Actinomyces timonensis sp. nov., isolated from a human clinical osteo-articular sample

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Gram-positive, non-spore-forming rods were isolated from a human osteo-articular sample (strain 7400942^T). Based on cellular morphology and the results of biochemical analysis, this strain was tentatively identified as a novel species of the genus *Actinomyces*. Phylogenetic analysis based on 16S rRNA gene sequence comparisons showed that the bacterium was closely related to the type strain of *Actinomyces denticolens* (96.9% 16S rRNA gene sequence similarity). A comparison of biochemical traits showed that strain 7400942^T was distinct from *A. denticolens* in a number of characteristics, i.e. in contrast with *A. denticolens*, strain 7400942^T was negative for nitrate reduction and for β -galactosidase, α -glucosidase and alanine arylamidase activities, it was positive for acid production from *N*-acetylglucosamine, melezitose and glycogen, and it was negative for acid production from turanose. Matrix-assisted laser-desorption/ionization time-of-flight MS protein analysis confirmed that strain 7400942^T represents a novel species, as scores obtained for its spectra were significant (>2.2) only with strain 7400942^T. On the basis of phenotypic data and phylogenetic inference, it is proposed that this strain should be designated *Actinomyces timonensis* sp. nov.; the type strain is strain 7400942^T (=CSUR P35^T=CCUG 55928^T).

The genus *Actinomyces* consists of high-G + C-containing, Gram-positive, anaerobic and aerotolerant, non-spore-forming, non-motile, rod-shaped micro-organisms. The natural habitats of members of the genus *Actinomyces* are the mucous membranes of humans and animals, particularly the oral mucosa, and urogenital and intestinal tracts (Acevedo *et al.*, 2008). Mucosal disruption leads to infection. Most commonly, actinomycosis involves the cervicofacial area, followed by the abdominal and thoracic areas (Acevedo *et al.*, 2008), but cutaneous, uterine, ocular, cerebrospinal and hepatic infections have also been described. Infection of bones is mainly due to adjacent tissue infection, but has also been described in some fractures or haematogenous spread. The lesions of actinomycosis are distinct from those observed in pyogenic osteomyelitis, multiple myeloma, fungal disease and osseous lesions of tuberculosis or metastatic cancer (Rothschild *et al.*, 2006). *Actinomyces naeslundii* has been reported to cause hip prosthesis infection (Funke *et al.*, 1997).

Abbreviation: MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight MS.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Actinomyces timonensis* strain 7400942^T is EU484334.

A supplementary figure showing the extended phylogenetic tree of representatives of the genus *Actinomyces* inferred from 16S rRNA gene sequence comparisons is available with the online version of this paper.

Many studies have been performed to characterize *Actinomyces* representatives using phenotypic approaches (Santala *et al.*, 2004; Miller *et al.*, 1995; Kerttula *et al.*, 2005), but their identification has remained problematic. Molecular approaches have been performed to detect, identify and clarify the taxonomic positions of putative pathogens (Hall *et al.*, 2001; Woo *et al.*, 2003; Song, 2005) and many novel species have been described from human or animal sources (Pascual *et al.*, 1999; Hall *et al.*, 2003a, b, 2005; An *et al.*, 2006), principally based on 16S rRNA gene sequence comparisons and supported by other taxonomic tools (Hall, 2008).

In this report, a strain of a novel species isolated from a human osteo-articular sample is described. It belongs to the genus *Actinomyces* and is closely related to the type strain of *Actinomyces denticolens* (96.9% 16S rRNA gene sequence similarity).

A 13-year-old girl was examined in January 2007 at the Timone Hospital in Marseilles, France. She had presented osteo-articular troubles for the past 5 years, which had never been resolved in spite of various treatments. At the present time, she suffered from a sacroiliitis, which was sampled for systematic bacteriological analysis. Acid-fast bacilli were found on direct examination, but no bacteria were cultivated on blood agar and PCR amplification remained negative. Nevertheless, a shell-vial culture assay (Gouriet *et al.*, 2005) was performed as this is done

routinely in our laboratory. A Gram-positive micro-organism, strain 7400942^T, was isolated and identified by 16S rRNA gene sequencing (Weisburg *et al.*, 1991) as a representative of the genus *Actinomyces*.

Antimicrobial susceptibility was determined according to the criteria of the Clinical and Laboratory Standards Institute, formerly the National Committee for Clinical Laboratory Standards. Strain 7400942^T was susceptible to penicillin G, amoxicillin-clavulanic acid, cefotetan, vancomycin and imipenem, but resistant to metronidazole.

Bacterial DNA was extracted using the MagNA Pure LC DNA isolation kit III (Roche) with the MagNA Pure LC instrument, as described by the manufacturer. PCR amplification of the 16S rRNA gene was performed using the universal primer pair fD1 and rp2 (Weisburg *et al.*, 1991). PCR products were purified using MultiScreen PCR (Millipore) and sequencing reactions were carried out using a DNA sequencing kit (BigDye Terminator Cycle Sequencing v1.1 Ready Reaction kit; PE biosystems) according to the manufacturer's instructions. Sequencing products were purified and electrophoresis was performed with the 3130 Genetic Analyzer (Applied Biosystems). The obtained sequences were compared with sequences deposited in GenBank by using the program BLAST through the NCBI server. Gene sequences were aligned using the multisequence 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. Bootstrap analysis (bootstrap values were obtained for a consensus tree based on 1000 randomly generated trees) was performed to investigate the stability of the trees obtained. Phylogenetic analysis demonstrated that our isolate is a member of the

genus *Actinomyces* and clustering of the novel isolate was the same with the two methods; it clustered with the type strain of *A. denticolens* (Fig. 1; see also Supplementary Fig. S1, available in IJSEM Online). 16S rRNA gene sequence similarities (%) were determined by using the EzTaxon server (Chun *et al.*, 2007); similarities between strain 7400942^T and the type strains of *A. denticolens*, *A. oricola*, *A. massiliensis*, *A. gerencseriae*, *A. dentalis*, *A. israelii* and *A. ruminicola* were 96.9, 93.7, 93.6, 92.6, 92.6, 92.6 and 92.3 %, respectively. These values were below 97 %, which is the recommended cut-off value for delineation of species, thus confirming that strain 7400942^T belongs to a novel species.

Catalase activity was determined by the ID colour catalase test kit (bioMérieux). Oxidase activity was assayed by applying the cells to moistened discs impregnated with dimethyl-*p*-phenylenediamine (bioMérieux). The commercially available API ZYM, API Rapid ID32A and API 50CH strips combined with API 50CHB/E medium (bioMérieux) were used to characterize the biochemical properties of the strain according to the manufacturer's instructions.

Phenotypic characteristics were compared with those of *A. oricola* CIP 107639^T, *A. israelii* CIP 103259^T, *A. gerencseriae* CIP 105418^T, *A. naeslundii* CIP 103128^T, *A. viscosus* CIP 103147^T, *A. catuli* CIP 106507^T, *A. denticolens* CIP 103126^T, *A. howellii* CIP 103127^T, *A. slackii* CIP 103130^T, *A. massiliensis* CSUR P18^T and *A. bowdenii* CIP 106647^T, which were the most closely related strains in terms of 16S rRNA gene sequence similarity. Characteristic traits are presented in Tables 1 and 2. Comparison of biochemical traits showed that strain 7400942^T was distinct from all other *Actinomyces* species. Characteristics that differentiated the novel isolate from *A. denticolens* were: nitrate reduction; α -galactosidase, β -galactosidase, α -glucosidase, β -glucosidase and alanine arylamidase activities; and acid production from D-mannose, D-mannitol, N-acetylglucosamine, arbutin, trehalose, melezitose, glycogen, turanose and D-arabitol. Other results are given in the species description.

Fig. 1. Phylogenetic tree of representatives of the genus *Actinomyces* inferred from 16S rRNA gene sequence comparisons (1360 nt fragment). Numbers at nodes are proportions of 1000 resamplings that support the topology shown. *Varibaculum cambriense* CCUG 44998^T (AM084231) was used as the out-group (not shown). Bar, 0.01 nucleotide changes per nucleotide position. An extended tree containing a wider selection of reference sequences is available as Supplementary Fig. S1 (available in IJSEM Online). Asterisks after the bootstrap values indicated the conserved nodes using the neighbour-joining and maximum-parsimony methods.

Table 1. Acid production from various substrates by strains of the *Actinomyces* species tested in this study

Strains: 1, *A. timonensis* 7400942^T; 2, *A. massiliensis* CCUG 53522^T; 3, *A. oricola* CIP 107639^T; 4, *A. israelii* CIP 103259^T; 5, *A. gerencseriae* CIP 105418^T; 6, *A. naeslundii* CIP 103128^T; 7, *A. viscosus* CIP 103147^T; 8, *A. catuli* CIP 106507^T; 9, *A. denticolens* CIP 103126^T; 10, *A. slackii* CIP 103130^T; 11, *A. bowdenii* CIP 106647^T; 12, *A. howellii* CIP 103127^T. +, Positive; –, negative; w, weakly positive; v, variable. Acid production was determined after 24 and 48 h: where one result is given, this was taken at 24 h and remained the same at 48 h; and where two results are given, the first was taken at 24 h and the second was taken at 48 h (i.e. 24 h/48 h).

Substrate	1	2	3	4	5	6	7	8	9	10	11	12
Glycerol	w / +	–	–	–	–	–	– / +	–	–	w / +	+	–
L-Arabinose	–	–	–	–	–	–	–	+	–	–	–	–
D-Ribose	w / +	– / w	–	+	–	–	–	+	w / +	–	+	+
D-Xylose	–	– / +	–	+	–	–	–	+	–	+	–	–
D-Adonitol	–	–	–	–	–	–	–	–	–	–	–	–
D-Galactose	+	+	–	+	–	–	+	+	+	+	+	– / w
D-Glucose	+	+	–	+	– / +	+	+	+	+	+	+	+
D-Fructose	+	+	–	+	–	+	+	+	+	+	+	– / w
D-Mannose	w / +	– / w	–	+	–	– / +	+	+	+	+	+	– / w
Inositol	+	–	–	– / +	–	– / +	+	–	+	+	+	–
D-Mannitol	+	–	–	– / +	–	–	–	–	w / +	–	–	–
Methyl α -D-glucopyranoside	–	–	–	–	–	–	–	– / +	–	–	–	– / +
N-Acetylglucosamine	+	–	–	–	–	+	– / +	+	–	–	–	–
Amygdalin	–	–	–	+	–	–	–	+	–	–	–	+
Arbutin	v	–	–	+	–	– / +	+	+	–	+	+	–
Aesculin	+	–	–	+	+	+	+	+	+	+	+	+
Salicin	+	–	–	+	w	+	+	+	+	+	+	–
Cellobiose	–	–	–	+	– / +	–	–	+	–	– / w	+	– / +
Maltose	+	+	–	+	– / +	+	+	+	+	+	+	+
D-Lactose	+	+	–	+	+	–	+	+	+	+	+	–
Melibiose	+	–	–	+	– / +	+	+	+	+	+	+	– / w
Sucrose	+	+	–	+	+	+	+	+	+	+	+	+
Trehalose	+	+	–	+	–	+	+	+	– / +	+	+	– / w
Melezitose	+	– / +	–	–	–	–	–	–	–	–	+	– / w
Raffinose	+	–	–	+	– / w	+	+	+	+	+	+	w / +
Starch	–	–	–	–	–	–	–	+	–	–	–	–
Glycogen	w / +	–	–	–	–	–	–	–	–	–	–	–
Gentiobiose	–	–	–	– / +	–	–	–	+	–	+	+	–
Turanose	–	– / +	–	–	– / w	–	–	+	w / +	+	+	+
D-Lyxose	–	– / +	–	– / +	–	–	–	+	–	+	–	–
L-Fucose	–	–	–	–	–	–	+	–	–	–	–	–
D-Arabitol	– / w	–	–	–	–	–	–	–	–	–	–	–
Potassium 5-ketogluconate	–	–	–	–	– / w	–	–	–	–	–	–	–

Matrix-assisted laser-desorption/ionization time-of-flight MS (MALDI-TOF MS) protein analysis was carried out. This new approach to the identification of bacteria based on bacterial protein composition analysis is simple and fast. It could be used for routine detection if high-quality reference databases were created (Mellmann *et al.*, 2008; Stingu *et al.*, 2008). Bacteria were grown in Schaedler broth supplemented with K3 (bioMérieux) for 24 h and washed twice in phosphate buffer. Smear bacterial material was deposited on each spot of a polished-steel target plate. After air-drying, 1.5 μ l matrix solution (saturated solution of α -cyanohydroxycinnaminic acid in 50 % aqueous acetonitrile containing 2.5 % trifluoroacetic acid) per spot was applied. MALDI-TOF MS was conducted using the Autoflex II mass spectrometer (Bruker Daltonics). All

spectra were recorded in linear, positive ion mode. The acceleration voltage was 20 kV. Spectra were collected as a sum of 675 shots across a spot. The manipulation was repeated 15 times for strain 7400942^T and the tested type strains. Pre-processing and identification steps were performed using the manufacturer's parameters. Consensus spectra were obtained. Scores obtained for the spectra of strain 7400942^T were significant (>2.2) only with strain 7400942^T, thus confirming that it represented a novel species, and a taxonomic tree was obtained using the principal component analysis clustering method (Fig. 2). Strain 7400942^T clustered with the type strain of *A. viscosus*.

Surface colonies on sheep blood agar (bioMérieux) were circular, white, dry, embedded in the agar and pin-point

Table 2. Diagnostic traits (API ZYM strips, API Rapid ID 32A strips and growth conditions) of strains of the *Actinomyces* species tested in this study

Strains: 1, *A. timonensis* 7400942^T; 2, *A. massiliensis* CCUG 53522^T; 3, *A. oricola* CIP 107639^T; 4, *A. israelii* CIP 103259^T; 5, *A. gerencseriae* CIP 105418^T; 6, *A. naeslundii* CIP 103128^T; 7, *A. viscosus* CIP 103147^T; 8, *A. catuli* CIP 106507^T; 9, *A. denticolens* CIP 103126^T; 10, *A. slackii* CIP 103130^T; 11, *A. bowdenii* CIP 106647^T; 12, *A. howellii* CIP 103127^T. +, Positive; –, negative; w, weakly positive; v, variable. API ZYM and Rapid ID 32A tests were all carried out for 4 h.

Characteristic/test	1	2	3	4	5	6	7	8	9	10	11	12
API ZYM:												
Alkaline phosphatase	–	–	–	–	–	–	–	–	–	–	–	+
Esterase (C4)	–	–	–	w	+	–	+	–	–	–	–	w
Esterase lipase (C8)	–	–	–	–	–	–	–	–	–	–	–	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	–
Valine arylamidase	–	+	–	–	+	–	–	–	–	–	–	–
α -Galactosidase	–	–	–*	–*	–*	+	–*	–*	–*	–*	+	–*
β -Galactosidase	–	+	–	+	+	+	+	+	+	+	–*	+
α -Glucosidase	–	+	+	+	+	–*	–*	+	+	+	+	+
β -Glucosidase	–*	–	+	+	+	+	+	+	+	+	+	–*
<i>N</i> -Acetyl- β -glucosaminidase	–	–	–	–	–	–	–	–*	–	–	–	+
α -Mannosidase	–	–	–	–	–	–	–	–	–	–	–	+
Rapid ID 32A:												
Arginine dihydrolase	–	–	–	+	+	–	–	+	–	+	+	–
α -Arabinosidase	–	–	–	+	+	–	–	+	–	+	–	–
β -Glucuronidase	–	–	–	–	–	–	–	+	–	–	–	–
Mannose fermentation	v	w	+	+	+	+	+	+	+	+	+	+
Raffinose fermentation	v	–	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	–	+	–	+	+	+	+	+	+	+	+	–
Arginine arylamidase	–	–	–	–	–	–	–	+	–	w	w	–
Proline arylamidase	+	+	+	+	+	+	+	+	+	+	+	–
Leucyl glycine arylamidase	–	–	–	–	–	–	–	–	–	–	+	+
Phenylalanine arylamidase	+	+	+	+	+	+	+	+	+	+	+	–
Pyroglutamic acid arylamidase	–	–	–	–	–	–	–	–	–	–	–	+
Tyrosine arylamidase	+	w	+	+	+	+	+	+	+	+	+	–
Alanine arylamidase	–	–	–	+	+	–	–	+	w	–	w	w
Glycine arylamidase	–	–	–	–	–	+	–	–	–	–	+	+
Histamine arylamidase	–	–	–	–	–	–	–	–	–	w	–	–
Serine arylamidase	–	–	–	–	–	–	–	–	–	–	–	w
Atmospheric conditions for growth:												
Aerobic	w	–	–	–	–	+	+	+	+	+	+	+
5 % CO ₂	+	+	–	–	w	+	+	+	+	+	+	+
Microaerophilic	+	+	w	w	w	+	+	+	+	+	+	+
Anaerobic	+	+	+	+	+	+	+	+	+	–	+	+
Growth at:												
25 °C	+	–	–	w	–	+	+	w	w	–	+	+
30 °C	+	w	–	w	+	+	+	+	+	–	+	+
37 °C	+	+	+	+	+	+	+	+	+	+	+	+
44 °C	–	–	–	–	–	+	+	–	–	–	+	w

*Results were negative with API ZYM strips, but positive with Rapid ID 32A strips.

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 under anaerobic and microaerophilic atmospheres, which were created using 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; growth occurred between 25 and 37 °C. The

strain was anaerobic and also grew under microaerophilic conditions and in 5 % CO₂. Growth in the presence of air was weak after 48 h incubation. The isolate was α -haemolytic after 48 h incubation. The size and ultrastructure of cells were determined by transmission electron microscopy. Cells were grown in trypticase soy broth for 48 h. A bacterial suspension was pre-fixed in 5 % (v/v) glutaraldehyde in phosphate buffer (Gibco) for at least 1 h

Fig. 2. Dendrogram generated by BIOTYPER software (version 2; Bruker Daltonics) showing the similarity of MALDI-TOF mass spectra of strain 7400942^T and representatives of the genus *Actinomyces*.

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. Results are given in the species description.

Based on results described above, the name *Actinomyces timonensis* sp. nov. is proposed to accommodate the novel isolate, with strain 7400942^T as the type strain.

Description of *Actinomyces timonensis* sp. nov.

Actinomyces timonensis (ti.mo.nen'sis. L. masc. adj. *timonensis* pertaining to la Timone, named after Hôpital de la Timone, the hospital in Marseilles, France, from where the type strain was isolated).

Cells are anaerobic, Gram-positive, straight rods, catalase-negative and oxidase-negative. Optimal growth occurs at 37 °C. After 48 h growth on blood sheep agar, surface colonies are pin-point. The rod size, estimated by electron microscopy, is 1.0–3.2 µm in length and 0.3–0.5 µm in diameter. Using API ZYM, leucine arylamidase activity is detected, but valine arylamidase, β-galactosidase, α-glucosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, 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. Using API 50CH strips after an incubation time of 24 h, acid is produced from D-galactose, D-glucose, D-fructose, inositol, D-mannitol, *N*-acetylglucosamine, aesculin

ferric citrate, salicin, maltose, D-lactose, melibiose, sucrose, trehalose, melezitose and raffinose. After an incubation time of 24 h, acid is produced weakly from glycerol, D-ribose, D-mannose and glycogen. After an incubation time of 48 h, acid is produced from glycerol, D-ribose, D-mannose and glycogen and acid is produced weakly from D-arabitol. Acid is not produced from erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, cellobiose, inulin, starch, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. After 24 h incubation, acid production is variable from arbutin. After 48 h incubation, acid production is variable from D-adonitol and arbutin. Using API Rapid ID32A: positive for β-glucosidase, proline arylamidase, phenylalanine arylamidase, leucine arylamidase and tyrosine arylamidase; variable for α-glucosidase, mannose fermentation and raffinose fermentation; and negative for urease, arginine dihydrolase, α-galactosidase, β-galactosidase-6-phosphate, β-galactosidase, α-arabinosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, glutamic acid decarboxylase, α-fucosidase, nitrate reduction, indole production, alkaline phosphatase, arginine arylamidase, leucyl glycine arylamidase, pyroglutamic acid arylamidase, alanine arylamidase, glycine arylamidase, histidine arylamidase, glutamyl glutamic acid arylamidase and serine arylamidase.

The type strain is 7400942^T (=CSUR P35^T=CCUG 55928^T), isolated from a human clinical osteo-articular sample.

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