Paenibacillus massiliensis sp. nov., *Paenibacillus sanguinis* sp. nov. and *Paenibacillus timonensis* sp. nov., isolated from blood cultures

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Gram-positive, spore-forming rods were isolated from blood cultures of three different patients. Based on phylogenetic analyses, these strains were placed within the *Paenibacillus* cluster and specific phenotypic characteristics for each strain were described. Levels of 16S rRNA gene sequence similarity between existing *Paenibacillus* species and the three novel strains 2301065^T, 2301032^T and 2301083^T were 87.6–94.4, 88.5–95.4 and 87.5–96.0%, respectively, and anteiso-branched C_{15:0} was the major fatty acid. On the basis of phenotypic data and phylogenetic inference, it is proposed that these strains should be designated *Paenibacillus massiliensis* sp. nov., *Paenibacillus sanguinis* sp. nov. and *Paenibacillus timonensis* sp. nov. The type strains are respectively strain 2301065^T (= CIP 107939^T = CCUG 48215^T), strain 2301083^T (= CIP 107938^T = CCUG 48214^T) and strain 2301032^T (= CIP 108005^T = CCUG 48216^T).

Gene comparison studies have shown that 16S rRNA is highly conserved within a species and among species of the same genus. Since the pioneering work of Woese (1987), identification and phylogenetic analysis of many bacteria have been performed and have allowed for the classification of non-cultivable organisms and organisms with ambiguous biochemical profiles. Based on phylogenetic studies using the 16S rRNA gene, the so-called rRNA group 3 bacilli were separated to form the novel genus *Paenibacillus* (Ash *et al.*, 1993); several novel species of *Paenibacillus* have recently been described (Aguilera *et al.*, 2001; Elo *et al.*, 2001; Meehan *et al.*, 2001; Dasman *et al.*, 2002; Takeda *et al.*, 2002; Yoon *et al.*, 2003).

Paenibacillus species have been isolated from human biological samples and the possibility that these bacteria may cause human infection has been considered. Prosthetic hip infection and endophthalmitis caused by *Paenibacillus alvei* (Antonello & Weinstein, 1989; Reboli *et al.*, 1989), brain abscess, catheter-associated infection and wound infection due to *Paenibacillus macerans* were reported (Bert *et al.*, 1995; Barrero *et al.*, 1996). Pseudobacteraemia due to *Paenibacillus* may also result from contamination of gloves, alcohol swabs, intravenous catheters or blood culture media

(CDC, 1974; Berger, 1983; York, 1990; Noskin *et al.*, 2001; Teng *et al.*, 2003).

The data presented in this paper describe three novel *Paenibacillus* species, two of which are related closely to *Paenibacillus macerans* (Ash *et al.*, 1993; Heyndrickx *et al.*, 1996); the third can be grouped with *Paenibacillus illinoisensis* (Shida *et al.*, 1997). These species were initially isolated from blood culture samples of patients suffering from carcinoma, chronic interstitial nephropathy or leukaemia.

Isolation and characterization of strains

Reference strains were obtained from the Pasteur Institute (Paris, France).

Paenibacillus macerans strain CIP 66.19^T, *Paenibacillus pabuli* strain CIP 103119^T and *Paenibacillus polymyxa* strain CIP 66.22^T, and clinical strains 2301083^T (= CIP 107938^T = CCUG 48214^T), 2301065^T (= CIP 107939^T = CCUG 48215^T) and 2301032^T (= CIP 108005^T = CCUG 48216^T), were isolated on Columbia blood agar plates (Oxoid) from blood samples in the bacteriology laboratory of the Timone hospital.

In October 1999, cervical adenopathy was detected in a 49-year-old man. Diagnosis of epidermoid carcinoma of the oropharynx was established and chemotherapy and radiotherapy were performed. In November 2002, at the time of hospitalization for chemotherapy, the patient was afebrile, but a blood sample was collected from which Gram-positive rods were isolated (strain 2301083^T).

In March 2001, acute lymphoblastic leukaemia was diagnosed

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of *P. massiliensis* strain 2301065^T, *P. sanguinis* strain 2301083^T and *P. timonensis* strain 2301032^T are respectively AY323608, AY323609 and AY323610 (clone 1), AY323611 (clone 2) and AY323612 (clone 3).

in a 13-year-old boy who received chemotherapy. In August 2001, allograft with compatible stem cells of umbilical blood was performed. At this time, cutaneous and hepatic graft versus host disease was noted. In October 2002, he was hospitalized with fever (38.5 °C) and neutropenia. Blood samples were obtained and Gram-positive rods (strain 2301065^T) were isolated from one of three blood samples.

In October 2002, strain 2301032^T was isolated from a 75-year-old woman who presented with chronic interstitial nephropathy that required haemodialysis from February 1989. At the time of the bacterial isolation, she was afebrile, but presented with loose cough and sputum.

All of the isolated bacteria were Gram-positive rods; their size was determined by transmission electron microscopy using cells from a 48 h culture. Bacterial suspension was pre-fixed in 5 % (v/v) glutaraldehyde in phosphate buffer (Gibco) for at least 1 h at room temperature, washed in the same buffer and stained with 1 % (w/v) phosphotungstic acid. Sporulation was studied by the same method. After the pre-fixation step, specimens were post-fixed with 4 % (w/v) osmium tetroxide for 1 h, dehydrated by using a graded series of acetone, transferred to 100 % acetone and embedded in Epon 812 (Fluka) substitute. Thin sections were cut with a diamond knife on a Leica Ultracut VCT microtome and stained with uranyl acetate and lead citrate. The grids were examined on a MORGANI 268 D (Philips) transmission electron microscope at an operating voltage of 60 kV. Bacteria of strain 2301065^T were 0.5 µm wide and 2.0–4.0 µm long. They formed translucent, flat colonies of beige colour after 24 h incubation at 30 °C. Cells of strain 2301083^T were 0.5 µm wide and 2.0–3.0 µm long, forming greyish colonies that were translucent, shiny, 1 mm in diameter after 24 h incubation at 30 °C, circular and regularly shaped. Isolated bacteria of strain 2301032^T were rods of 0.5 µm width and 2.0 µm length. Colonies were greyish, translucent, shiny, 1.5 mm in diameter, regularly shaped and low convex. Presence of spores was studied after 8 days growth at room temperature and was reported for the three isolates. Terminal or subterminal ellipsoidal spores were observed in swollen sporangia.

Growth temperatures tested were 30, 37, 44 and 50 °C, the optimum being 30–37 °C. Catalase activity was determined with the ID colour catalase test kit (bioMérieux). Oxidase activity was assayed by applying the cells to moistened discs that were impregnated with dimethyl-*p*-phenylene diamine (bioMérieux). Mobility was determined by light microscopy. An anaerobic atmosphere was created by using GENbag anaer (bioMérieux). Acid production from 49 carbon sources was tested at 30 °C under anaerobic conditions with an API 50CH kit combined with API 50CHB/E medium (bioMérieux). Other tests were completed with API 20 E (bioMérieux). Tolerance to NaCl was measured in trypticase soy broth that contained 1–5 % (w/v) NaCl. All three strains were motile by means of peritrichous flagella and facultatively anaerobic. With the exception of

strain 2301083^T, the isolates grew at 50 °C. None of the strains showed haemolytic activity on Columbia blood agar. ADH, LDC, ODC, CIT, H₂S, URE, TDA and IND tests in API 20 E strips were negative for all isolates tested. The three strains grew in the presence of 3 % NaCl, but strain 2301065^T also grew in the presence of 5 % NaCl. Diagnostic traits are specified in Table 1.

Preparation and determination of cellular fatty acids followed the procedures given for the Sherlock Microbial Identification system (MIDI). The major fatty acids found

Table 1. Diagnostic traits of the different species and isolates tested in this study

Strains: 1, *P. polymyxa* CIP 66.22^T; 2, *P. pabuli* CIP 103119^T; 3, *P. macerans* CIP 66.19^T; 5, *P. massiliensis* 2301065^T; 4, *P. sanguinis* 2301083^T; 6, *P. timonensis* 2301032^T. Mobility was positive for all strains.

Characteristic	1	2	3	4	5	6
Catalase	+	+	+	+	–	+
Oxidase	–	+	+	–	–	+
Growth at 50 °C	–	–	+	+	–	+
Growth at 44 °C	+	–	+	+	+	+
Anaerobic growth	–	+	+	+	+	+
Spore presence	+	+	–	+	+	+
Haemolysis	+	+	–	–	–	–
Growth in presence of 5 % NaCl	–	–	–	+	–	–
Nitrate reduction	+	+	+	+	–	w+*
Voges-Proskauer test	–	–	+	–	–	–
Gelatin liquefaction	+	–	–	–	–	–
Acid production from:						
Glycerol	+	+	+	+	–	–
D-Arabinose	–	–	+	–	–	–
Ribose	+	+	+	+	–	–
D-Xylose	+	+	+	–	+	+
Methyl β-D-xyloside	+	+	+	–	+	–
Glucose	+	+	+	+	–	+
Mannose	+	+	+	+	–	+
Rhamnose	–	–	+	–	–	–
Mannitol	+	+	+	+	+	–
Methyl α-D-glucoside	+	+	+	–	+	+
N-Acetylglucosamine	–	+	–	–	+	+
Sucrose	+	+	+	+	+	–
Inulin	–	+	+	–	+	–
Melezitose	–	+	+	–	+	–
Raffinose	+	+	+	+	+	–
Starch	+	+	+	+	–	+
Glycogen	+	+	+	+	–	+
Gentiobiose	+	+	+	+	–	+
D-Turanose	+	+	+	–	–	–
L-Fucose	–	–	+	–	–	–
D-Arabitol	–	–	+	–	–	–
Gluconate	–	–	–	–	–	+

*Weakly positive.

Table 2. Whole-cell fatty acid composition (%) of the novel isolates and related species

Strains: 1, *P. massiliensis* 2301065^T; 2, *P. sanguinis* 2301083^T; 3, *P. timonensis* 2301032^T; 4, *P. illinoisensis* DSM 11733^T; 5, *P. macerans* DSM 24^T; 6, *P. pabuli* DSM 3036^T; 7, *P. polymyxa* DSM 36^T.

Fatty acid	1	2	3	4*	5*	6*	7*
Straight-chain:							
C _{14:0}	2.9	0.5	1.3	2.8	6.9	3.9	1.5
C _{15:0}	1.6	2.8	2.0	0.7	1.0	<0.2	0.3
C _{16:0}	14.1	10.1	10.6	15.1	13.2	8.3	6.5
Iso-branched:							
C _{14:0}	8.0	1.7	8.2	2.1	4.8	3.1	1.9
C _{15:0}	5.4	6.8	10.1	5.8	7.6	11.5	7.8
C _{16:0}	13.6	13.4	15.5	5.5	25.7	4.4	11.3
C _{17:0}	1.0	5.8	1.9	3.9	6.2	2.9	4.3
Anteiso-branched:							
C _{15:0}	42.8	44.2	43.4	51.8	24.6	53.7	55.2
C _{17:0}	2.9	6.8	4.0	6.2	9.0	2.4	8.8

*Data from Elo *et al.* (2001).

are shown in Table 2 and compared with values that are available for closely related *Paenibacillus* species.

DNA analysis

Bacterial DNA was extracted by using the FastDNA kit (Bio101) as described by the manufacturer. PCR amplification of the 16S rRNA gene was done by using the universal primer pair fD1 and rp2 (Weisburg *et al.*, 1991). PCR products were purified by using MultiScreen PCR (Millipore) and sequencing reactions were carried out by using a DNA sequencing kit (BigDye Terminator Cycle Sequencing v2.0 Ready Reactions; PE Biosystems) as described by the manufacturer. Sequence products were purified and electrophoresis was performed with a 3100 Genetic Analyzer (Applied Biosystems).

PCR products were cloned in pGEM-T Easy Vector (Promega) as described by the manufacturer. Ten clones were cultivated in LB medium (USB) overnight and plasmid purification was performed by using the Wizard Plus SV Minipreps DNA purification system (Promega). Determined sequences were compared with those available in GenBank by using the BLAST program through the NCBI server.

16S rRNA gene sequences were aligned by using the multi-sequence alignment program CLUSTALX (1.8). Phylogenetic relationships between representatives of the genus *Paenibacillus* were determined by using MEGA version 2.1 (Kumar *et al.*, 2001). Distance matrices were determined following the assumptions described by Kimura (1980). These matrices were used to elaborate dendrograms by using the neighbour-joining method (Saitou & Nei, 1987). 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. Percentage similarities between 16S rRNA gene sequences were determined by using NALIGN in the PC/GENE software package (IntelliGenetics); similarities between existing *Paenibacillus* species and strains 2301065^T, 2301032^T and 2301083^T were 87.6–94.4, 88.5–95.4 and 87.5–96.0%, respectively.

It was impossible to determine the 3' end of the 2301032^T 16S rRNA gene sequence. We therefore cloned the PCR product and found that several sequences were present. Direct sequencing was impossible, due to mutations and insertions. For this isolate, at least three different sequences were characterized. Recently, whole genomes of *Bacillus* species, which are phylogenetically close to the genus *Paenibacillus*, were sequenced. In the genomes of *Bacillus subtilis*, *Bacillus cereus* and *Bacillus anthracis*, respectively 10, 13 and 11 genes that encode 16S rRNA were described (Kunst *et al.*, 1997; Ivanova *et al.*, 2003; Read *et al.*, 2003). The high number of 16S rRNA genes in the genomes of *Paenibacillus* species could explain the high diversity in the sequences of this gene for the same isolate. In fact, we observed this phenomenon for several representatives of the genus *Paenibacillus* (V. Roux, unpublished data).

Phylogenetic analysis, based on 16S rRNA gene sequence comparison, showed that isolate 2301065^T could be grouped with *Paenibacillus illinoisensis*, but the bootstrap value at the node was low (33 %) (Fig. 1). Isolates 2301083^T and 2301032^T were grouped with *Paenibacillus macerans* in the same cluster. Bootstrap values at the different nodes were high (82–100 %). The 16S rRNA gene sequences for strains 2301065^T, 2301083^T and 2301032^T were found to contain the *Paenibacillus*-specific 22 bp sequence in variable region V5 (Ash *et al.*, 1993).

Clinical signs of serious infection were not present in any of the three patients. It is, therefore, impossible to precisely determine the medical significance of the bacteria in blood-culture bottles. Skin or blood-bottle contamination, or asymptomatic bacteraemia in immunocompromised patients, are possible explanations. Antimicrobial sensitivity is presented in Table 3.

On the basis of the results described above, we propose that strains 2301065^T, 2301083^T and 2301032^T represent novel species of the genus *Paenibacillus*, for which the names *Paenibacillus massiliensis* sp. nov., *Paenibacillus sanguinis* sp. nov. and *Paenibacillus timonensis* sp. nov., respectively, are proposed.

Description of *Paenibacillus massiliensis* sp. nov.

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

Gram-positive, facultatively anaerobic rods (0.5 µm wide, 2.0–4.0 µm long). Ellipsoidal endospores are formed in

Fig. 1. Phylogenetic tree of bacteria that belong to the genus *Paenibacillus*, inferred from comparison of 16S rRNA gene sequences. Sequences (1440 nt) were aligned by using the multisequence alignment program CLUSTAL X (1.8). Phylogenetic relationships between representatives of the genus *Paenibacillus* were determined by using MEGA version 2.1. Distance matrices were determined by using Kimura's parameter. These matrices were used to elaborate dendrograms by using the neighbour-joining method. Numbers at nodes are proportions of 100 resamplings that support the topology shown. *Listeria monocytogenes* was used as the outgroup. Bar, 0.01 nucleotide changes per nucleotide position.

swollen sporangia. The organism grows on routine media and forms translucent, beige-coloured, flat colonies after incubation for 24 h at 30 °C. Bacteria are motile by means of peritrichous flagella. Catalase-positive, but oxidase-negative. Optimal growth occurs at 30–37 °C, but growth

Table 3. Antimicrobial susceptibility of the three novel *Paenibacillus* species

Strains: 1, *P. massiliensis* 2301065^T; 2, *P. sanguinis* 2301083^T; 3, *P. timonensis* 2301032^T. S, Sensitive; R, resistant; I, intermediate.

Antimicrobial	1	2	3
Amoxycillin	S	S	S
Gentamicin	S	S	S
Doxycycline	S	S	S
Erythromycin	S	S	R
Trimethoprim-sulfamethoxazole	S	S	R
Rifampicin	S	S	I
Vancomycin	S	S	S

at 50 °C is not inhibited. Growth occurs in the presence of 5 % (w/v) NaCl. Nitrate is reduced, but gelatin is not liquefied. Acid is produced from glycerol, L-arabinose, ribose, galactose, glucose, fructose, mannose, mannitol, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch, glycogen and gentiobiose. Acid is not produced from erythritol, D-arabinose, D-xylose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, inulin, melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate.

The type strain, which was isolated from blood culture, is strain 2301065^T (= CIP 107939^T = CCUG 48215^T).

Description of *Paenibacillus sanguinis* sp. nov.

Paenibacillus sanguinis (san'gui.nis. L. masc. gen. n. *sanguinis* of blood, referring to the fact that the type strain was isolated from a blood sample).

Gram-positive, facultatively anaerobic rods (0.5 µm wide, 2.0–3.0 µm long). Ellipsoidal endospores are formed in swollen sporangia. Colonies are greyish, translucent, shiny, circular, 1 mm in diameter after incubation for 24 h at 30 °C and regularly shaped. Bacteria are motile by means of peritrichous flagella. Catalase- and oxidase-negative. Optimal growth occurs at 30–37 °C and growth is inhibited at 50 °C. Growth does not occur in the presence of 5 % (w/v) NaCl. Nitrate is not reduced and gelatin is not liquefied. Acid is produced from L-arabinose, D-xylose, methyl β-D-xyloside, galactose, fructose, mannitol, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose and raffinose. Acid is not produced from glycerol, erythritol, D-arabinose, ribose, L-xylose, adonitol, glucose, mannose, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-mannoside, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate.

The type strain, which was isolated from blood culture, is strain 2301083^T (= CIP 107938^T = CCUG 48214^T).

Description of *Paenibacillus timonensis* sp. nov.

Paenibacillus timonensis (ti.mo.nen'sis. N.L. masc. adj. *timonensis* from the name Hôpital de la Timone, where the type strain was isolated).

Gram-positive, facultatively anaerobic rods (0.5 µm wide, 2.0 µm long). Ellipsoidal endospores are formed in swollen sporangia. Colonies are greyish, translucent, shiny, 1.5 mm in diameter, regularly shaped and low convex after incubation for 24 h at 30 °C. Bacteria are motile by means of peritrichous flagella. Catalase-positive, but oxidase-negative. Optimal growth occurs at 30–37 °C, but growth is not inhibited at 50 °C. Growth does not occur in the presence of 5 % (w/v) NaCl. Nitrate is reduced weakly, but gelatin is not liquefied. Acid is produced from L-arabinose, D-xylose, galactose, glucose, fructose, mannose, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, lactose, melibiose, trehalose, starch, glycogen, gentiobiose and gluconate. Acid is not produced from glycerol, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, mannose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, sucrose, inulin, melezitose, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate.

The type strain, which was isolated from blood culture, is strain 2301032^T (= CIP 108005^T = CCUG 48216^T).

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