND	ND	5·7	6·3	13·2	ND	4·9	3·2
P. alvei ATCC 6344^T	1·3	2·2	8·7	51·4	5·2	1·1	6·0	3·7	8·7	2·9	3·1	6·7

ND, Not detected.

* Mucoid and non-mucoid refer to the colony morphologies of the PAH-degrading *Paenibacillus* isolates. The mucoid strains represent the proposed *Paenibacillus naphthalenovorans* sp. nov., and the non-mucoid isolates are members of *P. validus*.

nated estuarine sediment and salt marsh rhizospheres by aerobic enrichment using either naphthalene, phenanthrene or biphenyl as the sole carbon source (Daane *et al.*, 2001). Strains PR-N1^T, PR-N5, PR-N16, PS-N1, PS-N6 and SA-N1 formed white, translucent, mucoid colonies when grown on solid medium. In contrast, a non-mucoid, mottled colony morphology was created by the remaining isolates (Table 1). Fig. 1 shows mucoid strain PR-N1^T observed by phase-contrast microscopy. Observation of high-density areas shows that the cells were separated from one another by capsular material (Fig. 1a). A cell with peritrichous flagellation is depicted in Fig. 1b. Spores in swollen sporangia are shown in Fig. 1c.

FAME analysis

FAME analysis suggested that the two morphological groups of PAH-degrading isolates were members of the genus *Paenibacillus* (Daane *et al.*, 2001). The non-mucoid isolates were identified as *P. validus*, having a strong match with the MIDI database (similarity index $\geqslant 0{\cdot}70$). In contrast, the mucoid isolates were only distantly related to either *P. validus*, *P. pabuli* or *P. alvei*. Table 2 lists the fatty acid compositions of the mucoid and non-mucoid isolates, and the type strains of several *Paenibacillus* species. The predominant cellular fatty acid for all of the tested isolates and type strains was 15:0 anteiso, comprising 45–55% of the total; this is indicative of the genus *Paenibacillus* (Ash *et al.*, 1993; Shida *et al.*, 1997a). However, the mucoid isolates differ from the closely related *P. validus* type strain and non-mucoid isolates in the relative abundance of several fatty acids (15:0 iso, 16:1ω11*c*, 16:0 and 17:1ω10), as highlighted in Table 2. The level of 15:0 iso in the mucoid group was $5{\cdot}6 \pm 0{\cdot}7$%, as opposed to 13·2% and $12{\cdot}5 \pm 1{\cdot}6$% for *P. validus* and the non-mucoid group, respectively. Similarly, the levels of 16:1ω11*c* and 16:0 were $12{\cdot}0 \pm 2{\cdot}6$% and $11{\cdot}2 \pm 2{\cdot}6$%, respectively, in the mucoid group, as opposed to 6·9% and 6·4%, respectively, for *P. validus*. In addition, the unsaturated acid, iso 17:1ω10, was not detected in any of the mucoid isolates; the levels were 1·7% and $0{\cdot}9 \pm 0{\cdot}4$% in the type strain of *P. validus* and the non-mucoid group, respectively.

A pairwise comparison Euclidean distance (ED) dendrogram based on whole-cell fatty acid compositions of the sediment and rhizosphere *Paenibacillus* isolates and ATCC type strains (data not shown) indicated that members of the mucoid-colony-morphology group form a tight cluster ($ED \leqslant 8{\cdot}0$) and are distantly related to their most closely identified *Paenibacillus* species ($ED = 13{\cdot}0$). In contrast, close clustering of the members of the non-mucoid-morphology group suggested conspecificity of these isolates with respect to the *P. validus* type strain ($ED \leqslant 5$). These results indicate that the members of the mucoid

cluster form a distinct group and may represent a new *Paenibacillus* species.

Phenotypic characterization

The mucoid, non-mucoid groups and type strains of selected *Paenibacillus* were differentiated phenotypically using the API 20E and 50CH test systems (Table 3). Acetoin (Voges–Proskauer test) was produced by the mucoid isolates and the *P. polymyxa* type strain, but not by the other organisms. The mucoid isolates were all unable to hydrolyse aesculin or produce acid from glycerol, D-ribose, D-raffinose or starch, unlike the non-mucoid isolates (D-raffinose variable) and the *Paenibacillus* type strains. Moreover, the mucoid isolates and the *P. alvei* type strain did not produce acid from D-xylose or glycogen; all other strains tested were positive for this trait. Lastly, while the mucoid isolates did not ferment inositol, the non-mucoid isolates and the *P. validus* type strain did. Other differences between the two isolate groups and the type strains are shown in Table 3.

Growth of the PAH-degrading strains on a variety of aromatic substrates is summarized in Table 4. The non-mucoid isolates utilized a greater number of aromatic substrates than the mucoid isolates. Of the substrates tested, gentisate, phthalate and *o*-hydroxybenzoate were not suitable growth substrates for either groups. Aromatics utilized by both groups included naphthalene, *p*-hydroxybenzoate and benzoate. Only the mucoid group utilized *m*-hydroxybenzoate. In contrast, phenanthrene and biphenyl were growth substrates only for the non-mucoid isolates, and not for the mucoid group.

Utilization of aromatic substrates by members of the genus *Paenibacillus* has been reported previously. Pichinoty *et al.* (1986) described *Bacillus gordonae* sp. nov. (emended as *P. validus* by Heyndrickx *et al.*, 1995), which utilized *p*-hydroxybenzoate, phthalate, isophthalate, protocatechuate, trimellitate, quinate, phenol, *p*-cresol and naphthalene as sole carbon sources. Recently, Meyer *et al.* (1999) reported the isolation of a PAH-degrading micro-organism, tentatively identified as a *Paenibacillus* sp., from tar oil-contaminated soil. In the present study, we found that naphthalene was a suitable growth substrate for *P. validus* but not *P. alvei*, *P. pabuli* or *P. polymyxa*.

Phylogenetic analyses

A dissimilarity matrix (not shown) of the rRNA sequences was calculated. Intragroup values ranged from 0·001 to 0·007 for the mucoid group, and from 0·001 to 0·012 for the non-mucoid group (which included *P. validus*). Intergroup values of 0·029–0·093 were measured between the mucoid or non-mucoid groups and the recognized *Paenibacillus* spp. These data suggested that the mucoid group was a distinct *Paenibacillus* species. Moreover, the non-mucoid isolates were shown to be strains of *P. validus*.

Detailed analyses using several inference methods generated trees that were congruent in topology for well-supported (> 70% by bootstrap analyses) branches (Fig. 2). The distinct grouping of the mucoid strains separately from all named *Paenibacillus* spp. is strongly supported (100% by maximum-likelihood and maximum-parsimony methods). Phylogenetic relationships between isolates within this group, however, were unresolved. Strong support was also found for a close relationship between the *P. validus* and the non-mucoid strains (DS-N1, PR-P1, PR-P9 and PR-N19). These two groups cluster together specifically, to the exclusion of all other *Paenibacillus* spp., with high bootstrap support (97/99%). These results support the conclusion that the mucoid isolates represent a distinct group of *Paenibacillus* that is closely related to *P. validus*. In addition, the PAH-degrading isolates, *P. validus*, *Paenibacillus chrondrotinus*, *Paenibacillus alginolyticus* and *P. larvae* form a coherent group to the exclusion of all other *Paenibacillus* species, with moderate (81/74%) bootstrap support.

DNA base composition of *Paenibacillus* isolates

The G+C content for the genus *Paenibacillus* ranges broadly from 39 mol% (reported for *Paenibacillus macquariensis*; Claus & Berkeley, 1986) to 55 mol% (for *Paenibacillus dendritiformis*; Tcherpakov *et al.*, 1999). The G+C content of the genomic DNA of members of the mucoid group and several isolates from the non-mucoid group is shown in Table 5. The results for both the mucoid and non-mucoid groups fall within the range for the genus *Paenibacillus*. The mean (±standard deviation) G+C content for the mucoid group was 48·8±1·9 mol%. This value is close to that found for *P. validus* ATCC 43897^T, but less than that of the non-mucoid group (50·6±2·2 mol%).

DNA hybridization experiments

Table 5 also summarizes the DNA similarity values obtained by hybridization using the S1 nuclease methodology. The similarity between reference PR-N1^T and other members of the mucoid group was high (71–97%), with the exception of strain SA-N1 (46%). A low similarity value of 6% was observed between strain PR-N1^T and the *P. validus* type strain. The reciprocal reaction, using *P. validus* as the reference DNA, revealed a low level of similarity to strain PR-N1^T (13%), and high levels of similarity to members of the non-mucoid group. These results show that the members of the mucoid group comprise a taxon distinct from the non-mucoid group and *P. validus*. DNA similarity analysis confirmed the identification of the non-mucoid PAH-degrading isolates as members of *P. validus*.

In summary, on the basis of the morphological, phenotypic and molecular data, the naphthalene-degrading mucoid isolates can be classified into a new species of *Paenibacillus*, for which we propose the name *P. naphthalenovorans*.

Table 3. Phenotypic characteristics of PAH-degrading strains isolated from sediment and rhizospheres, and closely related *Paenibacillus* strains

All strains were motile, catalase-positive, grew at 30 and 37 °C, did not grow at 5 or 55 °C, and did not grow with 5% NaCl. All isolates were negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S production and tryptophan deaminase. All isolates produced acid from maltose, saccharose and trehalose, but none produced acid from erythritol, L-xylose, sorbitol, methyl α-D-mannoside, D-lyxose, D-fucose, L-arabitol, 2-keto-gluconate and 5-keto-gluconate. +, 90% of strains reacted positively; v, less than 90% of strains reacted positively or negatively; −, 90% of strains reacted negatively.

Characteristic	*P. alvei* ATCC 6344^T	*P. pabuli* ATCC 43899^T	*P. polymyxa* ATCC 842^T	*P. validus* ATCC 43897^T	Group 1 (mucoid) ($n = 6$)	Group 2 (non-mucoid) ($n = 6$)
Anaerobic growth	+	−	+	−	−	−
Growth with 3% NaCl	−	+	+	−	v	−
Growth at 10 °C	−	+	−	−	−	−
Hydrolysis of:						
Casein	+	+	+	−	−	−
Starch	+	+	+	+	v	+
API 20E tests						
ONPG	+	+	+	−	−	−
Citrate	−	−	−	−	v	−
Urease	+	−	−	+	+	+
Indole production	+	−	−	−	−	−
Voges–Proskauer	−	−	+	−	+	−
Gelatin hydrolysis	+	−	+	−	−	−
Nitrate reduction	−	+	−	−	v	−
API 50CH tests						
Aesculin hydrolysis	+	+	+	+	−	+
Acid produced from:						
Glycerol	+	+	+	+	−	+
D-Arabinose	−	+	−	−	−	−
L-Arabinose	−	+	+	−	−	−
Ribose	+	+	+	+	−	+
D-Xylose	−	+	+	+	−	+
Adonitol	+	−	−	−	−	−
Methyl β-xyloside	−	+	+	−	−	−
Galactose	−	+	+	+	+	+
D-Glucose	−	+	+	+	+	+
D-Fructose	−	+	+	+	v	+
D-Mannose	−	+	+	+	+	v
L-Sorbose	−	−	−	−	v	−
Rhamnose	−	+	−	−	−	−
Dulcitol	−	−	−	−	−	+
Inositol	−	+	−	+	−	+
Mannitol	−	+	+	+	+	+
Methyl α-D-glucoside	−	+	+	+	v	+
N-Acetylglucosamine	+	+	−	−	−	−
Amygdalin	−	+	+	−	−	−
Arbutin	+	+	+	−	−	−
Salicin	−	+	+	−	−	−
Cellobiose	−	+	+	−	−	v
Lactose	−	+	+	−	−	−
Melibose	+	+	+	+	v	v
Inulin	−	+	+	−	−	−
Melezitose	−	+	+	−	−	−
D-Raffinose	+	+	+	+	−	v
Starch	+	+	+	+	−	+
Glycogen	−	+	+	+	−	+
Xylitol	−	−	−	−	v	−

Table 3 (*cont.*)

Characteristic	*P. alvei* ATCC 6344^T	*P. pabuli* ATCC 43899^T	*P. polymyxa* ATCC 842^T	*P. validus* ATCC 43897^T	Group 1 (mucoid) ($n = 6$)	Group 2 (non-mucoid) ($n = 6$)
β-Gentiobiose	+	+	+	−	−	V
D-Turanose	−	+	+	+	V	+
D-Tagatose	−	−	−	−	−	V
L-Fucose	−	+	−	−	−	−
D-Arabitol	−	−	−	−	−	V
Gluconate	−	+	−	−	−	−

Table 4. Growth of isolates from sediment and rhizospheres on aromatic substrates

Positive results were obtained for all strains for the utilization of naphthalene and benzoate. Negative results were obtained with all strains for the utilization of phthalate, gentisate and *o*-hydroxybenzoate. +, Growth of the isolate after 1 week of incubation at 30 °C; −, no growth; ND, not determined.

Substrate*	PAH-degrading *Paenibacillus* isolates†					
	Mucoid colony morphology		Non-mucoid colony morphology			
	PS-N1	PR-N1^T	PR-N5	PR-N4	PR-P1	PR-B2
Phenanthrene	−	−	−	+	+	−
Biphenyl	−	−	−	+	+	+
m-Hydroxybenzoate	−	+	+	−	−	ND
p-Hydroxybenzoate	−	+	+	+	+	ND

* Substrate concentrations: naphthalene and biphenyl were added as crystals in the Petri-dish lid; phenanthrene (1 mg ml^{-1}) was added as an agarose overlayer; organic acids were added to media at 5–10 mM.

† Mucoid isolates represent *P. naphthalenovorans* sp. nov.; non-mucoid strains were identified as *Paenibacillus validus*.

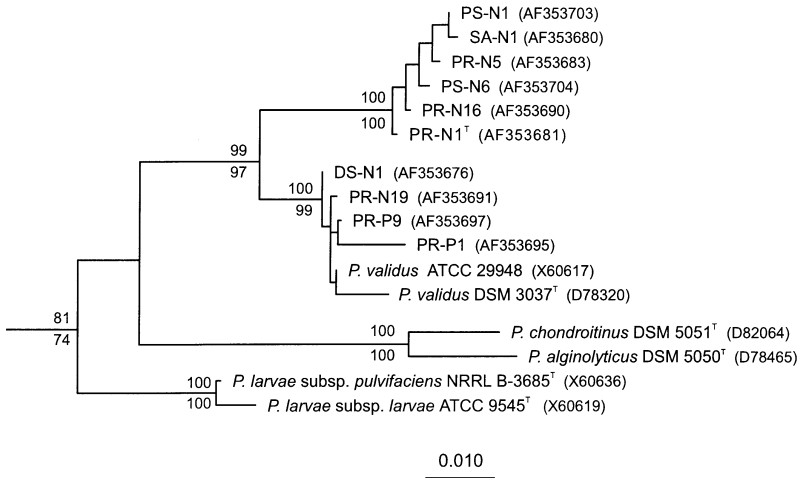

Fig. 2. Phylogenetic analysis of the polyaromatic hydrocarbon-degrading *Paenibacillus* isolates. The tree was inferred by maximum-likelihood analysis of homologous nucleotide positions of 16S rDNA sequence from each organism. An additional 21 sequences, representing the *Paenibacillus* type strains for which 16S rRNA sequences are available, were included in the analysis (not shown) and branched as an outgroup to the sequences shown. Numbers indicate percentages of bootstrap resamplings that support branches for maximum-likelihood/maximum-parsimony analyses. Bootstrap results are reported only for those branches that attained > 70% support with at least one method.

Description of *Paenibacillus naphthalenovorans* sp. nov.

Paenibacillus naphthalenovorans (naph.tha.le.no.vo'rans. L. neut. n. *naphthalene* from Persian *neft* naphtha; L. v. *vorare* to devour; N.L. part. adj. *naphthalenovorans* naphthalene-devouring).

Cells are rod-shaped and are 0·8 μm wide by 2·8–4·0 μm long, are motile by peritrichous flagella, and are

Table 5. G+C content and DNA binding data for PAH-degrading *Paenibacillus* isolates and *P. validus*

Strain	mol %G+C	DNA similarity (%) with:	
		PR-N1^T	*P. validus*
P. validus ATCC 43897^T	48·7	6·0	100
Mucoid			
PR-N1^T	46·6	100	13·2
PS-N1	51·5	97·0	ND
PR-N5	50·1	87·6	ND
PR-N16	49·9	87·3	ND
PS-N6	47·7	70·9	ND
SA-N1	47·2	45·5	ND
Non-mucoid			
PR-P1	50·1	ND	72·2
PR-N19	53·0	ND	79·0
DS-N1	48·8	ND	ND

ND, Not determined.

Gram-positive. Ellipsoidal spores are formed in swelling sporangia. Optimum growth occurs between 30 and 37 °C, but does not occur at either 10 or 55 °C. Does not grow in the presence of 5 % (w/v) NaCl, and growth with 3 % (w/v) NaCl is variable. Forms white, translucent, mucoid colonies when grown on solid media. Strictly aerobic. Catalase-positive. Casein hydrolysis is negative; starch hydrolysis is variable. Gelatin and ONPG are not hydrolysed, and arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase, hydrogen sulfide and indole are not produced. Citrate utilization and nitrate reduction are variable. Urease and acetoin are produced. Acid, but no gas, is produced from galactose, D-glucose, maltose, mannitol, D-mannose, saccharose and trehalose. Strain variation is observed for fermentation of D-fructose, melibose, methyl α-D-glucoside, L-sorbose, D-turanose and xylitol. Neither acid nor gas is produced from *N*-acetylglucosamine, adonitol, starch, amygdalin, D-arabinose, L-arabinose, D-arabitol, L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D-fucose, L-fucose, β-gentiobiose, gluconate, glycerol, glycogen, inositol, inulin, 2-keto-gluconate, 5-keto-gluconate, lactose, D-lyxose, melezitose, methyl α-D-mannoside, methyl β-xyloside, D-raffinose, L-rhamnose, D-ribose, salicin, sorbitol, D-tagatose, D-xylose and L-xylose. The predominant cellular fatty acids are 14:0 (2·9 %), 15:0 iso (5·6 %), 15:0 anteiso (50·5 %), 16:0 iso (5·2 %), 16:1ω11*c* (12·0 %), 16:0 (11·2 %), 17:0 iso (2·8 %) and 17:0 anteiso (6·5 %). The mean G+C content of the DNA is 49 mol %, as determined by HPLC. The habitats are estuarine sediments and salt marsh rhizospheres. Isolate PR-N1^T is the type strain (= ATCC BAA-206^T = DSM 14203^T). The description of this strain is essentially identical to that of the species. This strain does not grow in the presence of 3 % NaCl, is negative for starch hydrolysis, and does not utilize citrate or reduce nitrate. It produces acid, but not gas, from D-fructose, methyl α-D-glucoside, L-sorbose and D-turanose. No acid or gas is produced from melibose or xylitol.

ACKNOWLEDGEMENTS

This work was supported, in part, by a grant from the New Jersey Commission on Science and Technology. We wish to thank Jean-Alex Molina for assistance with French translations. We also thank Quinn Im, Michael Murillo, Lisa Newman and Bongkeun Song for helpful discussions and technical assistance. We acknowledge Cheryl Kuske for helpful suggestions regarding the manuscript and for the use of laboratory resources, computers and software for phylogenetic analyses.

REFERENCES

Alexander, B. & Priest, F. G. (1989). *Bacillus glucanolyticus*, a new species that degrades a variety of β-glucans. *Int J Syst Bacteriol* **39**, 112–115.

Ash, C., Farrow, J. A. E., Wallbanks, S. & Collins, M. D. (1991). Phylogenetic heterogeneity of the genus *Bacillus* revealed by comparative analysis of small-subunit-ribosomal RNA sequences. *Lett Appl Microbiol* **13**, 202–206.

Ash, C., Priest, F. G. & Collins, M. D. (1993). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie Leeuwenhoek* **64**, 253–260.

Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Daane, L. L., Harjono, I., Zylstra, G. J. & Häggblom, M. M. (2001). Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Appl Environ Microbiol* **67**, 2683–2691.

Gutell, R. R. (1993). Collection of small subunit (16S- and 16S-like) ribosomal RNA structures. *Nucleic Acids Res* **21**, 3051–3054.

Heyndrickx, M., Vandemeulebroecke, K., Scheldeman, P. & 7 other authors (1995). *Paenibacillus* (formerly *Bacillus*) *gordonae* (Pichinoty et al. 1986) Ash et al. 1994 is a later subjective synonym of *Paenibacillus* (formerly *Bacillus*) *validus* (Nakamura 1984) Ash et al. 1994: emended description of *P. validus*. *Int J Syst Bacteriol* **45**, 661–669.

Johnson, J. L. (1994a). Similarity analysis of DNAs. In *Methods for General and Molecular Bacteriology*, pp. 655–682. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Johnson, J. L. (1994b). Similarity analysis of rRNAs. In *Methods for General and Molecular Bacteriology*, pp. 683–700. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Kanzawa, Y., Harada, A., Takeuchi, M., Yokota, A. & Harada, T. (1995). *Bacillus curdlanolyticus* sp. nov. and *Bacillus kobensis* sp. nov., which hydrolyze resistant curdlan. *Int J Syst Bacteriol* **45**, 515–521.

Leifson, E. (1951). Staining, shape, and arrangement of bacterial flagella. *J Bacteriol* **62**, 377–389.

Logan, N. A. & Berkeley, R. C. W. (1984). Identification of *Bacillus* strains using the API system. *J Gen Microbiol* **130**, 1871–1882.

Maidak, B. L., Cole, J. R., Parker, C. T. & 11 other authors (1999). A new version of the RDP (Ribosomal Database Project). *Nucleic Acids Res* **27**, 171–173.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.

Meyer, S., Moser, R., Neef, A., Stahl, U. & Kämpfer, P. (1999). Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. *Microbiology* **145**, 1731–1741.

Montefusco, A., Nakamura, L. K. & Labeda, D. P. (1993). *Bacillus peoriae* sp. nov. *Int J Syst Bacteriol* **43**, 388–390.

Nakamura, L. K. (1984). *Bacillus pulvifaciens* sp. nov., nom. rev. *Int J Syst Bacteriol* **34**, 410–413.

Nakamura, L. K. (1987). *Bacillus alginolyticus* sp. nov. and *Bacillus chondroitinus* sp. nov., two alginate-degrading species. *Int J Syst Bacteriol* **37**, 284–286.

Olsen, G. J., Matsuda, H., Hagstrom, R. & Overbeek, R. (1994). fastDNAml: a tool for construction of phylogenetic trees of DNA sequences using maximum likelihood. *CABIOS* **10**, 41–48.

Pichinoty, F., Waterbury, J. B., Mandel, M. & Asselineau, J. (1986). *Bacillus gordonae* sp. nov., une nouvelle espèce appartenant au second groupe morphologique, dégrandant divers composés aromatiques. *Ann Inst Pasteur/Microbiol* (Paris) **137A**, 65–78.

Seldin, L., van Elsas, J. D. & Penido, E. G. C. (1984). *Bacillus azotofixans* sp. nov., a nitrogen-fixing species from Brazilian soils and grass roots. *Int J Syst Bacteriol* **34**, 451–456.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997a). Transfer of *Bacillus alginolyticus*, *Bacillus chondroitinus*, *Bacillus curdlanolyticus*, *Bacillus glucanolyticus*, *Bacillus kobensis*, and *Bacillus thiaminolyticus* to the genus *Paenibacillus* and emended description of the genus *Paenibacillus*. *Int J Syst Bacteriol* **47**, 289–298.

Shida, O., Takagi, H., Kadowaki, K., Nakamura, L. K. & Komagata, K. (1997b). Emended description of *Paenibacillus amylolyticus* and description of *Paenibacillus illinoisensis* sp. nov. and *Paenibacillus chibensis* sp. nov. *Int J Syst Bacteriol* **47**, 299–306.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

Song, B., Young, L. Y. & Palleroni, N. J. (1998). Identification of denitrifier strain T1 as *Thauera aromatica* and proposal for emendation of the genus *Thauera* definition. *Int J Syst Bacteriol* **48**, 889–894.

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

Tcherpakov, M., Ben-Jacob, E. & Gutnick, D. L. (1999). *Paenibacillus dendritiformis* sp. nov., proposal for a new pattern-forming species and its localization within a phylogenetic cluster. *Int J Syst Bacteriol* **49**, 239–246.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1999). The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876–4882.

Wilson, K. (1994). Preparation of genomic DNA from bacteria. In *Current Protocols in Molecular Biology*, vol. 1, pp. 2.4.1–2.4.5. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. New York: Wiley.

Yoon, J.-H., Yim, D. K., Lee, J.-S., Shin, K.-S., Sato, H. H., Lee, S. T., Park, Y. K. & Park, Y.-H. (1998). *Paenibacillus campinasensis* sp. nov., a cyclodextrin-producing bacterium isolated in Brazil. *Int J Syst Bacteriol* **48**, 833–837.