Bacillus massiliensis sp. nov., isolated from cerebrospinal fluid

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An unidentified Gram-negative-staining, aerobic, rod-shaped, spore-forming bacterium was isolated from a sample of cerebrospinal fluid. Based on comparative analysis of 16S rRNA gene sequences and phenotypic characteristics, the novel isolate was included in the *Bacillus sphaericus*-like group. The isolate was closely related to *Bacillus odysseyi* and *Bacillus silvestris*, with 96.2 and 94.4 % 16S rRNA gene sequence similarity, respectively. The major fatty acid was iso-C_{15:0} (48 %). The name *Bacillus massiliensis* sp. nov. is proposed for the novel isolate, with strain 4400831^T (= CIP 108446^T = CCUG 49529^T) as the type strain.

The species *Bacillus sphaericus* was described by Neide in 1904. Great genetic heterogeneity among *B. sphaericus*-like species has been observed, based on molecular biological techniques (Alexander & Priest, 1990; Aquino de Muro *et al.*, 1992; Woodburn *et al.*, 1995). Recently, based on 16S rRNA gene sequence comparisons, two novel species, *Bacillus fusiformis* (Priest *et al.*, 1988) and *Bacillus silvestris* (Rheims *et al.*, 1999), were included in the *B. sphaericus*-like group. Nakamura (2000) also proposed four novel potential species and the reclassification of earlier isolates based on the same technique. The *B. sphaericus*-like group consists of seven genetically distinct groups. The recognized species *B. fusiformis* and *B. sphaericus* were placed in groups 2 and 3, respectively, whereas *B. silvestris* appeared to be in a separate cluster. In 2002, the same author (Nakamura *et al.*, 2002) described two novel species, *Bacillus pycnus* and *Bacillus neidei*, belonging to groups 6 and 7, respectively. Most of the strains included in the *B. sphaericus*-like group were isolated from soil, marine environments, mud or different types of insects, with some strains being pathogenic for their host (Nakamura, 2000). Usually, *Bacillus* spp. are considered as contaminants when they are isolated from biological samples but, in immunocompromised patients, they can cause significant disease. Bacteraemia due to *B. sphaericus* has been described in children receiving antineoplastic treatment or bone-marrow transplantation (Castagnola *et al.*, 2001). In this paper, we describe a novel species of the genus *Bacillus* that was isolated from human cerebrospinal fluid (CSF).

Abbreviation: CSF, cerebrospinal fluid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 4400831^T is AY677116.

Transmission electron micrographs of cells of strain 4400831^T are available as supplementary material in IJSEM Online.

Strain isolation and characterization

A 54-year-old man with cerebellar syndrome and diarrhoea episodes was diagnosed with Whipple's disease in January 2004 by PCR amplification of *Tropheryma whippelii* DNA from the CSF. The patient was treated with ceftriaxone, in association with trimethoprim-sulfamethoxazole. The treatment with trimethoprim-sulfamethoxazole was continued for 6 months. After 3 months of antibiotic therapy, a sample of CSF was taken and cell culture was performed. The presence of a Gram-negative-staining bacillus was detected in the supernatant using a Gram-stain kit (Color Gram 2; bioMérieux), according to the manufacturer's instructions. Subcultivation was carried out using sheep-blood agar (bioMérieux). The isolate, strain 4400831^T, could not be identified using a phenotypic approach, but was identified as a strain of *Bacillus* at the molecular biology laboratory of Timone Hospital (Marseille, France), based on 16S rRNA gene sequence comparison. The antimicrobial susceptibility of the novel isolate was determined according to the NCCLS criteria and is presented in the species description.

After growth for 24 h on sheep-blood agar at 30 °C, surface colonies of strain 4400831^T were circular, white-greyish, smooth and shiny and approximately 1–2 mm in diameter. Growth also occurred in trypticase soy broth (TSB; Becton Dickinson). The ability of the strain to grow at different temperatures (25, 30, 37, 45 and 50 °C) was investigated using sheep-blood agar. Growth occurred at 25–45 °C, with optimum growth at 30–37 °C. Growth also occurred in the presence of air, 5 % CO₂ and a microaerophilic atmosphere that was created using a GENbag microaer (bioMérieux), but did not occur in an anaerobic atmosphere created using a GENbag anaer (bioMérieux), similar to other representatives of the *B. sphaericus*-like group (Reva *et al.*, 2001).

The size and ultrastructure of the cells and spores were determined by electron microscopy. Cells were grown in

TSB for 24 h at 30 °C and stained with 1 % (w/v) phosphotungstic acid. The samples were examined using a Morgagni 268D (Philips) electron microscope at an operating voltage of 60 kV. The cells were rods of 1.5–4 µm in length and 0.3–0.5 µm in width, occurring singly or in groups, and had peritrichous flagella (Supplementary Fig. S1 in IJSEM Online). Catalase activity was determined by using an ID color catalase test kit (bioMérieux) and oxidase activity was assayed by application of cells to moistened discs impregnated with dimethyl-*p*-phenylene diamine (bioMérieux). The novel isolate was catalase- and oxidase-positive. Tolerance of high NaCl concentrations was tested in TSB supplemented with 2, 5, 7 and 10 % (w/v) NaCl. The bacterium was able to grow in 5 % NaCl, but not in 7 %. This observation differed from that for *B. silvestris* (Table 1). The bacterium was motile, as determined by light microscopy.

API 20E and API 50 CH test strips, combined with API 50 CHB/E medium (bioMérieux), and the Biolog identification system were used to characterize the biochemical properties of the strain, according to the manufacturers' instructions. The API 20E and API 50 CH strips were incubated at 30 °C and the Biolog plates at 33 °C. The results of these tests are given in the species description.

Preparation of cellular fatty acids and determination of their composition were carried out according to the methods of the Sherlock Microbial Identification System (MIDI Inc.). The major fatty acids were iso-C_{15:0} (48 %), anteiso-C_{15:0} (15.3 %) and iso-C_{16:0} (13.5 %). Smaller amounts of anteiso-C_{17:0} (5.6 %), iso-C_{17:0} (3.7 %), C_{15:0} (3.2 %), iso-C_{14:0} (3.1 %), C_{16:1ω7c} alcohol (2.6 %), C_{16:0} (1.5 %), C_{16:1ω11c} (0.7 %) and C_{18:1ω9c} (0.7 %) were also present. The predominance of iso-C_{15:0} was similar to other members of the *B. sphaericus*-like group; the composition and amounts of other fatty acids are characteristic for each group of Nakamura (2000) and for *B. silvestris* (Rheims *et al.*, 1999).

Bacterial DNA was extracted using a FastDNA kit (BIO 101), as described by the manufacturer. PCR of the 16S rRNA gene was performed using the universal primer pair fD1 and rp2 (Weisburg *et al.*, 1991). The PCR products were purified using MultiScreen PCR (Millipore) and sequencing was carried out using a DNA sequencing kit (BigDye Terminator Cycle sequencing version 2.0 ready reactions; PE Biosystems), according to the manufacturer's instructions. The sequence products were purified and electrophoresis was performed using a 3100 Genetic Analyzer (Applied

Table 1. Characteristics that differentiate *B. massiliensis* sp. nov. from other closely related species

Strains: 1, *B. massiliensis* 4400831^T; 2, *B. silvestris* CIP 106059^T; 3, *B. sphaericus* CIP 65.30^T; 4, *B. neidei* CIP 107432^T; 5, *B. pycnus* CIP 107434^T; 6, *B. odysseyi* CIP 108263^T. ND, Not determined.

Characteristic	1	2	3	4	5	6
β-Galactosidase (ortho nitrophenyl β-D-galactopyranosidase)	–	–	–	+	–	–
Urease	+	+	+	+	–	–
Tryptophan deaminase	+	–	–	–	–	–
Gelatinase	–	–	+	+	+	–
Growth in:						
5 % NaCl	+	+	+	+	–*	+
7 % NaCl	–	+	–	ND	–*	ND
Oxidation of:						
Acetate	+	+	+	ND	+	+
Pyruvate	+	+	+	–*	+	+
α-Hydroxybutyrate	–	–	–†	ND	–	–
β-Hydroxybutyrate	–	+	–†	–*	+	+
Methyl pyruvate	+	+	+	ND	+	+
L-Alanine	–	+	+	–*	+	+
Glycyl L-glutamate	–	+	+	+	–	+
Adenosine	–	+	+	+	+	+
2'-Deoxyadenosine	–	+	+	+	+	+
Inosine	–	+	+	+	+	+
AMP	–	+	+	+	–	–
UMP	–	–	+	+	–	–
16S rRNA gene sequence similarity with strain 4400831 ^T (%)	(100)	94.4	95.3	92.1	93.1	96.2

*Data from Nakamura *et al.* (2002).

†Data from La Duc *et al.* (2004).

Fig. 1. Phylogenetic tree based on 16S rRNA gene nucleotide sequence comparison (1398 nt) indicating the position of *B. massiliensis* sp. nov. 4400831^T within representatives of the *B. sphaericus*-like group. Numbers at nodes are bootstrap values based on 100 resamplings. *Bacillus thuringiensis* 97-27 was used as the outgroup. Bar, 0.01 nucleotide changes per nucleotide position.

Biosystems). Gene sequences were aligned using the multi-sequence alignment program CLUSTAL X (1.8). Phylogenetic relationships with closely related species were determined using MEGA version 2.1 (Kumar *et al.*, 2001). Distance matrices were determined following the assumptions described by Kimura (1980) and were used to elaborate dendrograms using the neighbour-joining method (Saitou & Nei, 1987). The maximum-parsimony algorithm was also used to infer phylogenetic relationships. A bootstrap analysis was performed to investigate the stability of the trees obtained. Bootstrap values were obtained for a consensus tree based on 100 randomly generated trees. The tree organization was the same with the two methods. The phylogenetic analysis showed that the novel isolate is related to *Bacillus odyseeyi*, which was isolated from the Mars Odyssey spacecraft (La Duc *et al.*, 2004), and *B. silvestris* (Fig. 1). This cluster branched between the cluster including representatives of groups 1, 2 (*B. fusiformis*) and 3 (*B. sphaericus*) and the cluster including groups 6 (*B. pycnus*) and 7 (*B. neidei*). The percentage similarity between 16S rRNA gene sequences was determined using NALIGN in the PC/GENE software package (IntelliGenetics). The sequence similarity between *B. odyseeyi* and strain 4400831^T was 96.2%; lower sequence similarities were obtained with all other recognized species of the genus *Bacillus*.

Based on the results described above, we propose that the novel isolate represents a novel species with the name *Bacillus massiliensis* sp. nov.

Description of *Bacillus massiliensis* sp. nov.

Bacillus massiliensis (mas.si.li.en'sis. L. masc. adj. *massiliensis* of Massilia, the ancient Greek and Roman name for Marseille, France, where the type strain was isolated).

Cells are aerobic, endospore-forming, motile, Gram-negative-staining straight rods, with peritrichous flagella. Growth occurs on sheep-blood agar and in TSB. After growth for 24 h on sheep-blood agar, colonies are circular, white-greyish, smooth, shiny, and approximately 1–2 mm in diameter. The temperature range for growth is 25–45 °C, with optimum growth occurring at 30–37 °C. After growth for 24 h in TSB, rods are 1.5–4 × 0.3–0.5 µm. Grows in 5% NaCl in TSB. Catalase- and oxidase-positive. Voges-Proskauer test is positive. Using the API 20E strip,

activities of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease are positive, and citrate utilization is positive. Gelatin is not liquefied and ONPG hydrolysis, hydrogen sulfide production and indole production are negative. D-Glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose, amygdalin and L-arabinose are not fermented. No sugar is fermented in the API 50 CH strips, using CHB/E suspension medium. Acetate, pyruvate and methyl pyruvate are oxidized, but α-hydroxybutyrate, β-hydroxybutyrate, L-alanine, glycyl L-glutamate, adenosine, 2'-deoxyadenosine, inosine, AMP and UMP are not. The fatty acid profile is characterized by the predominance of iso-C_{15:0} (48%), followed by anteiso-C_{15:0} (15.3%) and iso-C_{16:0} (13.5%). Smaller amounts of anteiso-C_{17:0} (5.6%), iso-C_{17:0} (3.7%), C_{15:0} (3.2%), iso-C_{14:0} (3.1%), C_{16:1ω7c} alcohol (2.6%), C_{16:0} (1.5%), C_{16:1ω11c} (0.7%) and C_{18:1ω9c} (0.7%) are also present. Sensitive to amoxycillin, augmentin, ceftriaxone, ticarcillin, claventin, imipinem, ciprofloxacin, gentamicin (10 IU), amikacin, tobramycin, trimethoprim-sulfamethoxazole, rifampicin, ofloxacin, colistin, piperacillin-tazobactam, isepamycin, chloramphenicol, tetracycline and streptomycin, moderately sensitive to cefepime and ceftiofime, and resistant to ceftazidime, fosfomycin and aztreonam.

The type strain, 4400831^T (= CIP 108446^T = CCUG 49529^T), was isolated from human CSF.

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