Actinomyces massiliensis sp. nov., isolated from a patient blood culture

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Gram-positive, non-spore-forming rods (strain 4401292^T) were isolated from a human blood sample. Based on cellular morphology and the results of biochemical tests, this strain was tentatively identified as belonging to an undescribed species of the genus *Actinomyces*. Phylogenetic analysis based on 16S rRNA gene sequence comparison showed that the bacterium was related closely to *Actinomyces gerencseriae* (95.1 % 16S rRNA gene sequence similarity), *Actinomyces israelii* (95.2 %), *Actinomyces oricola* (95.2 %), *Actinomyces ruminicola* (93.3 %) and *Actinomyces dentalis* (91.4 %). The predominant fatty acids were C18:1 ω 9c and C16:0. On the basis of phenotypic data and phylogenetic inference, the novel species *Actinomyces massiliensis* sp. nov. is proposed; the type strain is 4401292^T (=CSUR P18^T =CCUG 53522^T).

The genus *Actinomyces* consists of a heterogeneous group of Gram-positive, anaerobic and aerotolerant, non-spore-forming, non-motile rods that have a high G+C content. The natural habitats of *Actinomyces* are the mucous membranes of humans and animals, particularly the oral mucosa. They are aetiological agents of actinomycosis, which is a polymorphous disease that can be acute or chronic. Classical forms of the disease are cervicofacial, thoracic and abdominal. However, uterine, ocular, cerebrospinal and hepatic infections have also been described. Identification of representatives of the genus *Actinomyces* is often problematic in clinical laboratories (Sarkonen *et al.*, 2001). Many studies based on phenotypic identification of members of the genus *Actinomyces* have been performed (e.g. Miller *et al.*, 1995; Santala *et al.*, 2004). Some of them proposed flowcharts for accurate differentiation (e.g. Sarkonen *et al.*, 2001). However, molecular approaches are often more useful to identify and clarify the taxonomic positions of putative pathogens (Hall *et al.*, 2001; Woo *et al.*, 2003; Song, 2005). Many novel *Actinomyces* species have been described in recent years, most of which originated from human sources (Hall *et al.*, 2003a, b, 2005; An *et al.*, 2006). It remains important to clarify their roles in both ecology and infections (Clarridge & Zhang, 2002).

In this report, we describe a novel species belonging to the genus *Actinomyces* that is related closely to *Actinomyces gerencseriae* and was isolated from a human blood sample.

Isolation and characterization of the strain

A 38-year-old man was admitted to hospital in December 2003 for haemoptysis. He presented with cough, flu syndrome, fever (38.5 °C), diarrhoea, asthenia and weight loss. Computed tomography examination showed pulmonary excavated lesions, which were treated by surgery. The patient was readmitted to hospital in April 2004, 1 month following pulmonary surgery, presenting with malaise, dyspnoea and basal thoracic pain. Blood cultures remained sterile, but a pleuropneumonia was diagnosed and the patient was treated empirically with 3 g cotrimoxazole day⁻¹ and 2 g ciprofloxacin day⁻¹ for 10 days. His condition improved quickly. Blood cultures were performed to follow the patient evolution and an anaerobic bacterium was isolated (4401292^T). Phenotypic identification was impossible to obtain, but genomic identification was obtained by 16S rRNA gene sequence comparison. Strain 4401292^T was identified as a representative of the genus *Actinomyces*.

Antimicrobial susceptibility was determined according to the criteria of the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical Laboratory Standards (NCCLS). Strain 4401292^T was susceptible to penicillin G, amoxicillin, cefotetan, vancomycin, imipenem, rifampicin, erythromycin, doxycycline, gentamicin and trimethoprim/sulfamethoxazole.

Surface colonies on sheep-blood agar (bioMérieux) were circular, white, shiny and pinpoint after 48 h anaerobic incubation. Growth and haemolytic activity were tested at 37 °C on 5 % sheep-blood agar. Growth was tested in the presence of air, in 5 % CO₂ and in anaerobic and microaerophilic atmospheres that were created by using

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Actinomyces massiliensis* 4401292^T is EF558367.

GENbag anaer and GENbag microaer (bioMérieux), respectively. The ability of the strain to grow at different temperatures (25, 30, 37, 44 and 50 °C) was investigated. Optimum growth was obtained at 37 °C, but occurred between 30 and 37 °C. The strain was anaerobic and also grew in microaerophilia and in 5 % CO₂. The bacteria were non-haemolytic. The size and ultrastructure of cells were determined by transmission electron microscopy. Cells were grown on 5 % sheep-blood agar for 24 h. A 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. Samples were examined on a Morgagni 268D (Philips) electron microscope at an operating voltage of 60 kV. The rods were 0.5–1.7 µm long and 0.35–0.74 µm wide.

Catalase activity, as determined by an ID Color Catalase test kit (bioMérieux), was negative. Oxidase activity, assayed by applying the cells to moistened discs impregnated with dimethyl-*p*-phenylenediamine (bioMérieux), was negative.

The commercially available API ZYM, API Rapid ID 32 A and API 50 CH strips, combined with API 50CHB/E medium (all from bioMérieux), were used to characterize the biochemical properties of the strain according to the manufacturer's instructions.

Phenotypic characteristics were compared with those of *Actinomyces oricola* CIP 107639^T, *Actinomyces israelii* CIP 103259^T, *A. gerencseriae* CIP 105418^T, *Actinomyces naeslundii* CIP 103128^T, *Actinomyces viscosus* CIP 103147^T and *Actinomyces catuli* CIP 106507^T, which were the most closely related species when 16S rRNA gene sequences were compared. Characteristic traits are presented in Table 1. Results of other tests for the type strain are given in the species description.

Preparation and determination of cellular fatty acids were carried out by following the procedures given for the Sherlock Microbial Identification system (MIDI). The major fatty acids were C18:1 ω 9 c (41.2 %), C16:0 (34.0 %), C18:0 (10.9 %) and a mix of C18:0 anteiso and 18:2 ω 6,9 c (10.9 %). The predominance of C16:0 and C18:1 ω 9 c was similar to the fatty acid profiles of other closely related members of the genus *Actinomyces*. However, the presence of a mix of C18:2 ω 6,9 c and C18:0 (10.9 %) was characteristic of our isolate (Table 2).

Genotypic characterization

Bacterial DNA was extracted by using a MagNA Pure LC DNA isolation kit III (Roche) with a MagNA Pure LC instrument, as described by the manufacturer. PCR amplification of the 16S rRNA gene was performed 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 v1.1 Ready Reactions; PE Biosystems) according

to the manufacturer's instructions. Sequencing products were purified and electrophoresis was performed with a 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with sequences deposited in GenBank by using the BLAST program through the NCBI server. Gene sequences were aligned by using the multiple sequence alignment program CLUSTAL_X (1.8). Phylogenetic relationships with closely related species were determined by using MEGA version 4.0 (Tamura *et al.*, 2007). Distance matrices were determined following the assumptions described by Kimura (1980) and were used to elaborate a dendrogram using the neighbour-joining method (Saitou & Nei, 1987). The maximum-parsimony algorithm was also used to infer phylogenetic analysis. A bootstrap analysis (bootstrap values were obtained for a consensus tree based on 100 randomly generated trees) was performed to investigate the stability of the trees obtained. The clustering of the novel isolate was the same with the two methods. The phylogenetic analysis demonstrated that our isolate is a member of the genus *Actinomyces*. It clustered with *A. gerencseriae* and *A. israelii* (Fig. 1) and three other species: *A. oricola*, *A. ruminicola* and *A. dentalis*. Percentage similarities between 16S rRNA gene sequences were determined by using NALIGN in the PC/GENE software package (IntelliGenetics). Similarities between strain 4401292^T and *A. gerencseriae* DSM 6844^T, *A. israelii* CIP 103259^T, *A. oricola* CCUG 46090^T, *A. ruminicola* B71^T and *A. dentalis* R18165^T were 95.1, 95.2, 95.2, 93.3 and 91.4 %, respectively. All of these values are <97 %, confirming that strain 4401292^T belongs to a novel species (Stackebrandt *et al.*, 2002) in the genus *Actinomyces*.

Based on the results described above, we propose the name *Actinomyces massiliensis* sp. nov. for the novel bacterium, with 4401292^T as the type strain.

Description of *Actinomyces massiliensis* sp. nov.

Actinomyces 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).

Cells are anaerobic, Gram-positive, straight rods that are catalase- and oxidase-negative. Optimal growth occurs at 37 °C. After 48 h growth on sheep-blood agar, surface colonies are pinpoint. The rods are 0.5–1.7 µm in length and 0.35–0.74 µm in diameter, as estimated by electronic microscopy. By using API ZYM, leucine arylamidase, valine arylamidase, β -galactosidase and α -glucosidase activities are detected. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α -chemotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -glucuronidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase activities are not detected. By using API 50 CH strips after an incubation time of 24 h, acid is produced from D-galactose, D-glucose, D-fructose, maltose, lactose, sucrose and trehalose. After an incubation time of 48 h, acid is produced from D-xylose, melezitose, turanose

Table 1. Diagnostic traits of species tested in this study

Taxa: 1, *A. massiliensis* 4401292^T; 2, *A. oricola* CIP 107639^T; 3, *A. israelii* CIP 103259^T; 4, *A. gerencseriae* CIP 105418^T; 5, *A. naeslundii* CIP 103128^T; 6, *A. viscosus* CIP 103147^T; 7, *A. catuli* CIP 106507^T. w+, Weakly positive. Acid production results were read after 24 h; some strains showed a different result at 48 h (in parentheses). API ZYM and API 32 ANA results were read after 4 h. All taxa were positive for proline arylamidase and growth at 37 °C.

Test	1	2	3	4	5	6	7
Acid production from:							
Glycerol	–	–	–	–	–	– (+)	–
L-Arabinose	–	–	–	–	–	–	+
D-Ribose	– (w+)	–	+	–	–	–	+
D-Xylose	– (+)	–	+	–	–	–	+
D-Galactose	+	–	+	–	–	+	+
D-Glucose	+	–	+	– (+)	+	+	+
D-Fructose	+	–	+	–	+	+	+
D-Mannose	– (w+)	–	+	–	– (+)	+	+
Inositol	–	–	– (+)	–	– (+)	+	–
D-Mannitol	–	–	– (+)	–	–	–	–
Methyl- α -D-glucopyranoside	–	–	–	–	–	–	– (+)
N-Acetylglucosamine	–	–	–	–	+	– (+)	+
Amygdalin	–	–	+	–	–	–	+
Arbutin	–	–	+	–	– (+)	+	+
Aesculin	–	–	+	+	+	+	+
Salicin	–	–	+	w+	+	+	+
Cellobiose	–	–	+	–	–	–	+
Lactose	+	–	+	+	–	+	+
Melibiose	–	–	+	–	+	+	+
Trehalose	+	–	+	–	+	+	+
Melezitose	– (+)	–	–	–	–	–	–
Raffinose	–	–	+	– (w+)	+	+	+
Starch	–	–	–	–	–	–	+
Gentiobiose	–	–	– (+)	–	–	–	+
Turanose	– (+)	–	–	– (w+)	–	–	+
D-Lyxose	– (+)	–	– (+)	–	–	–	+
L-Fucose	–	–	–	–	–	+	–
Potassium 5-ketogluconate	–	–	–	– (w+)	–	–	–
API ZYM:							
Esterase (C4)	–	–	w+	+	–	+	–
Valine arylamidase	+	–	–	+	–	–	–
α -Galactosidase	–	–	–	–	+	–	–
β -Galactosidase	+	–	+	+	+	+	+
α -Glucosidase	+	+	+	+	–	–	+
β -Glucosidase	–	+	+	+	+	+	+
API 32 ANA:							
Arginine dihydrolase	–	–	+	+	–	–	+
α -Galactosidase	–	w+	+	+	+	+	+
β -Glucosidase	–	+	+	+	+	+	+
α -Arabinosidase	–	–	+	+	–	–	+
β -Glucuronidase	–	–	–	–	–	–	+
N-acetyl- β -glucosaminidase	–	–	–	–	–	–	+
Glycine arylamidase	–	–	–	–	+	–	–
Histamine arylamidase	–	–	–	–	+	–	–
Raffinose fermentation	–	+	+	+	+	+	+
Indole production	–	–	–	–	–	–	+

Table 1. cont.

Test	1	2	3	4	5	6	7
Growth in/at:							
Aerobic atmosphere	—	—	—	—	+	+	+
5 % CO ₂	+	—	—	w +	+	+	+
Microaerophilic atmosphere	+	w +	w +	w +	+	+	+
Anaerobic atmosphere	+	+	+	+	+	+	+
25 °C	—	—	w +	—	+	+	w +
30 °C	w +	—	w +	+	+	+	+
44 °C	—	—	—	—	+	+	—

Table 2. Whole-cell fatty acid compositions (%) of the novel *Actinomyces* isolate and related species

ND, Not detected. Data for *A. massiliensis* are from the current study; data for *A. oricola* are from Hall *et al.* (2003a); data for *A. ruminicola* and *A. israelii* are from An *et al.* (2006); data for *A. nasicola* are from Hall *et al.* (2003b).

Species	C16:0	Mix of C18:2 ω 6,9c and C18:0 anteiso	C18:1 ω 9c	C18:0
<i>A. massiliensis</i>	34.0	10.9	41.2	10.9
<i>A. oricola</i>	44	ND	37	14
<i>A. ruminicola</i>	34.64	ND	25.33	15.91
<i>A. nasicola</i>	29	ND	26	11
<i>A. israelii</i>	38.9	ND	33.4	2.5

Fig. 1. Phylogenetic tree of representatives of the genus *Actinomyces* inferred from 16S rRNA gene sequence comparison (1358 nt fragment). Numbers at nodes are proportions of 100 resamplings that support the topology shown; values >50 % are indicated. *Varibaculum cambriense* was used as the outgroup. Bar, 0.01 nucleotide change per nucleotide position.

and D-lyxose. After an incubation time of 48 h, acid is produced weakly from D-ribose and D-mannose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, methyl β -D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, cellobiose, melibiose, inulin, raffinose, starch, glycogen, xylitol, gentiobiose, D-tagatose, D-fucose, L-fucose, D-arabinol, L-arabinol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. By using API Rapid ID 32 A, the following tests were positive: β -galactosidase, α -glucosidase, reduction of nitrates, proline arylamidase, phenylalanine arylamidase and leucine arylamidase. Mannose fermentation and tyrosine arylamidase were weakly positive. The remaining tests were negative: urease, arginine dihydrolase, α -galactosidase, β -galactosidase 6-phosphate, β -glucosidase, α -arabinosidase, β -glucuronidase, N-acetyl- β -glucosaminidase, raffinose fermentation, glutamic acid decarboxylase, α -fucosidase, indole production, alkaline phosphatase, arginine arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glycine arylamidase, histamine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase. The fatty acid profile is characterized by the presence of C12:0, C14:0, C16:1 ω 9c, C16:1 ω 7c, C16:0, a mix of C18:2 ω 6,9c and C18:0 anteiso, C18:1 ω 9c and C18:0.

The type strain, which was isolated from a human blood sample, is 4401292^T (=CSUR P18^T=CCUG 53522^T).

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