Paenibacillus jamilae sp. nov., an exopolysaccharide-producing bacterium able to grow in olive-mill wastewater

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Endospore-forming strains were isolated from corn-compost treated with olive-mill wastewater (‘alpechín’). The strains were taxonomically studied and proposed as a novel Paenibacillus species. These organisms (strains B.3T, B.7 and B.9) were particularly distinguishable from other aerobic spore-forming species by their ability to grow optimally in 100% (v/v) olive-mill wastewater at 30 °C and pH 7-0 and concomitant production of an interesting exopolysaccharide. Chemotaxonomic analysis revealed that MK-7 was the predominant menaquinone, the major fatty acid was anteiso C15:0 and the cell wall contained meso-diaminopimelic acid. The DNA G+C content was 40-7 mol%. Comparative sequence analysis of 16S rDNA with different reference species from the genera Bacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Alicyclobacillus, Halobacillus, Virgibacillus, Amphibacillus, Coprobacillus and Gracilibacillus indicated that the isolated strains were highly related to the genus Paenibacillus. Strain B.3T formed an evolutionary lineage distinct from other species within the evolutionary radiation encompassing the genus Paenibacillus. Strain B.3T was a close relative of Paenibacillus polymyxa, but DNA–DNA relatedness data with this species was very low (relative binding ratio <16%). Based on the morphological and physiological characteristics, as well as on the phylogenetic position determined by 16S rDNA analysis and DNA–DNA relatedness data, it is concluded that these strains should be designated a novel species, for which the name Paenibacillus jamilae sp. nov. is proposed. The type strain is B.3T (=CECT 5266T = DSM 13815T).

Keywords: Paenibacillus jamilae sp. nov., olive-mill wastewater, exopolysaccharide, 16S rDNA analysis

INTRODUCTION

Olive-mill wastewater (OMWW), known as ‘alpechín’, is a toxic aqueous residue produced in olive oil extraction. The most important olive oil-producing countries are Spain and Italy. Bioremediation of alpechín by using the residue as a culture medium for some micro-organisms can also provide new resources, such as exopolysaccharides (EPSs), which are highly interesting biopolymers due to their potential applications (Ramos-Cormenzana et al., 1995). Developments in molecular biological methods have suggested that the genus Bacillus is a phylogenetically heterogeneous taxon. For example, analyses showed a wide range of G+C contents (32–69 mol%) among the DNAs of Bacillus species (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991; Stackebrandt & Liesack, 1993). DNA reassociation studies have shown that many Bacillus species are composites of several genetically unrelated species (Priest, 1981; Slepecky & Hemphill, 1991; Nakamura, 1996; Shida et al., 1997b). Resulting phylogenetic studies by 16S rRNA gene sequencing revealed that the genus Bacillus could be

Abbreviations: EPS, exopolysaccharide; OMWW, olive-mill wastewater; RBR, relative binding ratio.
The GenBank/EMBL/DDJ accession number for the 16S rDNA sequence of strain B.3T is J271157.
separated into several phylogenetically distinct genera such as *Alicyclobacillus* (Wisotzkey et al., 1992), *Paenibacillus* (Ash et al., 1993), *Aneurinibacillus* (Shida et al., 1996), *Brevibacillus* (Shida et al., 1996), *Halobacillus* (Spring et al., 1996), *Gracilibacillus* and *Salibacillus* (Waino et al., 1999), and *Coprobacillus* (Kageyama & Benno, 2000).

In the present study, three strains (B.3, B.7 and B.9) of Gram-variable, spore-forming bacilli were isolated from a corn-compost treated with OMWW. The strains were able to grow in 100% (v/v) OMWW and to produce an heteropolysaccharide consisting of fucose, xylose, rhamnose, arabinose, mannose, galacto- and glucose as sugar components (V. Guerra, M. Monteoliva-Sánchez, M. Aguilera & A. Ramos-Cormenzana, unpublished data). This EPS has been shown to enhance the immune response in mice (Ramos-Cormenzana & Monteoliva-Sánchez, 2000). Based on phenotypic properties and DNA G+C content, these organisms were tentatively identified as *Bacillus* species, closely related to *Bacillus firmus*, *Brevibacillus laterosporus*, *Bacillus lentus* and *Paenibacillus polymyxa*. To establish their correct taxonomic position and to rectify the tenuous classification, strains B.3, B.7 and B.9 were further characterized by 16S rDNA sequencing. Data suggested that the strains B.3, B.7 and B.9 could be a *Paenibacillus* species. After carefully studying the data obtained by phenotypic characterization, DNA G+C content, DNA–DNA relatedness and 16S rDNA analysis, a novel species, *Paenibacillus jamilae* sp. nov., is proposed.

**METHODS**

**Bacterial strains and culture conditions.** Strains B.3, B.7 and B.9 were isolated from a sample of corn-compost treated with OMWW. The following reference strains were included in the study: *Bacillus firmus* CCM 2213T, *Bacillus lentus* CCM 2214T, *Brevibacillus laterosporus* CCM 2116T and *P. polymyxa* CECT 153. All strains were grown on YM broth (Difco) and 15 g agar (Difco) l−1 (if needed). Strains B.3, B.7 and B.9 were cultivated at 30 °C for 24 h on TSA (Difco) and TSB (Difco) supplemented with 0.5% (w/v) glycine for the isolation of DNA.

**Morphological and physiological tests.** The morphology of cells, motility, shape of spores, swollen sporangia and parasporal crystal were examined by phase-contrast microscopy. The presence of EPS and size of the cells were examined by transmission electron microscopy using cells extracted by treating 500 mg lyophilized cells grown in YM broth with 150 ml chloroform: methanol (2:1, v/v) for 2 h, using a reciprocal shaker (120 strokes min⁻¹) at room temperature. The extracted solution was concentrated using liquid N₂ and transferred by re-dissolving in acetone. The resulting solution was evaporated, separated by TLC using n-hexane: dimethyl ether (85:15, v/v) as the solvent and recovered from TLC plates with acetone. Isoprenoid quinones in the cell wall was performed by TLC (DC-Alufoline Cellulose; Merck) as described by Yamada & Komaga (1970).

**Chemotaxonomic characterization**

Analysis of isoprenoid quinones. Isoprenoid quinones were extracted by treating 500 mg lyophilized cells grown in YM broth with 150 ml chloroform: methanol (2:1, v/v) for 2 h, using a reciprocal shaker (120 strokes min⁻¹) at room temperature. The extracted solution was concentrated using liquid N₂ and transferred by re-dissolving in acetone. The resulting solution was evaporated, separated by TLC using n-hexane: dimethyl ether (85:15, v/v) as the solvent and recovered from TLC plates with acetone. Isoprenoid quinones thus obtained were analysed by HPLC (Waters) as described by Yamada et al. (1998).

Analyses of cellular fatty acids and the cell wall. For cellular fatty acid analysis, cells were cultivated in YM broth until the late exponential phase of growth. Methods for fatty acid extraction, methyl ester preparation, methyl ester separation by GC and identification of fatty acids were previously described (Monteoliva-Sánchez et al., 1993). Identification of meso-diaminopimelic acid in the cell wall was performed by TLC (DC-Alufoline Cellulose; Merck) as described by Yamada & Komaga (1970).

**Isolation of DNA and determination of G+C content.** Chromosomal DNA was isolated by the method of Marmur (1961). DNA preparations were analysed by electrophoresis through 0.8% (w/v) agarose gel, spectrophotometrically quantified at 260 nm (Biophotometer-Eppendorf) and stored at −20 °C. The G+C content was determined by the method of Marmur & Doty (1962), based on the determination of DNA TₘDNA from *Escherichia coli* was included in each set of analyses as a reference standard. The G+C content of *E. coli* DNA was regarded as 51.7 mol%.

**DNA–DNA reassociation and 16S rDNA sequencing.** The hybridization of genomic DNA from strain B.3T with B.7, B.9, *Bacillus firmus*, *B. lentus*, *B. laterosporus* and *P. polymyxa* was performed according to the method of Ziemke et al. (1998).

Purified genomic DNA was used for the amplification of 16S rDNA genes via PCR with the forward primer 16F27 (5’-AGAGTTTGATCMTGGCTCAG-3’) and the reverse primer 16R1525 (5’-AAGGAGGTGWTCCARCC-3’). PCR reactions were carried out under the conditions described by Saiki et al. (1988). Amplification products were purified with Microcon-100 concentrators (Amicon) and directly sequenced using an Applied Biosystems 373A DNA sequencer and the manufacturer’s protocols for Taq cycle-sequencing with fluorescent dye-labelled dideoxynucleotides (Perkin-Elmer). The primers used for the sequencing reactions were previously described (Lane, 1991).

**Phylogenetic analysis.** To establish the relatives of strain B.3T based on 16S rDNA sequence similarity, preliminary searches in different databases were performed with the program BLAST. Sequences of close relatives (*Paenibacillus*) were retrieved from databases and were aligned with the sequences of other *Bacillus*, *Lactobacillus*, *Alicyclobacillus*, *Aneurinibacillus*, *Virgibacillus*, *Brevibacillus*, *Amphibacillus*, *Halobacillus*, *Gracilibacillus* and *Coprobacillus* species using
RESULTS AND DISCUSSION

Morphological and physiological characteristics

The cells of strains B.3\textsuperscript{T}, B.7 and B.9 were rod-shaped measuring 0.5–1.2 x 4.5–6.5 μm after growing for 48 h at 30 °C and pH 7.0. They produced ellipsoidal spores in swollen sporangia. Colonies were convex, mucoid and opaque. The colonies of strain B.7 were greater in size (> 4 mm) than those of strains B.9 and B.3\textsuperscript{T} (< 4 mm). All strains formed motile microcolonies while growing on wet agar plates. The strains were facultatively anaerobic, Gram-variable and motile by means of peritrichous flagella. Strains had catalase activity, but no oxidase activity. These strains did not grow in the presence of 5% (w/v) NaCl, which is the case with other Paenibacillus species (Shida et al., 1997a). Gelatin, casein and starch were hydrolysed. The optimum growth temperature was 30 °C, as it is for all Paenibacillus species except Paenibacillus macquariensis which has an optimum growth temperature of 20–23 °C (Shida et al., 1997a). The optimum pH for growth was 7.0, but it was able to grow in a wide range of pH (5–12). Members of the genus Paenibacillus grow optimally at the same pH (Shida et al., 1997a). The strains B.3\textsuperscript{T}, B.7 and B.9 grew in 100% (v/v) OMWW, which was an important phenotypic difference that distinguished our strains from other known Paenibacillus species. All the physiological and biochemical characteristic which are identical among the isolated B.3\textsuperscript{T}, B.7 and B.9 are given below in the description of the novel species. Only strain B.3\textsuperscript{T} was positive for ONPG hydrolysis and results obtained for acid production from several carbohydrates showed that utilization of methyl α-D-mannoside was positive only for strain B.3\textsuperscript{T}, methyl α-D-glucoside was positive for strains B.3\textsuperscript{T} and B.7, and utilization of both inulin and gentiobiose was positive for strains B.7 and B.9. Paenibacillus strain B.3\textsuperscript{T} can be differentiated from other related Paenibacillus species as shown in Table 1.

Chemotaxonomic characteristics

meso-Diaminopimelic acid was the diamino acid found in the cell-wall peptidoglycan of strains B.3\textsuperscript{T}, B.7 and B.9. This diamino acid is common to the members of the genus Paenibacillus and to other aerobic endospore formers such as Bacillus, Sporolactobacillus and Amphi-

| Table 1. Differentiation of strain B.3\textsuperscript{T} from closely related Paenibacillus species

| Strains | B.3\textsuperscript{T}; 2, P. polymyxa; 3, P. peoriae; 4, P. azotofixans. | Positive; −, negative; v, variable; ND, not determined. |
|---------|---------------------------------------------------------------|
| Characteristic | 1 | 2\* | 3\* | 4\* |
| Growth in 0.001% lysozyme | + | v | + | – |
| Acid from: | | | | |
| Arabinose | + | + | + | – |
| Xylose | + | + | + | – |
| Glycero | + | + | – | – |
| Trehalose | + | + | – | ND |
| Methyl β-xyloside | – | + | ND | ND |
| Gas from carbohydrate | – | + | + | + |
| Starch hydrolysis | + | + | – | – |
| Citrate utilization | – | + | – | – |
| Nitrate reduced to nitrite | + | + | + | – |
| Casein hydrolysis | + | + | + | – |
| G + C content (mol%) | 40–43 | 43–46 | 45–47 | 48–53 |

* Data from Montefusco et al. (1993).

| Table 2. G + C content, 16S rDNA similarity and DNA–DNA hybridization data for some strains studied

<table>
<thead>
<tr>
<th>Strain</th>
<th>(T_m) (°C)</th>
<th>G + C content (mol%)</th>
<th>16S rDNA similarity (%)</th>
<th>Hybridization to DNA from B.3\textsuperscript{T} (RBR: %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.3\textsuperscript{T}</td>
<td>85.98</td>
<td>40.6</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B.7</td>
<td>85.87</td>
<td>40.5</td>
<td>98</td>
<td>75</td>
</tr>
<tr>
<td>B.9</td>
<td>86.05</td>
<td>40.8</td>
<td>98</td>
<td>73</td>
</tr>
<tr>
<td>Bacillus firmus</td>
<td>85.78</td>
<td>40.2</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Bacillus lentus</td>
<td>85.56</td>
<td>39.6</td>
<td>ND</td>
<td>13</td>
</tr>
<tr>
<td>Brevibacillus laterosporus</td>
<td>85.31</td>
<td>39.1</td>
<td>ND</td>
<td>21</td>
</tr>
<tr>
<td>P. polymyxa</td>
<td>87.5</td>
<td>44.5</td>
<td>98</td>
<td>15</td>
</tr>
<tr>
<td>P. peoriae</td>
<td>88.16</td>
<td>46.0</td>
<td>96</td>
<td>ND</td>
</tr>
<tr>
<td>P. azotofixans</td>
<td>90.01</td>
<td>50.5</td>
<td>95</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, Not determined.
bacillus (Shida et al., 1997a). The major isoprenoid quinone was MK-7, which is the major menaquinone generally found in aerobic, endospore-forming rods. Anteiso-C<sub>15</sub>:0, the predominant fatty acid found in this genus (Shida et al., 1997a), was also the major fatty acids component of strains B.3<sup>T</sup>, B.7 and B.9.

### DNA base composition and DNA–DNA reassocation

The G+C contents of strains B.3<sup>T</sup>, B.7 and B.9 were 40.6, 40.7 and 40.8 mol%, respectively. These G+C contents lie within the range found in the members of genus Paenibacillus (Shida et al., 1997a). DNA G+C contents of Bacillus firmus, Bacillus lentus, Brevibacillus laterosporus and P. polymyxa, Paenibacillus peoriae, Paenibacillus azotofixans and their relative binding ratios (RBRs) of DNA–DNA hybridization are compiled in Table 2. Strains B.3<sup>T</sup>, B.7 and B.9 belonged to the same species because their RBRs to each other were > 70%. However, it was concluded that these strains were a separate species from Bacillus firmus, Brevibacillus laterosporus, Bacillus lentus and Paenibacillus polymyxa because the RBRs with these species were < 22%.

### Phylogenetic analysis

Although the preceding biochemical, chemotaxonomic, G+C content and hybridization analysis did not establish a definite taxonomic position for the...
strains B.3³, B.7 and B.9, these data suggested *Paenibacillus* as a possible classification. 16S rDNA was sequenced to verify the suggestion. An almost complete 16S rDNA sequence (1525 bp) of strain B.3³, which corresponded to a region between positions 1 and 1524 by comparison with other eubacteria 16S rRNA genes, was obtained. The phylogenetic tree (Fig. 1) constructed from the sequence data showed that strain B.3³ appeared within the evolutionary radiation encompassing genus *Paenibacillus* and occupied a distinct phylogenetic position within the genus. The phylogenetic study clearly established that our strains were a *Paenibacillus* species. Moreover, there was a consensus signature sequence (PAEN515F) of *Paenibacillus* genus (5'-GAGTAACACTGCTACAAGAGTGCAGGTACCTGAGAAGAAAGCCCC-3') within the 16S rRNA sequence of our strains (Shida et al., 1997a).

The results of this polyphasic study supports strains B.3³, B.7 and B.9 being the same species and belonging to the genus *Paenibacillus*. Their genetic distinctiveness inferred from the phylogenetic study warrants the proposal of these strains as a novel species, *Paenibacillus jamilae* sp. nov.

**Description of *Paenibacillus jamilae* sp. nov.**

*Paenibacillus jamilae* (ja.mi’lae. N.L. fem. n. jamilae residual water of olive oil production, from jamila specific term of Arabic origin commonly used in Andalusia, Spain).

Cells are rod-shaped (0.5–1.2 x 4.5–6.5 μm) and motile by means of peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Colonies are convex, mucoid and opaque. Forms motile microcolonies on wet agar plates. Facultatively anaerobic and Gram-variable. Catalase reaction is positive, whereas oxidase activity is negative. Gelatin, casein and starch are hydrolysed. Acid but not gas is produced from glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-mannose, D-mannitol, amygdaline, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, glycerogen and D-turanose. Erythritol, D-arabinose, L-xylose, adonitol, methyl β-D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, sorbitol, N-acetylglucosamine, melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate and 5-keto-gluconate are not utilized as sole carbon sources. Citrate and propionate are not utilized. Acetyl methylcarbinol is positive (as determined by the Voges–Proskauer reaction). Nitrate is reduced to nitrite. Growth occurs at temperatures of 30–40 °C and at pH 5–12. Optimum growth occurs at 30 °C and at pH 7.0. Growth occurs in the presence of 2% NaCl and 0.001% lysozyme, whereas it is inhibited by 5% NaCl. The major fatty acid is anteiso-C₁₅:₀. The major quinone is MK-7. The mean DNA G+C content is 40.6–40.8 mol%. The type strain is B.3³ (= CECT 5266T = DSM 13815T).

**ACKNOWLEDGEMENTS**

The authors would like to thank Dr Antonio Lario (Institute of Parasitology and Biomedicine Lopez-Neyra) for his help with DNA sequencing, and the Technical Services of University of Granada for its help with electron microscopy. This research was supported by a grant from the Ministerio de Educación y Ciencia, Spain (Project no. OL96-2189).

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