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Paenibacillus agarexedens sp. nov., nom. rev., and *Paenibacillus agaridevorans* sp. nov.

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Twenty-two agarolytic, aerobic, spore-forming strains were characterized taxonomically by DNA–DNA reassociation experiments, riboprint analyses, 16S rDNA sequencing and phenetic similarity analyses. Based on riboprint analyses, the strains formed eight ribogroups, six of which contained 2–6 strains and two encompassed single strains. Within the multi-strain ribogroups, similarities ranged from 91–99 %. Phylogenetic analyses of representatives of the eight groups by 16S rDNA sequence analysis showed that the strains were affiliated to the genus *Paenibacillus*, but relatedness to described *Paenibacillus* species was only moderate (<97.8 % sequence similarity). Published DNA–DNA similarity values for most of the agarolytic strains, supplemented with new data, supported the distinctiveness of the eight ribogroups. Intragroup DNA–DNA similarity values ranged from 80 to 104 %, while intergroup DNA–DNA similarities were <35 %. Based on genomic distinctiveness and supported by the presence of distinguishing phenotypic properties, multi-strain groups 1 and 2 are proposed as novel species, *Paenibacillus agarexedens* sp. nov., nom. rev. (type strain, DSM 1327^T = CIP 107437^T) and *Paenibacillus agaridevorans* sp. nov. (type strain, DSM 1355^T = CIP 107436^T).

INTRODUCTION

In 1941, Wieringa isolated and described agarolytic, spore-forming organisms and proposed a novel species with the name '*Bacillus agar-exedens*' (Wieringa, 1941). As these strains were lost (K. T. Wieringa, personal communication), this species was not included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1989) and therefore has no standing in nomenclature. A new attempt to reisolate Wieringa's agarolytic spore-forming strains led to the collection of a considerable number of organisms (Hunger & Claus, 1978). Some of these isolates (designated growth requirement group I) matched the original description of '*Bacillus agar-exedens*'. These organisms were characterized

by inhibition of growth by peptone on neutral to slightly acid mineral/glucose medium, an effect that was neutralized by the addition of urea to the medium. A second group of agarolytic strains was characterized by lack of growth inhibition by peptone and a moderate to strong requirement for carbohydrates (designated growth requirement group II); these strains matched the description of '*Bacillus palustris* var. *gelaticus*' (Sickles & Shaw, 1934). Within groups I and II, phenotypic properties varied and the G + C content of the DNA covered a broad range (Hunger, 1978; Hunger & Claus 1978, 1981). By extensive DNA–DNA hybridization studies, the genomic diversity of the isolates was confirmed and group I was divided into DNA subgroups 1, 2, 3, 4 and 5, and group II into DNA subgroups 6 and 7. Hunger & Claus (1981) concluded that each of these groups might represent a novel species, but did not validly publish names for them.

Based on the results of Hunger & Claus (1978, 1981), the agarolytic strains were subjected to a renewed taxonomic study, using classical methods and novel genomic approaches that did not exist 25 years ago.

METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. Twenty strains had been isolated during 1970–1976 at the DSMZ. One strain had been obtained

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Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences determined in this study are AJ345017 (DSM 1352), AJ345018 (DSM 6358), AJ345019 (DSM 1482), AJ345020 (DSM 1327^T), AJ345021 (DSM 1474), AJ345022 (DSM 34), AJ345023 (DSM 1355^T) and AJ345024 (DSM 1481).

Micrographs of cells and spores of strains DSM 1327^T and DSM 1355^T are available as supplementary data in IJSEM Online.

Table 1. List of bacterial strains used in this study and differentiation of groups of agarolytic *Paenibacillus* strains

Growth requirement groups I and II according to Hunger & Claus (1978); members of group I are inhibited by peptone; members of group II are not inhibited by peptone; NA, not applicable; ND, not determined.

Strain	Source	Isolated by	DNA similarity group (intragroup hybridization, %)	DNA G + C content (mol%)	Ribopattern group
Growth requirement group I					
DSM 1327 ^T =strain 10 ^T	Meadow soil, Göttingen, Germany	I. Miehlmann, 1972	1 (80–104)	47–49	1
DSM 1473 ^T =strain 4	Compost, Göttingen, Germany	D. Claus, 1970			
DSM 1475 = strain 12	Ditch mud, Ossendorf, Germany	I. Miehlmann, 1972			
DSM 1477 = strain 26b	Field soil, Yugoslavia	D. Claus, 1970			
DSM 1478 = strain 31a	Forest soil, Lüneburg, Germany	I. Miehlmann, 1972			
DSM 1479 = strain 31b	Forest soil, Lüneburg, Germany	I. Miehlmann, 1972	2 (96–102)	50–52	2
DSM 1355 ^T =strain 65 ^T	Volcanic soil, Paricutin volcano, Mexico	W. Hunger, 1975			
DSM 1483 = strain 62	Garden soil, Göttingen, Germany	W. Hunger, 1974			
DSM 1484 = strain 64	Forest soil, Göttingen, Germany	W. Hunger, 1974			
DSM 1485 = strain 68	Savanna soil, Tuxtla, Mexico	W. Hunger, 1975			
DSM 1486 = strain 69	Garden soil, Göttingen, Germany	W. Hunger, 1976	3 (97–104)	52–53	3
DSM 1354 = strain 34	Forest soil, Celle, Germany	I. Miehlmann, 1972			
DSM 1474 = strain 8	Lawn soil, Hannover, Germany	I. Miehlmann, 1972			
DSM 1480 = strain 33	Forest soil, Dransfeld, Germany	I. Miehlmann, 1972			
DSM 1356 = strain 35	Forest soil, Göttingen, Germany	I. Miehlmann, 1972			
DSM 1482 = strain M	Contaminated agar plate, Göttingen, Germany	K. A. Malik, 1972	4 (97–100)	44–45	4
DSM 1352 = strain 39	Garden soil, Karlsruhe, Germany	D. Claus, 1970	5 (NA)	44	5
Growth requirement group II					
DSM 1358 = strain 19	Forest soil, Göttingen, Germany	I. Miehlmann, 1972	6 (97–100)	49–50	6
DSM 1481 = strain 44	Field soil, Rothamsted, England	D. Claus, 1970	7 (98–100)	51	7
DSM 34 = strain 32829 = ATCC 14176	Decayed tree stump	M. Shaw (<i>B. palustris</i> var. <i>gelaticus</i>), 1934			
DSM 1487 = strain 70	Lawn soil, Göttingen, Germany	W. Hunger, 1976			
No growth requirement group					
DSM 6358	Contaminant on agar plate	D. P. Stahly, Univ. Iowa, USA	– (NA)	ND	8

from the American Type Culture Collection (ATCC), Manassas, VA, USA, and one strain was donated in 1991 by D. P. Stahly, University of Iowa, IA, USA. Cultures were grown in nutrient broth medium supplemented with 0.1 % (w/v) urea. The pH after autoclaving was 7.3–7.5. Sterile glucose solution was added to this basal medium to a final concentration of 1 % (w/v). For solid media, 1.5 % (w/v) agar was added. Incubation was carried out at 30 °C.

Phenotypic tests and cell wall analysis. Physiological characterizations were based on the methods of Gordon *et al.* (1973), with the modifications described by Hunger & Claus (1981). Other tests were done as described by Hunger & Claus (1981). Several additional tests and repetitions were performed in the present study: hydrolysis of starch [using Lugol's iodine solution as well as ethanol precipitation according to Gordon *et al.* (1973)], hydrolysis of urea and pectin (Wieringa, 1947) and production of dextranase (Mencier, 1972). Diaminopimelic acid (A₂pm) was determined by the method described by Schleifer & Kandler (1972). For microphotographs, cultures were prepared as described by Claus & Berkeley (1986).

DNA–DNA hybridization and ribotyping. DNA was isolated by chromatography on hydroxyapatite, as described by Cashion *et al.* (1977). DNA–DNA hybridizations were carried out according to De Ley *et al.* (1970). Renaturation rates were calculated using the computer program TRANSFER.BAS (Jahnke, 1992). Automated ribotyping of the isolates was accomplished by using the DuPont Qualicon RiboPrinter system (Bruce, 1996) and *EcoRI* as the standard restriction enzyme for cutting genomic DNA.

16S rDNA sequence determination and analysis. Genomic DNA extraction, PCR-mediated amplification of 16S rDNA and purification of PCR products were carried out as described by Rainey *et al.* (1996). Purified PCR products were sequenced with *Taq* DyeDeoxy Terminator Cycle Sequencing kits (Applied Biosystems), as directed in the manufacturer's protocol. An Applied Biosystems 373A DNA sequencer was used for electrophoresis of the sequence reaction products. The ae2 alignment editor (Maidak *et al.*, 1999) was used to align the 16S rDNA sequences determined in this study against those of representatives of the main bacterial lineages, available from public databases. Pairwise evolutionary distances were computed by using the correction of Jukes & Cantor (1969). The phylogenetic dendrogram was reconstructed from a distance matrix using the treeing algorithm of DeSoete (1983) and Felsenstein (1993). Strains and accession numbers are indicated in Fig. 2.

RESULTS AND DISCUSSION

Riboprint analysis

The 22 strains fell into eight ribogroups. Within the six groups that were defined by more than one strain, the patterns were almost identical and displayed similarities of >91 %. Similarity increased to >95 % when the comparison was based on the arithmetic mean of the ribogroup pattern. Low similarity values (10–30 %) separated these groups. Convincing congruence was found between the results of two methods that targeted the genome, i.e. DNA–DNA hybridization and analysis of the *rrn* operon. All previous groupings of strains revealed by DNA–DNA hybridization (80–104 %; systematic error of ± 5 %) were mirrored completely in corresponding ribogroups. The ribogroup of isolate DSM 6358, not investigated by Hunger & Claus (1981), was unique (Table 1, Fig. 1).

Fig. 1. Congruence of riboprint pattern and DNA–DNA similarity groups.

DNA–DNA reassociation

Table 2 is a modification of a table shown by Hunger & Claus (1981), supplemented with new data. Intragroup DNA similarities were >80 %, while intergroup similarities were <35 %. Riboprint analysis assigned the phenotypically similar strains DSM 1486 and DSM 1355^T to ribogroup 2; in contrast, DNA–DNA hybridization of these two strains (Hunger & Claus, 1981) revealed only 63 % similarity. Repetition of the DNA reassociation experiment gave a value of 98 %, indicating that these strains are indeed genomically closely related. Separation of ribogroups 1 and 7 was obvious from phenotypic results, but had not been verified by DNA–DNA hybridization. DSM 34 (ribogroup 7) shared only 20 % DNA similarity with DSM 1327^T (ribogroup 1), thus supporting the results obtained by analysis of the *rrn* operon.

16S rDNA analysis

Phylogenetic positions, based on the almost-complete 16S rDNA sequences of one representative of each group, are shown in Fig. 2. Binary similarity values are indicated in Table 2. Levels of 16S rDNA similarity range from 98.7 % (DSM 1355^T versus DSM 6358) to 91.4 % (DSM 1352 versus DSM 34). All strains are members of the genus *Paenibacillus* and each representative occupies a distinct phylogenetic position. Similarity values obtained for each of the representative strains and all type strains of *Paenibacillus* species varied between 90.5 and 97.3 %, indicating that none of the agarolytic isolates is a member of a described species. These results suggest that all agarolytic strains in the present study should be renamed as *Paenibacillus* species. Interestingly, several of the known *Paenibacillus* species have been described as being capable of degrading unusual

Table 2. Intragroup (diagonal) and intergroup (upper-right triangle) DNA–DNA hybridization values, together with binary 16S rDNA sequence similarity values of representative strains of each group (lower-left triangle)

Representative strains of each DNA subgroup for 16S rDNA binary similarity analysis: DNA subgroup 1, DSM 1327^T; 2, DSM 1355^T; 3, DSM 1474; 4, DSM 1482; 5, DSM 1352; 6, DSM 1481; 7, DSM 34; 8, DSM 6358. All values are percentages. NA, Not applicable; ND, not determined; <xx, highest value determined.

DNA subgroup	1 (n=6)	2 (n=5)	3 (n=3)	4 (n=2)	5 (n=1)	6 (n=2)	7 (n=2)	8 (n=1)
1	80–104	<32	<32	29	22	<20	20	ND
2	95·3	96–102	<34	24	21	<31	15	ND
3	95·7	97·6	97–104	ND	ND	22	ND	ND
4	93·5	92·2	92·8	97–100	14	26	ND	ND
5	95	94·7	93·6	91·3	NA	21	ND	ND
6	98·7	96·2	96	93·5	94·9	97–100	24	ND
7	91·6	91·6	91·8	96	90	91·6	98–100	ND
8	95·4	98·7	97·3	91·9	95	95·9	91·4	NA

Fig. 2. Neighbour-joining tree based on 16S rDNA data, showing the phylogenetic positions of strains DSM 1327^T, DSM 1355^T, DSM 1474, DSM 1352, DSM 1481, DSM 34 and DSM 6358 within the genus *Paenibacillus*. Sequences in the tree were selected from a comparison of 16S rDNA sequence from all *Paenibacillus* spp. with validly published names. Sequences of members of the *Bacillus*/*Streptococcus* group were used to root the dendrogram. Bootstrap analyses were made with 500 resamplings; bootstrap values > 75% are indicated on branching nodes. Bar, 5% sequence divergence.

polysaccharides and are named accordingly, e.g. *Paenibacillus alginolyticus*, *Paenibacillus chondroitinus*, *Paenibacillus curdlanolyticus* and *Paenibacillus glucanolyticus*.

Cell-wall analysis

Whole-cell lysates were examined for the presence or absence of A₂pm. A number of strains were found not to contain A₂pm, but results were not consistent within subgroups. Repetition of the analysis using purified cell walls did improve the picture, but still not satisfactorily. Inconsistent detection of A₂pm may be due to a very thin peptidoglycan layer in these organisms, as suggested by uneven or even Gram-negative staining.

Phenotypic characterization

Some of the key phenotypic traits that distinguish members of individual subgroups of growth requirement groups I and II (Hunger, 1978), were repeated in the present study. A complete listing of reactions is given by Hunger (1978) and Hunger & Claus (1981). While the results for pectin hydrolysis and dextranase activity were confirmed, those for starch hydrolysis only partially matched previous findings. Urea hydrolysis could not be confirmed at all. Different findings for starch hydrolysis were probably due to different methodologies: the method described by Gordon *et al.* (1973) resulted in a positive reaction for all strains. However, when starch hydrolysis was visualized by flooding the agar with Lugol's iodine solution, the results of Hunger & Claus (1981) were confirmed, i.e. subgroups 1, 5, 6 and 7 were positive, while subgroups 2, 3 and 4 were negative. The rationale for Hunger & Claus (1981) not to describe novel species for the emerging groups was the physiological inactivity and phenotypic similarity of the strains enclosed. Only a few properties were found that were coherent for members of a DNA subgroup, but discriminatory between the DNA subgroups (Table 3). By and large, re-examination of a selection of those properties of Hunger & Claus (1981) in the present work generally confirmed their findings.

Strains DSM 1487 and DSM 34 define subgroup 7. The

Table 3. Phenotypic characteristics that differentiate between eight DNA subgroups of agarolytic *Paenibacillus* strains

+, Positive; –, negative; v, variable among strains; n, number of strains tested.

Test	Growth requirement group I					Growth requirement group II		
	1 (n=6)	2 (n=5)	3 (n=3)	4 (n=2)	5 (n=1)	6 (n=2)	7 (n=2)	8 (n=1)
Starch hydrolysis (Lugol)*	+	–	–	–	+	+	+	–
Pectin hydrolysis	–	–	+	+	–	–	+	–
Dextranase	–	+	–	v	+	+	–	–
Pigment formation	–	–	–	–	+	–	–	–
Deamination of phenylalanine	v	–	+	+	–	–	–	–

*When starch hydrolysis was visualized using ethanol, all strains showed a positive reaction.

latter strain was originally described as '*Bacillus palustris* var. *gelaticus*' by Sickles & Shaw (1934). Later, this name was accepted as being synonymous with *Bacillus circulans* and thus strain DSM 34 was included in this species by Gordon *et al.* (1973). However, the high G+C content of its DNA (51 mol%) and the high 16S rDNA sequence similarity to *Paenibacillus* species clearly exclude a close relationship to *B. circulans sensu stricto*, as defined by Nakamura & Swezey (1983a, b). The type strain of *B. circulans*, DSM 11^T, has a DNA G+C content of 36 mol% and is an authentic member of the genus *Bacillus*.

The properties described by Wieringa (1941) are difficult to interpret today and can hardly be compared with modern results. According to findings obtained by Wieringa (1941), growth of all strains of '*Bacillus agar-exedens*' is inhibited by peptone and the effect is reversed by the addition of urea. Members of DNA subgroups 1–5 do indeed react this way. Wieringa further described the strains as being strong starch hydrolysers, which is not true for members of DNA subgroups 2–4. This leaves members of DNA subgroups 1 and 5 as candidates to match the original description of '*Bacillus agar-exedens*'. As the single strain of DNA subgroup 5, DSM 1352, forms a yellowish pigment on mineral/glucose/yeast medium and Wieringa did not mention any pigmentation of the isolates studied, it is concluded that the six strains of DNA subgroup 1 most closely resemble Wieringa's original description.

16S rDNA and DNA–DNA similarities determined in the present study clearly point towards the presence of eight novel genospecies of the genus *Paenibacillus*. Additional ribotyping experiments on all genospecies confirm their distinctiveness and show, for those taxa that contain more than a single strain, that they are genomically homogeneous. Agarolytic activity, the salient phenotypic characteristic of the strains, has not been described so far for any of the *Paenibacillus* species with validly published names, and thus makes the novel species easily distinguishable from all of them. However, we refrain from describing as species those of our genospecies that contain three or fewer strains. It is suggested that additional strains in groups 3–8 should

be isolated, to enable broadened investigation on physiological properties in order to better separate these putative species, and to search for intraspecies variation. For an overview of phenotypic traits of species of the genus *Paenibacillus*, see Heyndricks *et al.* (1996), Shida *et al.* (1997) and Petterson *et al.* (1999).

As already suggested by Hunger & Claus (1981), the specific epithet *agar-exedens* (ex Wieringa 1941) should be revived for a novel *Paenibacillus* species that embraces the six strains of DNA subgroup 1. We propose *Paenibacillus agarexedens* sp. nov., nom. rev., with the spelling corrected according to Rule 12a of the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992). A detailed phenotypic description is given below.

We also propose *Paenibacillus agaridevorans* sp. nov. for the five members of DNA subgroup 2, which are physiologically distinguishable from all other agarolytic strains by the combination of characteristics shown in Table 3. A detailed phenotypic description is given below.

Description of *Paenibacillus agarexedens* (ex Wieringa 1941) sp. nov., nom. rev.

Paenibacillus agarexedens [a.gar.ex.e'dens. Malayan n. *agar* agar (algal polysaccharide); N.L. n. *agarum* agar; L. v. *exedere* to eat up, utilize; N.L. part. adj. *agarexedens* agar-utilizing].

Motile rods, width 0.5–1.4 µm, length 2–8 µm (type strain: 0.5–0.8 µm, 3–5 µm). Unstained cells are not granulated. Gram reaction in 12 h cultures is uneven (dappled); after 38 h, cells are Gram-negative. Spores are ellipsoidal and most (> 50 %) of the sporangia are not swollen. Colonies on agar media sink into the agar within a few days (see Fig. 3); no liquefaction of agar occurs. Colonies on peptone/urea agar are whitish and round with entire margins; no pigmentation occurs on mineral/glucose/yeast extract medium. Chemo-organotrophic. Growth is inhibited by peptones; inhibition may be neutralized by urea. Catalase- and oxidase-positive. Mesophilic; maximum temperature for growth is 40 °C (type strain: 35 °C). Positive for hydrolysis of agar, starch, hippurate and aesculin. Acid is

Fig. 3. Colonies of agarolytic organisms sinking into agar. Photograph taken from Hunger & Claus (1978), p. 107; with kind permission of Kluwer Academic Publishers.

produced from agar and glucose. Shows weak aminopeptidase activity. Negative for anaerobic growth, growth at pH 5.7 and in 5% NaCl, Voges–Proskauer test, urease, nitrate reduction, activities of egg-yolk lecithinase, dextranase, DNase and lysine decarboxylase, hydrolysis of poly- β -hydroxybutyric acid, casein, pectin, Tween 80 and chitin, production of indole, dihydroxyacetone and dextrin crystals, anaerobic gas production from nitrate, alkali or acid production in litmus milk, liquefaction of gelatin and resistance to lysozyme and sodium lauryl sulfate. Variable reactions are observed for deamination of phenylalanine, tyrosine degradation (type strain is positive) and methylene blue reduction (type strain is negative). The G+C content of the DNA is 47–49 mol% (type strain, 47 mol%), as determined by the thermal denaturation method.

Type strain is 10^T=DSM 1327^T=CIP 107437^T. Isolated from meadow soil in Göttingen, Germany, in 1972.

Description of *Paenibacillus agaridevorans* sp. nov.

Paenibacillus agaridevorans [a.ga.ri.de.vo'rans. Malayan n. agar agar (algal polysaccharide); N.L. n. *agarum* agar; L. part. adj. *devorans* consuming, devouring; N.L. part. adj. *agaridevorans* agar-devouring].

Motile rods, width 0.6–0.8 μ m, length 2–5 μ m. Unstained cells are not granulated. Gram-reaction in 12 h cultures is uneven (dappled); after 38 h, cells are Gram-negative. Spores are ellipsoidal and most of the sporangia (>50%) are not swollen. Colonies on agar media sink into the agar within a few days; no liquefaction of agar occurs. Colonies on peptone/urea agar are whitish and round with entire margins; no pigmentation occurs on mineral/glucose/yeast extract medium. Chemo-organotrophic. Growth is inhibited by peptones; inhibition may be neutralized by urea.

Catalase- and oxidase-positive. Mesophilic; maximum temperature for growth is 35 °C. Positive for hydrolysis of agar, dextran, hippurate and aesculin. Acid is produced from agar and glucose. Negative for aminopeptidase, anaerobic growth, growth at pH 5.7 and in 5% NaCl, Voges–Proskauer test, nitrate reduction, urease, activities of egg-yolk lecithinase, DNase and lysine decarboxylase, hydrolysis of starch (ethanol precipitation, positive; flooding with Lugol's iodine solution, negative), poly- β -hydroxybutyric acid, casein, pectin, Tween 80 and chitin, tyrosine degradation, deamination of phenylalanine, production of indole, dihydroxyacetone and dextrin crystals, anaerobic gas production from nitrate, alkali or acid production in litmus milk, methylene blue reaction, liquefaction of gelatin and resistance to lysozyme and sodium lauryl sulfate. The G+C content of the DNA is 50–52 mol% (type strain, 51 mol%) as determined by the thermal denaturation method.

Type strain is 65^T=DSM 1355^T=CIP 107436^T. Isolated from volcanic soil, Paricutin volcano, Mexico, in 1975.

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