

Noncontiguous finished genome sequences and descriptions of *Actinomyces ihuae*, *Actinomyces bouchesdurhonensis*, *Actinomyces urinae*, *Actinomyces marseillensis*, *Actinomyces mediterranea* and *Actinomyces oralis* sp. nov. identified by culturomics

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Abstract

The taxonogenomic approach, including the culturomics techniques, is now currently used to isolate and characterize new bacteria. These approaches notably allowed us to discover six new species of the *Actinomyces* genus: *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. Each is the type strain of the corresponding bacterial species. 16S ribosomal RNA gene sequence comparison was used to classify these strains among the *Actinomyces* genus. These strains are all Gram positive, rod shaped and facultative aerobic. We describe the main characteristics of each bacterium and present their complete genome sequence and annotation.

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Introduction

Known bacteria of the genus *Actinomyces* are all Gram positive. The majority are facultative anaerobes, but some are strict anaerobes. Members of the genus *Actinomyces* have a high DNA G+C content [1]. The genus *Actinomyces* was first established in 1877 by Harz and colleagues as a member of the *Actinobacteria* phylum [2]. The first isolated species of this genus was *Actinomyces bovis* [3]. Today 47 species have been identified with validly published names with standing in nomenclature. The

number of discovered *Actinomyces* species increased considerably from the 1980s, corresponding with the start of utilization of PCR (Fig. 1). This date was a turning point in the characterization of *Actinomyces* species, which now is not just only based on phenotypic observation but also on genetic analysis. Now, the development of quick and low-price genome sequencing and annotating allow us to go further in the characterization of bacterial species. *Actinomyces* species are known to be ubiquitous, colonizing soil, animals or humans. In humans, they are particularly present in the oral mucosa or urogenital and intestinal tracts [4]. Several of them, such as *Actinomyces israeli* [5–7], are present in the ground and can play an important role in the decomposition of organic matter and biotechnological studies. However, bacteria of the genus *Actinomyces* are also part of the normal flora of the oral cavity and respiratory tract, and can be implicated in blood and organ infections [8,9].

In this study, we used a new approach, including matrix-assisted desorption ionization–time of flight mass spectrometry

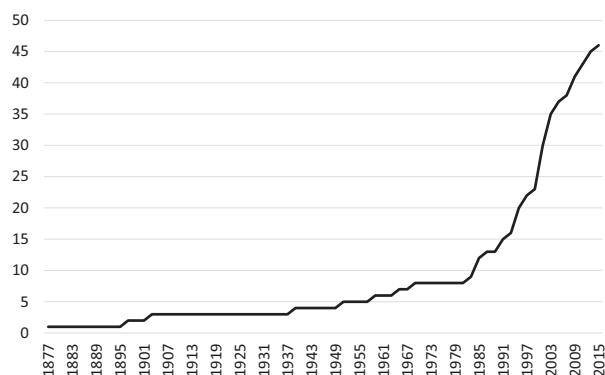


FIG. 1. Identification of *Actinomyces* new species since first one, *Actinomyces bovis*, was described by Harz and collaborators in 1877 [3].

(MALDI-TOF MS), phenotypic description and genome sequencing [10–14] to describe six new *Actinomyces* species: *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. These are all Gram positive, rod shaped and facultative aerobic. They were respectively isolated from sputum sample of healthy persons living in Marseille (France) (*A. marseillensis* and *A. oralis*), stool sample of an HIV-infected man (*A. ihuae*), urine sample of a girl with nephrotic syndrome (*A. urinae*), duodenum wash sample of a woman with oesophagitis (*A. mediterranea*) and stomach wash sample of a man with iron-deficiency anaemia (*A. bouchesdurhonensis*) (Table 1). These new bacterial species were part of a culturomics study which aimed to explore the diversity in the whole human microbiota using multiple culture conditions [15,16].

On the basis of the results of phenotypic, genomic and phylogenetic analyses, these strains are considered to represent new species of the *Actinomyces* genus. However, our study uses a new concept of bacterial description combining a proteomics analysis with the MALDI-TOF MS profile [17] associated with phenotypic and genomic descriptions of these six new species.

Here we present a summary of classification, main features and complete genomic sequencing and annotation of the present type strains of these six *Actinomyces* new species: *Actinomyces ihuae* strain SD1 (= CSUR P2006 = DSM 100538), *Actinomyces bouchesdurhonensis* strain Marseille-P2825 (= CSUR P2825 = DSM 103075), *Actinomyces urinae* strain Marseille-P2225 (= CSUR P2225 = DSM 100700), *Actinomyces marseillensis* strain Marseille-P2818 (= CSUR P2818 = CCGU 71898), *Actinomyces mediterranea* strain Marseille-P3257 (= CSUR P3257 = CCGU 70143) and *Actinomyces oralis* strain Marseille-P3109 (= CSUR P3109 = DSM 103942). These characteristics support the creation of these six new species.

TABLE 1. Sample information

Characteristic	A	B	C	D	E	F
Sample origin	Human stomach wash	Human stool	Human sputum	Human duodenum wash	Human sputum	Human urine
Patient information	60-year-old man with iron-deficiency anaemia	50-year-old HIV-infected man	Healthy Marseille woman	76-year-old woman with oesophagitis	Healthy Marseille man	13-year-old girl with nephritic syndrome
Authorization/consent	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)	No. 09-022 (IFR 48, Marseille)
Storage	-80°C	-80°C	-80°C	-80°C	-80°C	-80°C
Isolation conditions	COS medium day 3 anaerobic 37°C	COS medium day 2 microaerobic 37°C	Haemoculture + rumen day 30 anaerobic 30°C	Haemoculture + rumen + sang day 7 anaerobic 37°C	Haemoculture + rumen day 15 aerobic 37°C	Haemoculture + rumen day 10 anaerobic 37°C
A, <i>Actinomyces bouchesdurhonensis</i> strain Marseille-P2825; B, <i>A. ihuae</i> strain SD1; C, <i>A. marseillensis</i> strain Marseille-P2818; D, <i>A. mediterranea</i> strain Marseille-P3257; E, <i>A. oralis</i> strain Marseille-P3109; F, <i>A. urinae</i> strain Marseille-P2225.						

Materials and methods

Strain identification and phylogenetic analysis. Culturomics methodology, previously described by Lagier *et al.* [18], doubles the number of bacteria species isolated at least once from the human gut [16]. We used this methodology to isolate these strains from several diverse human samples. Samples, conditions of isolation and origins are summarized in Table 1. All patients provided informed consent, and the study was validated by the ethics committee of the Institut Fédératif de Recherche 48, Faculty of Medicine, Marseille, France, under agreement 09-022.

Purified colonies were identified by MALDI-TOF MS using a Microflex LT spectrometer and a MSP 96 MALDI-TOF target plate (Bruker Daltonics, Bremen, Germany), as previously described [17]. The obtained spectra were imported into MALDI Biotyper 3.0 software (Bruker) and analysed by standard pattern matching (with default parameter settings) against the main spectra of the 7537 bacteria included in the databases (constantly updated Bruker and Unité des Maladies Infectieuses et Tropicales Emergentes (URMITE) databases). The resulting score enabled the identification (or not) of tested species: a score of ≥ 2 with a validly published species enabled identification at the species level; a score of ≥ 1.7 but < 2 enabled identification at the genus level; and a score of < 1.7 did not enable any identification. Any significant score has been obtained for our six strains, suggesting that the isolates were not members of known species.

We thus realized sequencing of 16S rRNA genes in order to identify these strains. DNA was previously extracted by EZ1 DNA Tissue Kit using BioRobot EZ1 Advanced XL (Qiagen, Courtaboeuf, France). The amplification and purification of the 16S rRNA gene was done as previously described by using the universal primer pair fD1 and rP2 (Eurogentec, Angers, France). Sequencing was then done using the Big Dye Terminator v1.1 Cycle Sequencing Kit and ABI Prism 3130xl Genetic Analyzer capillary sequencer (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA), as previously described [19]. The 16S rRNA nucleotide sequences were assembled and corrected using CodonCode Aligner software (<http://www.codoncode.com>), and BLASTn searches were performed against the GenBank National Center for Biotechnology Information (NCBI) database (<http://blast.ncbi.nlm.nih.gov/gate1.inist.fr/Blast.cgi>) to determine the percentage of similarity with the closest bacteria. A similarity threshold $< 98.7\%$ allows the definition of a new species, whereas a threshold $< 95\%$ allows the definition of a new genus without performing DNA-DNA hybridization [20]. A custom Python script was used to automatically retrieve all species from the same family of the new species and to download 16S sequences from NCBI by parsing NCBI eUtils results and the NCBI taxonomy page, which only keeps sequences from type strains. In cases

of multiple sequences for one type strain, it selects the sequence obtaining the best identity rate from the BLASTn alignment with our sequence. The script then separates 16S gene sequences in two groups: one containing the sequences of strains from the same genus (group A) and one containing the others (group B). It finally only keeps the 48 closest strains from group A and the closest three strains from group B. Different species are selected because they are the closest species of each five studied strains.

All the spectra were integrated into the URMITE database (<http://www.mediterranee-infection.com/article.php?laref=256&titre=urms-database>). We compared the proteomic profiles between our strains and their closest species.

Phenotypic features. Optimal growth conditions of our strains were determined by testing five growth temperatures (20, 25, 30, 37 and 45°C) in an aerobic atmosphere with or without 5% CO₂ and under anaerobic and microaerophilic conditions using the GENbag anaer and GENbag microaer systems, respectively (bioMérieux, Marcy l'Etoile, France). Phenotypic characteristics such as Gram staining, motility, sporulation, and catalase and oxidase activities were tested as previously described [14]. Negative staining was done in order to observe cell morphology. Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1 hour at 4°C. A drop of cell suspension was deposited for approximately 5 minutes on glow-discharged formvar carbon film on 400 mesh nickel grids (FCF400-Ni; Electron Microscopy Sciences (EMS), Hatfield, PA, USA). The grids were dried on blotting paper, and the cells were negatively stained for 10 seconds with 1% ammonium molybdate solution in filtered water at room temperature. Electron micrographs were acquired with a Tecnai G20 Cryo (FEI Company, Limeil-Brevannes, France) transmission electron microscope operated at 200 keV.

Biochemical analysis of SD1, Marseille-P2825, Marseille-P2225, Marseille-P2818, Marseille-P3257 and Marseille-P3109 was carried out using API 50CH, API 20A and API ZYM strips according to manufacturer's instructions (bioMérieux). Table 2 lists data of our six new species compared to published data of type strains of close species: *Actinomyces oricola* strain CIP 107639 [21], *A. gerencseriae* strain CIP 105418 [22], *A. naeslundii* strain CIP 103128 [22,23], *A. timonensis* strain 7400942 [24] and *A. massiliensis* strain 4401292 [25].

Cellular fatty acid methyl ester (FAME) analysis was performed by gas chromatography/mass spectrometry (GC/MS). FAMES were prepared as described by Sasser [26]. GC/MS analyses were carried out as previously described [27]. Briefly, FAMES were separated using an Elite 5-MS column and monitored by mass spectrometry (Clarus 500-SQ 8 S; PerkinElmer, Courtaboeuf, France). A spectral database search was

TABLE 2. Comparison of phenotypic characteristics

Characteristic	A	B	C	D	E	F	G	H	I	J	K
Optimal temperature	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C	37°C
Atmosphere	Anaerobic	Aerobic	Aerobic	Anaerobic	Aerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic	Anaerobic
pH range	5.0–8.0	5.0–8.0	6.0–8.5	5.0–8.0	6.0–8.5	6.0–7.5	NA	5.7 - 6.6	NA	NA	NA
Colony aspect	Smooth and grey	Bright grey	Smooth and shiny	Smooth and white colour	Smooth and grey	Translucent, beige micro colonies	Pin point, breadcrumb like, white and nonhaemolytic	Circular, peaked to pulvinate, lumpy, opaque and white	NA	Circular, white, dry, embedded in the agar and pin point	Circular, white, shiny and pinpoint
Cell shape	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped	Rod shaped
Cell size (µm)	1.5–1.9	0.7–1	2.0–2.2	2.3–2.6	1.6–1.8	0.4–0.6	NA	NA	NA	1.0–3.2	0.5–1.7
Cell diameter (µm)	0.5–0.6	0.5–0.7	0.4–0.5	0.5–0.6	0.6–0.7	0.2–0.4	NA	NA	NA	0.3–0.5	0.35–0.74
Gram stain	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Salt tolerance (g.L-1)	<10%	<10%	5	<10%	5	10–15%	NA	NA	NA	NA	NA
Motility	No	No	No	No	No	No	No	No	No	NA	NA
Endospore formation	No	No	No	No	No	No	No	No	No	No	No
Major cellular fatty acid	18:1n9	16:0	18:1n9	18:1n9	16:0	16:0	16:0	NA	NA	NA	18:1v9c
Production of:											
Alkaline phosphatase	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	NA	-	NA	-	-
Nitrate reductase	+	-	+	-	+	-	-	+	+	-	+
Urease	-	-	+	+	+	+	-	-	-	-	-
β-Galactosidase	+	+	+	+	-	-	-	+	+	-	+
N-Acetylglucosamine	+	-	+	-	+	+	-	-	-	-	-
Acid from:											
L-Arabinose	-	-	-	+	-	+	-	-	-	-	-
Ribose	-	-	+	+	+	+	-	-	-	w/+	-/w
Mannose	-	+	+	+	+	+	-	-/+	+	w/+	-/w
Mannitol	-	-	-	-	-	+	-	-	-	+	-
D-Saccharose	+	+	+	+	+	+	NA	+	NA	NA	NA
D-Glucose	+	-	+	+	+	+	-	-/+	+	+	+
D-Fructose	+	-	+	+	+	+	-	-	+	+	+
D-Maltose	+	-	+	-	+	+	-	-/+	+	+	+
D-Lactose	+	+	-	+	+	+	-	+	-	+	+
Habitat	Human stomach	Human gut	Human lung	Human duodenum	Human lung	Human bladder	Human dental abscess	Human parotid abscess	Human sinus	Human clinical osteo-articular	Human blood

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225; G, *A. oricola* strain CIP 107639T; H, *A. gerencseriae* strain CIP 105418T; I, *A. naeslundii* strain CIP 103128T; J, *A. timonensis* strain 7400942T; K, *A. massiliensis* strain 4401292T.
 +, positive result; -, negative result; w, weakly positive result; NA, data not available.

performed using MS Search 2.0 operated with the Standard Reference Database IA (National Institute of Standards and Technology, Gaithersburg, MD, USA) and the FAME mass spectral database (Wiley, Chichester, UK). Antibiotic susceptibility was tested using the disc diffusion method [28] and according to European Committee on Antimicrobial Susceptibility Testing 2015 recommendations.

Genome description and comparison. Genomic DNA (gDNA) of *Actinomyces urinae*, *Actinomyces mediterranea*, *Actinomyces oralis* and *Actinomyces marseillensis* were first extracted by a mechanical treatment by acid-washed glass beads (G4649-500g; Sigma, St Louis, MO, USA) using a FastPrep BIO 101 instrument (Qbiogene, Strasbourg, France) at maximum speed (6.5) for 3×30 seconds. Then, for all the *Actinomyces* strains, successive pretreatments by a lysozyme incubation at 37°C for 2 hours (3 hours for *Actinomyces oralis*) were done, followed by proteinase K for 3 hours for *Actinomyces ihuae* only. gDNA was then extracted on the EZ1 biorobot (Qiagen) with the EZ1 DNA tissues kit. The elution volume was 50 μ L. gDNA was quantified by a Qubit assay with a high-sensitivity kit (Life Technologies, Carlsbad, CA, USA) (Supplementary Table S1).

The genome's assembly was performed with a pipeline that enabled us to create an assembly with different software (Velvet [29], Spades [30] and Soap Denovo [31]) on untrimmed data. gDNA was sequenced by MiSeq Technology (Illumina, San Diego, CA, USA) with the mate-pair strategy for all the *Actinomyces* strains, and paired end for *Actinomyces urinae*, *Actinomyces mediterranea* and *Actinomyces oralis*. The gDNA was barcoded in order to be mixed with 11 other projects with the Nextera Mate Pair sample prep kit (Illumina).

The mate-pair library was prepared with 1.5 μ g of gDNA using the Nextera mate-pair Illumina guide. The gDNA sample was simultaneously fragmented and tagged with a mate-pair junction adapter. The pattern of the fragmentation was validated on an Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) with a DNA 7500 labchip. The DNA fragments ranged in size from 1 to 11 kb (with an optimal size at 2.458, 2.458, 6.284, 5.830, 9.683 and 6.518kb for *A. urinae*, *A. mediterranea*, *A. oralis*, *A. ihuae*, *A. bouchedurhonensis* and *A. marseillensis*, respectively). No size selection was performed, and 600 ng (for *A. marseillensis* and *A. ihuae*), 284.7 ng (for *A. bouchedurhonensis*), 96.1 ng (for *Actinomyces urinae*), 112 ng (for *A. mediterranea*) and 250.7 ng (for *Actinomyces oralis*) of tagged fragments were circularized. The circularized DNA was mechanically sheared to small fragments, with an optimum at 1163, 1041, 406, 736, 1033 and 949 bp for *A. urinae*, *A. mediterranea*, *A. oralis*, *A. ihuae*, *A. bouchedurhonensis* and *A. marseillensis*, respectively, on the Covaris device S2 in T6 tubes (microtubes for *A. oralis* and *A. marseillensis*) (Covaris,

Woburn, MA, USA). The library profile was visualized on a High Sensitivity Bioanalyzer LabChip (Agilent Technologies), and the final concentration library was measured (Supplementary Table S1). The libraries were normalized at 2 nM (4 nM for *A. ihuae*) and pooled. After a denaturation step and dilution at 15 pM, the pool of libraries was loaded onto the reagent cartridge and then onto the instrument along with the flow cell. Automated cluster generation and a sequencing run were performed in a single 39-hour run in a 2×251 bp read length (2×250 bp read length for *A. oralis*, *A. marseillensis* and *A. marseillensis*). The paired reads were finally trimmed and assembled. For each assembly performed, GapCloser [31] was used to reduce gaps. Complementary information is provided in Supplementary Table S1.

To prepare the paired end library, dilution was performed to require 1 ng of each genome as input. The tagmentation step fragmented and tagged the DNA. Then limited-cycle PCR amplification (12 cycles) completed the tag adapters and introduced dual-index barcodes. After purification on AMPure XP beads (Beckman Coulter, Brea, CA, USA), the libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina). Normalized libraries were pooled for sequencing on the MiSeq device. Automated cluster generation and paired-end sequencing with dual index reads were performed in a single 39-hour run in 2×250 bp read length. Complementary information is available in Supplementary Table S1.

Open reading frames (ORFs) were predicted using Prodigal [32] with default parameters, but the predicted ORFs were excluded if they spanned a sequencing gap region. The predicted bacterial protein sequences were searched against the GenBank [33] and the Clusters of Orthologous Groups database (COGs) using BLASTP (E value $1e-03$, coverage 0.7 and identity percentage of 30%). If no hit was found, it was searched against the NR database using BLASTP with an E value of $1e-03$, coverage of 0.7 and an identity percentage of 30%, and if the sequence length was smaller than 80 aa, we used an E value of $1e-05$. The tRNAScanSE tool [34] was used to find tRNA genes, while ribosomal RNAs were found using RNAmmer [35]. Lipoprotein signal peptides and the number of transmembrane helices were predicted using Phobius [36]. Mobile genetic elements were predicted using PHAST [37] and RAST [38]. ORFans were identified if all the BLASTP performed did not give positive results (E value smaller than $1e-03$ for ORFs with sequence size larger than 80 aa or E value smaller than $1e-05$ for ORFs with sequence length smaller than 80 aa). Such parameter thresholds have already been used in previous studies to define ORFans. Artemis [39] and DNA Plotter [40] were used for data management and the visualization of genomic features, respectively. The Mauve alignment tool

(version 2.3.1) was used for multiple genomic sequence alignment [41]. PFAM-A conserved domains were searched on each protein with the HMMscan of the HMMER3 suite [42]. PKS and NRPS were searched against the ClusterMine360 [43] database. Resistome was analysed by using the ARG-ANNOT database [44]. The closest species for genomic comparison were identified in the 16S rRNA tree using Phylopattern software [45]. For each selected genome, the complete genome sequence, proteome genome sequence and ORFeome genome sequence were retrieved from the FTP of NCBI.

An annotation of the entire proteome was performed to define the distribution of functional classes of predicted genes according to the COGs of proteins (using the same method as for the genome annotation). Annotation and comparison processes were performed in the multiagent software system DAGOBAN [46], which includes Figenix [47] libraries that provide pipeline analysis. To evaluate the genomic similarity between studied genomes, we determined two parameters, digital DNA-DNA hybridization (dDDH), which exhibits a high correlation with DNA-DNA hybridization (DDH) [20,48], and average genomic identity of orthologous gene sequences (AGIOS), which was designed to be independent from DDH. The AGIOS score is the mean value of nucleotide similarity between all couples of orthologous proteins between the two studied genomes [49].

Results

Strain identification and phylogenetic analysis

A phylogenetic tree of our strains is provided in Fig. 2. Strain SDI (accession no. LN866997) exhibited a 98.6% 16S rRNA gene sequence identity with *Actinomyces radingae* strain APL1 (accession no. NR_026169), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces ihuae* strain SDI (= CSUR P2006 = DSM 100538). Strain Marseille-P2825 (accession no. LT576385) revealed a 98.33% sequence similarity with the 16S rRNA of *Actinomyces odontolyticus* strain JCM 14871 (accession no. AJ234040), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces bouchesdurhonensis* strain Marseille-P2825 (= CSUR P2825 = DSM 103075). Strain Marseille-P2225 (accession no. LN870295) revealed an exhibited 98.45% sequence identity with *Actinomyces europaeus* strain CCUG 32789A (accession no. NR_026363), the closest species with a validly published name. We therefore suggested that our strain is a representative

strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces urinae* strain Marseille-P2225 (= CSUR P2225 = DSM 100700). Strain Marseille-P2818 (accession no. LT576400) exhibited a 98.1% 16S rRNA gene sequence identity with *Actinomyces odontolyticus* strain JCM 14871 (accession no. AJ234040), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces marseillensis* strain Marseille-P2818 (= CSUR P2818 = CCUG 71898). Furthermore, *Actinomyces bouchesdurhonensis* strain Marseille-P2825 and *Actinomyces marseillensis* strain Marseille-P2818 showed only 97.55% of 16S gene sequence identity, indicating that there are two distinct species of *Actinomyces odontolyticus*. Strain Marseille-P3257 (accession no. LT598588) exhibited 93.94% 16S rRNA gene sequence identity with *Actinomyces hyovaginalis* strain BM 1192/5 (accession no. X69616), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces mediterranea* strain Marseille-P3257 (= CSUR P3257 = CCUG 70143). Strain Marseille-P3109 (accession no. LT627670) revealed a 98.5% of sequence similarity with the 16S rRNA of *Actinomyces naeslundii* strain JCM 8349 (accession no. NR_113326), the closest species with a validly published name. We therefore suggested that our strain is a representative strain of a new species within the genus *Actinomyces*, for which we suggest the name *Actinomyces oralis* strain Marseille-P3109 (= CSUR P3109 = DSM 103942).

The analysis of the gel view shows that all the profiles of our studied strains have similar general characteristics with the other *Actinomyces* species used for the comparison (Fig. 3). Furthermore, the outsider species *Actinobaculum urinale* strain DSM 15805 profile shows several unique differences.

Phenotypic features

The main phenotypic results of each studied strains are presented in Table 2. Results show that these six new species are all Gram positive and rod shaped. These observations have been permitted by electronic microscopy (Fig. 4), which reveals similar morphologies characteristic of the *Actinomyces* genus.

The cellular fatty acid composition of our strains is provided in Table 3, and the antibiotic analyses are presented in Table 4. As Table 3 indicates, 9-octadecenoic acid and hexadecanoic acid were the two most abundant cellular fatty acid of all the bacteria. 9-Octadecenoic acid was the most abundant for the strains Marseille-P2825, Marseille-P2818 and Marseille-P3257; and hexadecanoic acid was the second most abundant. Hexadecanoic acid was the most abundant for the strains Marseille-P2225, SDI and Marseille-P3109; and 9-octadecenoic acid was

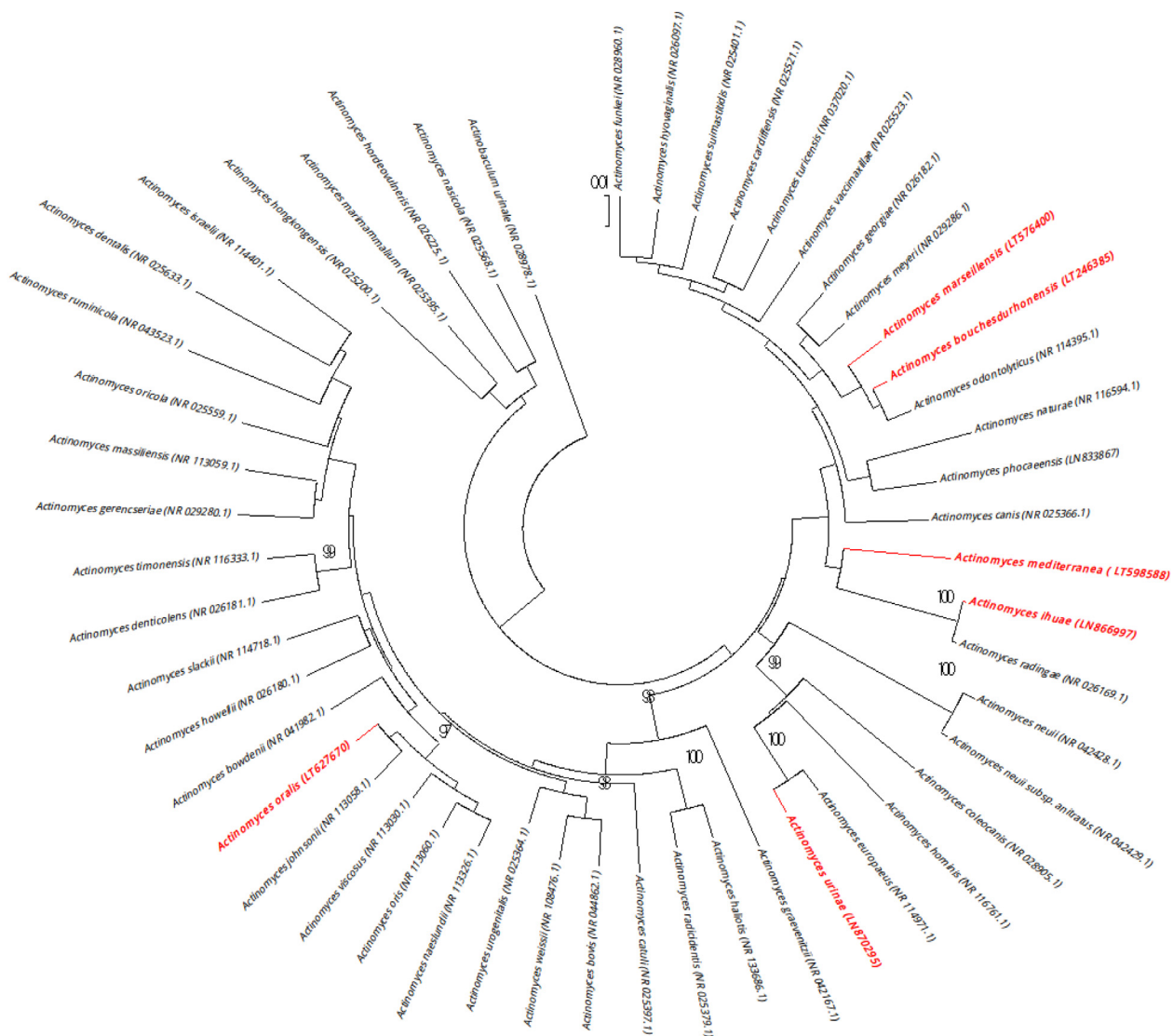


FIG. 2. Phylogenetic tree highlighting position of *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 relative to other type strains within *Actinomyces* genus. Strains and their corresponding GenBank accession numbers for 16S rRNA genes sequences are indicated in brackets. Sequences were aligned using CLUSTALW (<http://www.clustal.org/clustal2/>), and phylogenetic inferences were obtained using maximum-likelihood method within MEGA 6 (<http://www.megasoftware.net/mega.php>). Numbers at nodes are percentages of bootstrap values obtained by repeating analysis 1000 times to generate majority consensus tree. *Actinobaculum urinale* (NR 028978.1) was used as outgroup. Scale bar = 1% nucleotide sequence divergence.

the second most abundant. Table 4 shows the results of antibiotic susceptibility tests. The strains were all susceptible to amoxicillin 25 µg, rifampicin 30 µg and gentamicin 500 µg and were resistant to colistin 50 µg and metronidazole 4 µg. These results support the notion that these strains are all members of the same *Actinomyces* genus.

Genome description and comparison

Maps of genomes of our different strains are presented in Supplementary Fig. S1. The properties and statistics of the

genomes are summarized in Table 5, and the distribution of predicted genes of our strains according to COGs categories are shown in Table 6. We can observe that for all the 25 general COGs functional categories, values of our six new *Actinomyces* species are in the same range. Genomic characteristics of our strains are compared to those of closely related species with an available genome in Table 7. Although the genome *A. urinae* is smaller than the other species, it has the same GC percentage range, between 49.60% and 72.58%, as the other characterized genomes of known *Actinomyces* species

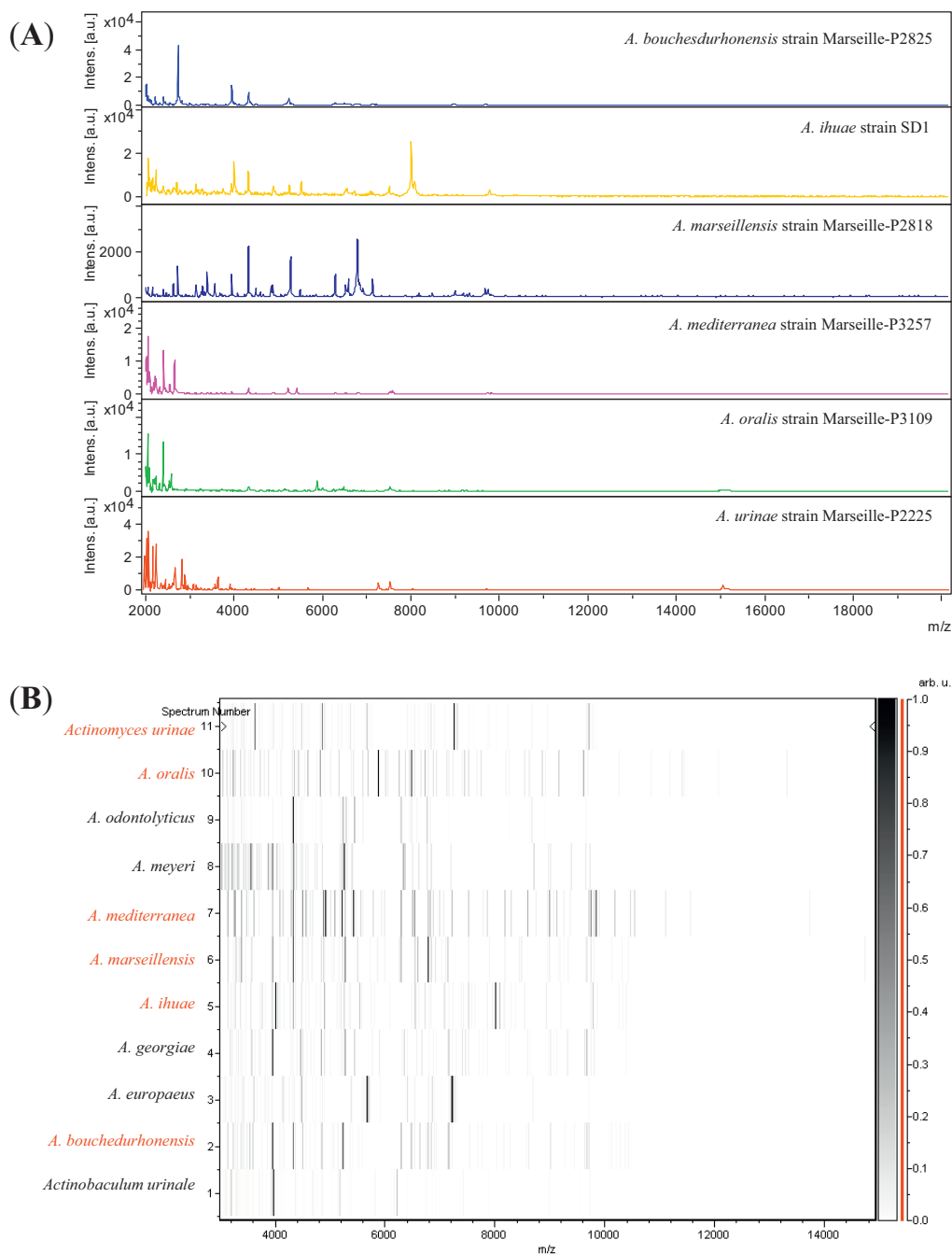


FIG. 3. Reference mass spectra from *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109. Spectra from 12 individual colonies were compared and each reference spectrum generated (A). Gel view comparing *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 to other species within genus *Actinomyces*. Gel view displays raw spectra of loaded spectrum files arranged in pseudo-gel-like look; x-axis indicates *m/z* value and left y-axis running spectrum number originating from subsequent spectra loading. Peak intensity expressed by greyscale scheme code. Colour bar and right y-axis indicate relation between colour peak, with peak intensity in arbitrary units. Displayed species are indicated at left (B).

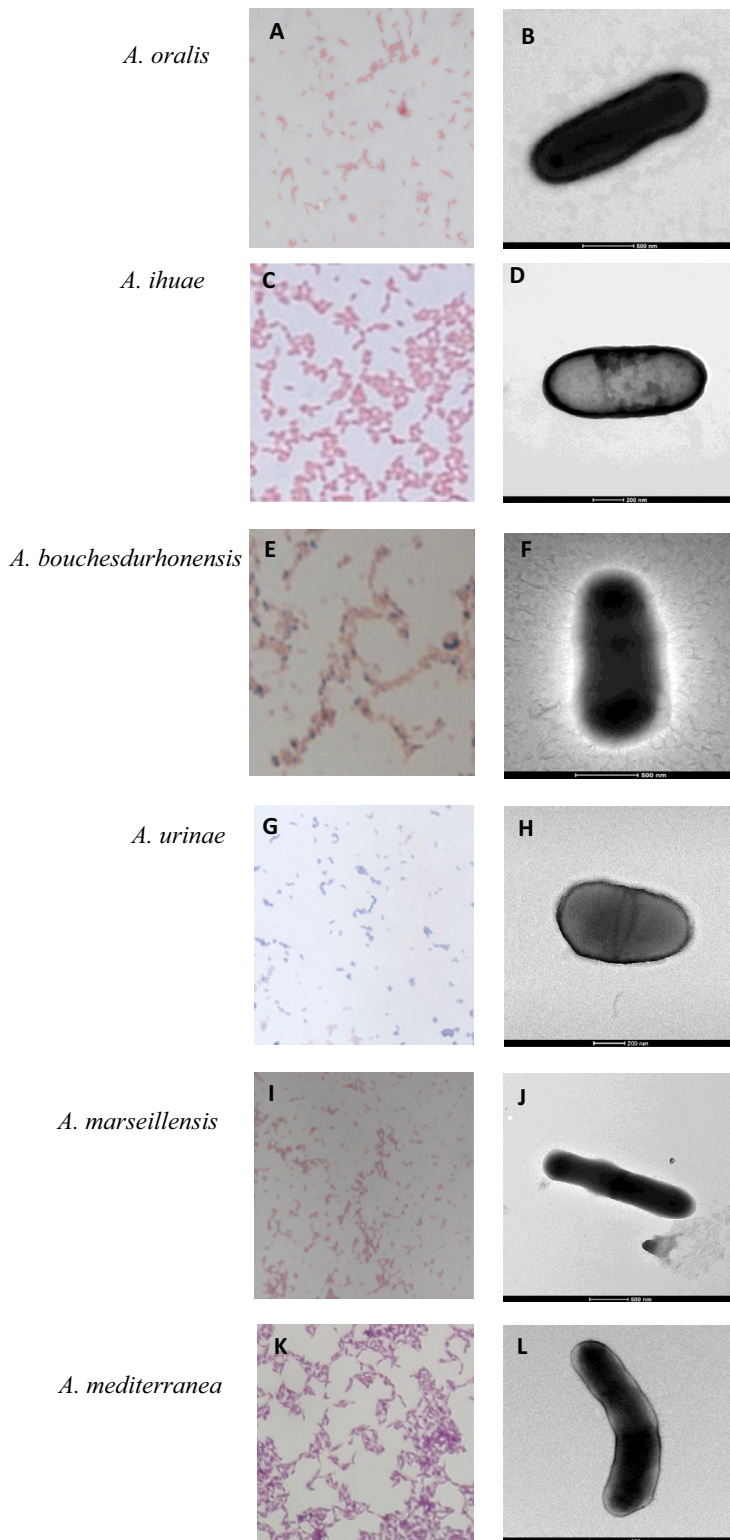


FIG. 4. Gram staining and electron micrographs, respectively, of *Actinomyces oralis* strain Marseille-P3109 (A, B), *Actinomyces ihuae* strain SD1 (C, D), *Actinomyces bouchedurhonensis* strain Marseille-P2825 (E, F), *Actinomyces urinae* strain Marseille-P2225 (G, H), *Actinomyces marseillensis* strain Marseille-P2818 (I, J) and *Actinomyces mediterranea* strain Marseille-P3257 (K, L).

TABLE 3. Cellular fatty acid composition (in %^a)

Fatty acid	IUPAC name	A	B	C	D	E	F
12:0	Dodecanoic acid	1.0 ± 0.2	TR	TR	TR	1.6 ± 0.3	TR
14:0	Tetradecanoic acid	8.4 ± 1.5	1.2 ± 0.1	3.8 ± 0.3	2.3 ± 0.3	3.9 ± 0.2	1.9 ± 0.1
16:0	Hexadecanoic acid	30.1 ± 1.9	32.3 ± 0.1	27.9 ± 3.5	32.2 ± 0.5	55.0 ± 0.2	44.9 ± 0.3
16:1n7	9-Hexadecenoic acid	TR	TR	No	TR	2.1 ± 0.2	No
17:0	Heptadecanoic acid	TR	1.2 ± 0.1	TR	TR	TR	TR
18:0	Octadecanoic acid	14.3 ± 2.5	18.0 ± 1.2	13.2 ± 0.6	20.1 ± 0.3	3.7 ± 0.5	18.1 ± 0.8
18:1n5	13-Octadecenoic acid	No	No	No	TR	No	2.5 ± 0.1
18:1n6	12-Octadecenoic acid	No	No	1.1 ± 0.1	No	No	No
18:1n7	11-Octadecenoic acid	3.0 ± 0.2	3.1 ± 0.3	1.4 ± 0.2	1.4 ± 0.1	No	1.5 ± 0.2
18:1n9	9-Octadecenoic acid	32.4 ± 1.7	23.8 ± 0.6	44.9 ± 3.8	35.1 ± 1.0	31.4 ± 0.2	21.4 ± 0.8
18:2n6	9,12-Octadecadienoic acid	8.7 ± 0.4	16.8 ± 0.3	5.8 ± 1.0	6.8 ± 0.1	2.1 ± 0.3	7.8 ± 0.3

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
 TR, trace amounts <1%.
^aMean peak area percentage ± standard deviation.

[50,51]. Furthermore, dDDH values (Table 8) are less than 70% and confirm that all the studied species are distinct species [20,48]. These results are supported by AGIOS values (Supplementary Table S2), which demonstrated that comparison between our strains and other species within *Actinomyces* genus resulted in a similar range of values compared to the comparison between the same species except our strains, thus confirming their new species status.

Conclusion

In this study, we used the polyphasic approach developed in our laboratory to describe six new species of the *Actinomyces* genus. This concept is based on genome sequences, MALDI-TOF MS identification and main phenotypic characteristics of the studied new species. As previously observed, the presented strains, which have been isolated from diverse origin, possess close

morphologic properties. Their cellular fatty acid composition and their profile of resistance to antibiotics support that these five strains belong to the same genus. Their 16S rRNA gene sequencing, supported by genome analysis compared to other characterized strains of the *Actinomyces* genus, indicated that *Actinomyces ihuae* strain SD1, *Actinomyces bouchesdurhonensis* strain Marseille-P2825, *Actinomyces urinae* strain Marseille-P2225, *Actinomyces marseillensis* strain Marseille-P2818, *Actinomyces mediterranea* strain Marseille-P3257 and *Actinomyces oralis* strain Marseille-P3109 are all members of the *Actinomyces* genus.

Description of ‘*Actinomyces ihuae*’ sp. nov.

Cells are Gram positive and rod shaped, with a length of 0.7 to 1 µm and a width of 0.5 to 0.7 µm. This strain exhibited no catalase or oxidase activity. ‘*Actinomyces ihuae*’ is nonmotile and non-spore forming. Colonies are bright grey, with a diameter of 1 to 2 mm. Optimum growth occurs at 37°C in an aerobic

TABLE 4. Antibiotic resistance tests

Characteristic	A		B		C		D		E		F	
	IMC	Status	IMC	Status	IMC	Status	IMC	Status	IMC	Status	IMC	Status
Gentamicin 15 µg	3	R	9	R	11.1	I	7	R	24.6	S	0	R
Gentamicin 500 µg	16	I	25	S	30.6	S	35	S	34.3	S	21.5	S
Amoxicillin/clavulanic acid 30 µg	20	I	26	S	30.0	S	30	S	41.9	S	9.8	R
Ceftriaxone 30 µg	21	R	20	R	14.4	R	>30	S	40.4	S	19.1	R
Colistin 50 µg	0	R	0	R	0	R	0	R	0	R	0	R
Penicillin G 10 IU	21	I	27	I	20.2	I	28	I	33.9	S	16.1	R
Imipenem 10 µg	26	S	30	S	31.1	S	>30	S	22.6	I	22.4	I
Trimethoprim/sulfamethoxazole 25 µg	6	R	5	R	30.7	S	11	I	0	R	0	R
Clindamycin 15 µg	30	S	24.1	S	23.9	S	25	S	7.4	R	10.9	R
Metronidazole 4 µg	6	R	10	R	19.8	R	5	R	6.5	R	0	R
Tobramycin 10 µg	7	R	10.9	R	23.7	S	12	R	0	R	0	R
Rifampicin 30 µg	25	S	28	S	19.4	S	>30	S	40.0	S	14.4	I
Fosfomycin 50 µg	7	R	12	R	6.5	R	>30	S	13.5	R	9.4	R
Amoxicillin 25 µg	22	I	29.4	S	31.5	S	>30	S	36.3	S	27.0	S
Vancomycin 30 µg	13	R	20	S	27.6	S	22	S	32.4	S	17.2	S
Doxycycline 30 IU	3	R	29.2	S	28.3	S	>30	S	8.9	R	27.2	S
Erythromycin 15 IU	24	S	10	R	30.6	S	>30	S	19.4	I	9.4	R

A, *Actinomyces bouchesdurhonensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.
 I, indeterminate; R, resistant; S, susceptible; IMC, inhibitory minimal concentration.

TABLE 5. Nucleotide content and gene counts levels of genome

Characteristic	A		B		C		D		E		F	
	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a
Scaffolds; contigs	31; 114		5; 5		2; 18		4; 4		8; 8		3; 4	
Size (bp)	2 304 249	100.0	2 458 350	100.0	2 007 553	100.0	2 395 621	100.0	3 149 233	100	1 946 897	100.0
G+C content (%)	1 436 338	63.3	1 447 601	58.9	1 131 738	57.1	1 528 162	63.8	2 154 177	68.4	1 092 830	56.1
Coding region (bp)	1 612 522	70.0	2 240 376	91.1	1 692	100.0	2 151 624	89.8	2 750 321	87.3	1 787 920	91.8
Total genes	1821	100.0	2 200	100.0	1754	100.0	2133	100.0	2639	100.0	1756	100.0
Protein-coding genes	1766	100.0	2135	100.0	1692	100.0	2081	100.0	2579	100.0	1701	100.0
RNA genes	55	3.0	65	3.0	62	3.5	52	2.4	60	2.3	55	3.1
Proteins with function prediction	1257	71.2	1533	71.8	1279	75.6	1571	75.5	2013	78.1	1257	73.9
Proteins assigned to COGs	1016	57.5	1324	62.0	1091	64.5	1312	63.0	1615	62.6	1166	68.5
Proteins with peptide signals	181	10.2	205	9.6	203	12.0	239	11.5	287	11.1	179	10.5
No. of proteins associated with ORFan	89	5.0	142	6.7	33	2.0	100	4.8	55	2.1	37	2.2
Genes associated with PKS or NRPS	2	0.1	3	0.1	4	0.2	8	0.4	12	0.5	4	0.2
No. of antibiotic resistance genes	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0
No. of genes associated with Pfam-A domains	1519	83.0	1936	88.0	1549	88.0	1 89	88.0	2381	90.0	1583	90.0

A, *Actinomyces bouchesdurhanensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.

COGs, Clusters of Orthologous Groups database.

^aTotal is based on either size of genome (bp) or total number of protein-coding genes in annotated genome.

TABLE 6. Number of genes associated with 25 general COGs functional categories

Code	Description	A		B		C		D		E		F	
		Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a	Value	% of total ^a
J	Translation	145	8.21	156	7.31	163	9.63	166	7.98	165	6.40	158	9.29
A	RNA processing and modification	1	0.07	1	0.04	1	0.06	1	0.05	1	0.04	1	0.06
K	Transcription	65	3.69	91	4.26	70	4.14	98	4.71	145	5.62	76	4.47
L	Replication, recombination and repair	48	2.72	71	3.33	64	3.78	68	3.27	72	2.79	65	3.82
B	Chromatin structure and dynamics	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
D	Cell cycle control, mitosis and meiosis	19	1.08	22	1.03	18	1.06	19	0.91	20	0.78	21	1.23
Y	Nuclear structure	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0
V	Defense mechanisms	37	2.10	41	1.92	36	2.13	43	2.07	68	2.64	34	2.0
T	Signal transduction mechanisms	32	1.81	41	1.92	31	1.83	42	2.02	67	2.60	32	1.88
M	Cell wall/membrane biogenesis	55	3.11	59	2.76	49	2.90	72	3.46	88	3.41	60	3.53
N	Cell motility	6	0.34	6	0.28	6	0.35	8	0.38	8	0.31	7	0.41
Z	Cytoskeleton	0	0.0	0	0.0	0	0.0	0	0.0	1	0.04	0	0.0
W	Extracellular structures	3	0.17	4	0.19	1	0.06	4	0.19	4	0.16	4	0.24
U	Intracellular trafficking and secretion	15	0.85	17	0.80	17	1.00	16	0.77	19	0.74	19	1.12
O	Posttranslational modification, protein turnover, chaperones	56	3.17	61	2.86	59	3.49	64	3.08	64	2.48	60	3.53
X	Mobilome: prophages, transposons	51	2.89	21	0.98	2	0.12	19	0.91	27	1.05	13	0.77
C	Energy production and conversion	63	3.57	81	3.79	73	4.31	91	4.37	92	3.57	81	4.77
G	Carbohydrate transport and metabolism	89	5.04	218	10.21	118	6.97	186	8.94	205	7.95	151	8.88
E	Amino acid transport and metabolism	98	5.55	126	5.90	113	6.68	131	6.30	169	6.55	111	6.53
F	Nucleotide transport and metabolism	50	2.83	73	3.42	51	3.01	67	3.22	71	2.75	67	3.94
H	Coenzyme transport and metabolism	62	3.51	74	3.47	59	3.49	63	3.03	102	3.96	66	3.89
I	Lipid transport and metabolism	34	1.93	38	1.78	51	3.01	49	2.35	65	2.52	37	2.18
P	Inorganic ion transport and metabolism	51	2.89	72	3.37	67	3.96	60	2.88	91	3.53	70	4.12
Q	Secondary metabolites biosynthesis, transport and catabolism	16	0.91	17	0.80	14	0.83	18	0.86	35	1.36	19	1.12
R	General function prediction only	74	4.19	99	4.64	84	4.96	112	5.38	149	5.78	77	4.53
S	Function unknown	53	3.00	67	3.14	54	3.19	51	2.45	79	3.06	53	3.12
—	Not in COGs	750	42.47	811	38.0	601	35.52	769	36.95	964	37.39	535	31.45

A, *Actinomyces bouchesdurhanensis* strain Marseille-P2825; B, *A. ihuae* strain SD1^T; C, *A. marseillensis* strain Marseille-P2818; D, *A. mediterranea* strain Marseille-P3257; E, *A. oralis* strain Marseille-P3109; F, *A. urinae* strain Marseille-P2225.

COGs, Clusters of Orthologous Groups database.

^aTotal is based on either size of genome (bp) or total number of protein-coding genes in annotated genome.

TABLE 7. Genome comparison of closely related species.

Organism	Strain	INSDC	Size (Mb)	G+C%	Total genes
<i>Actinotignum massiliense</i>	FC3	CYUL00000000	2.067	60.17	1771
<i>Actinobaculum suis</i>	CI-22-1	MASX00000000	2.23	57.75	1611
<i>Actinomyces bouchesdurhonensis</i>	Marseille-P2825	FQSA00000000	2.3	63.28	1766
<i>Actinomyces cardiffensis</i>	F0333	AQHZ00000000	2.19	61.49	1983
<i>Actinomyces coleocanis</i>	DSM 15436	ACFG00000000	1.72	49.60	1546
<i>Actinomyces europaeus</i>	ACS-120-V-Col10b	AGWN00000000	1.911	56.65	1670
<i>Actinomyces georgiae</i>	DSM 6843	AUBM00000000	2.5	69.87	2103
<i>Actinomyces graevenitzi</i>	C83	ACRN00000000	2.21	57.80	1853
<i>Actinomyces ihuae</i>	SD1	CZPX00000000	2.45	58.89	2135
<i>Actinomyces israelii</i>	DSM 43320	JONS00000000	4.03	71.44	3387
<i>Actinomyces johnsonii</i>	F0542	AWSE00000000	3.33	67.45	3324
<i>Actinomyces marseillensis</i>	Marseille-P2818	FTLP00000000	2.01	57.09	1692
<i>Actinomyces mediterranea</i>	Marseille-P3257	FTPB00000000	2.4	63.79	2081
<i>Actinomyces meyeri</i>	W712	CP012072	2.05	65.51	1667
<i>Actinomyces naeslundii</i>	Howell 279	ALJK00000000	3.11	67.85	2930
<i>Actinomyces neuii</i> subsp. <i>neuii</i>	DSM 8576	ATUW00000000	2.27	56.18	2013
<i>Actinomyces odontolyticus</i>	F0309	ACYT00000000	2.43	65.25	2372
<i>Actinomyces oralis</i>	Marseille-P3109	OCHN00000000	3.15	68.40	2579
<i>Actinomyces oris</i>	K20	BABV00000000	2.87	67.80	3006
<i>Actinomyces radidentis</i>	CCUG 36733	CP014228	3.05	72.58	2342
<i>Actinomyces slackii</i>	ATCC 49928	AUAK00000000	3.17	70.14	2611
<i>Actinomyces suimastitidis</i>	DSM 15538	AUBF00000000	2.29	56.43	1943
<i>Actinomyces turicensis</i>	ACS 279 V Col4	AGWQ00000000	1.95	57.18	1717
<i>Actinomyces urinae</i>	Marseille-P2225	FPKP01000000	1.95	56.13	1701
<i>Actinomyces urogenitalis</i>	DORA 12	AZLV00000000	2.6	68.66	3034
<i>Actinomyces viscosus</i>	C505	ACRE00000000	3.13	68.60	2587
<i>Isoptericola dokdonensis</i>	DS-3	CP014209	3.83	73.81	3403
<i>Isoptericola variabilis</i>	JZ7	LWGM00000000	3.48	74.58	3529
<i>Mobiluncus curtisii</i> subsp. <i>curtisii</i>	ATCC 43063	CP001992	2.15	55.41	1909
<i>Mobiluncus mulieris</i>	28-1	ADBR00000000	2.45	55.07	2321
<i>Varibaculum cambriense</i>	DORA 20	AZMI00000000	2.28	52.76	2025

INSDC, International Nucleotide Sequence Database Collaboration.

atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain SD1 was susceptible to gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL), rifampicin (30 µg/mL), amoxicillin (25 µg/mL), doxycycline (30 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid. The genome of strain SD1^T is 2 458 350 bp long with 58.89% G+C content. The 16S rRNA gene and genome sequences are available in the European Molecular Biology Laboratory (EMBL)-EBI (European Bioinformatics Institute) database under accession numbers LN866997 and CZPX00000000, respectively.

We propose the new species '*Actinomyces ihuae*' (i.hu.ae, N.L. gen. adj., *ihuae*, which is based on the acronym IHU, for Institut Hospitalo-Universitaire in Marseille, France, where the strain was isolated), isolated from stool. Strain SD1^T is the type strain of the new species '*Actinomyces ihuae*' (= CSUR P2006 = DSM 100538).

Description of '*Actinomyces bouchesdurhonensis*' sp. nov.

Cells are Gram-positive bacilli with a length of 1.5 to 1.9 µm and a width of 0.5 to 0.6 µm. This strain did not exhibit catalase and oxidase activity. *Actinomyces bouchesdurhonensis* is nonmotile and non-spore forming. Colonies are smooth and grey with a diameter of 0.6 to 1.5 mm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth.

Marseille-P2825 was susceptible to rifampicin (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and erythromycin (15 IU). The major fatty acid is 9-octadecenoic acid.

The genome of strain Marseille-P2825 is 2 304 249 bp long with 63.28% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT576385 and FQSA01000000, respectively.

We propose the new species '*Actinomyces bouchesdurhonensis*' sp. nov. (bou.ches.du.rho.nen'sis, N.L. neut. adj. *bouchesdurhonensis*, pertaining to Boûches du Rhône, the name of the French territory where the strain was isolated) was isolated from a stomach wash. Strain Marseille-P2825^T is the type strain of the new species '*Actinomyces bouchesdurhonensis*' (= CSUR P2825 = DSM 103075).

Description of '*Actinomyces urinae*' sp. nov.

Marseille-P2225 is a Gram-positive rod-shape with a length of 400 to 600 nm and width of 200 to 400 nm. This strain no catalase and no oxidase activity. '*Actinomyces urinae*' is nonmotile and non-spore forming. Colonies are translucent, beige microcolonies, with a diameter of 100 µm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 48 hours' growth.

Strain Marseille-P2225 was susceptible to gentamicin (500 µg/mL), amoxicillin (25 µg/mL), doxycycline (30 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid.

TABLE 8. dDDH comparison^a

	A	B	C	D	E	F	G	H	I	J	K
A	100% ± 0.0										
B	20.80% ± 2.35	100% ± 0.0									
C	22.30% ± 2.35	21.70% ± 2.35	100% ± 0.0								
D	21.40% ± 2.35	21.30% ± 2.35	22.90% ± 2.40	100% ± 0.0							
E	20.70% ± 2.30	22.10% ± 2.35	22.00% ± 2.35	18.80% ± 2.40	100% ± 0.0						
F	21.40% ± 2.35	27.50% ± 2.40	26.20% ± 2.40	18.10% ± 2.25	23.10% ± 2.40	100% ± 0.0					
G	21.30% ± 2.35	20.20% ± 2.30	21.60% ± 2.35	23.70% ± 2.40	19.80% ± 2.30	30.90% ± 2.45	100% ± 0.0				
H	20.40% ± 2.20	24.90% ± 2.40	23.70% ± 2.40	19.50% ± 2.30	25.10% ± 2.40	21.80% ± 2.35	22.70% ± 2.35	100% ± 0.0			
I	20.60% ± 2.35	22.10% ± 2.35	21.90% ± 2.35	19.60% ± 2.3	34.80% ± 2.45	21.80% ± 2.35	19.00% ± 2.30	22.30% ± 2.35	100% ± 0.0		
J	20.60% ± 2.35	22.10% ± 2.35	22.40% ± 2.35	19.90% ± 2.35	35.00% ± 2.50	24.50% ± 2.40	19.90% ± 2.30	25.00% ± 2.40	23.50% ± 2.40	100% ± 0.0	
K	25.90% ± 2.40	19.70% ± 2.30	22.60% ± 2.40	20.90% ± 2.30	20.10% ± 2.30	20.10% ± 2.30	21.50% ± 2.35	20.40% ± 2.35	20.20% ± 2.30	21.40% ± 2.35	100% ± 0.0

A. *Actinomyces boitesdufortensis* strain Marseille-P2825; B. *A. ihuae* strain SD^T; C. *A. marseillensis* strain Marseille-P2818; D. *A. mediterranea* strain Marseille-P2818; E. *A. oralis* strain Marseille-P3257; F. *A. urinae* strain Marseille-P2225; G. *A. cardiffensis*; H. *A. europaeus*; I. *A. johnsonii*; J. *A. naeslundii*; K. *A. odontolyticus*.
dDDH, digital DNA-DNA hybridization; GGDC, genome-to-genome distance; HSP, high-scoring pair.
^aConfidence intervals indicate inherent uncertainty in estimating dDDH values from intergenomic distances based on empirical test data sets (which are always limited in size). These results are in accordance with 16S rRNA (Fig. 1) and phylogenetic analyses as well as GGDC results.

The genome of strain Marseille-P2225 is 1 946 897 bp long with 56.13% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LN870295 and FPKP01000000, respectively.

We propose the creation of the new species '*Actinomyces urinae*' sp. nov. (u.ri.na'e, N.L. gen. fem. *urinae*, 'of urine'), which was isolated from urine. Strain Marseille-P2225^T is the type strain of the new species '*Actinomyces urinae*' (= CSUR P2225 = DSM 100700).

Description of '*Actinomyces marseillensis*' sp. nov.

Cells are Gram positive and rod shaped, with a length of 2.0 to 2.2 µm and a width of 0.4 to 0.5 µm. This strain exhibited no catalase or oxidase activity. '*Actinomyces marseillensis*' is nonmotile and non-spore forming. Colonies are smooth and shiny with a diameter of 0.5 to 1.5 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth.

Strain Marseille-P2818 was susceptible to gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), amoxicillin (25 µg/mL), tobramycin (30 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL), rifampicin (30 µg/mL), doxycycline (30 IU) erythromycin (15 IU) and vancomycin (30 µg/mL). The major fatty acid is 9-octadecenoic acid.

The genome of strain Marseille-P2818 is 2 007 553 bp long with 57.09% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT576400 and FTLP00000000, respectively.

'*Actinomyces marseillensis*' sp. nov. is proposed as new species (*mar.sei.ll.en'sis*, L. gen. neut. adj., from *marseillensis*, pertaining to Marseille, where the strain was isolated). It was isolated from the sputum. Strain Marseille-P2818^T is the type strain of the new species '*Actinomyces marseillensis*' (= CSUR P2818 = CCUG 71898).

Description of '*Actinomyces mediterranea*' sp. nov.

Marseille-P3257 cells are Gram-positive bacilli with a length of 2.3 to 2.6 µm and a width of 0.53 to 0.61 µm. This strain did not exhibit catalase and oxidase activity. '*Actinomyces mediterranea*' is nonmotile and non-spore forming. Colonies are smooth and white in colour, with a diameter of 0.4 to 1.1 mm. Optimum growth occurs at 37°C in an anaerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain Marseille-P3257 was susceptible to rifampicin (30 µg/mL), fosfomycin (50 µg/mL), doxycycline (30 IU), erythromycin (15 IU), gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), ceftriaxone (30 µg/mL), amoxicillin (25 µg/mL), clindamycin (15 µg/mL), imipenem (10 µg/mL) and

vancomycin (30 µg/mL). The major fatty acid is 9-octadecenoic acid. The genome of strain Marseille-P3257 is 2 395 621 bp long with 63.79% GC content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT598588 and FTPB00000000, respectively.

We propose the new species *Actinomyces mediterranea* sp. nov. (me.di.ter.ra.ne.a, L. fem. adj. *mediterranea*, the Latin name of the Mediterranean Sea bordering Marseille, where the strain was isolated), which was isolated from the duodenum wash. Strain Marseille-P3257^T is the type strain of the new species '*Actinomyces mediterranea*' (= CSUR 3257 = CCUG 70143).

Description of '*Actinomyces oralis*' sp. nov.

Cells are Gram positive and rod shaped, with a length of 1.6 to 1.8 µm and a width of 0.6 to 0.7 µm. This strain no exhibited catalase or oxidase activity. '*Actinomyces oralis*' is nonmotile and non-spore forming. Colonies are smooth and grey, with a diameter of 0.3 to 1.2 mm. Optimum growth occurs at 37°C in an aerobic atmosphere on Colombia agar enriched with 5% sheep's blood after 24 hours' growth. Strain Marseille-P3109 was susceptible to rifampicin (30 µg/mL), gentamicin (15 µg/mL), gentamicin (500 µg/mL), amoxicillin/clavulanic acid (30 µg/mL), ceftriaxone (30 µg/mL), amoxicillin (25 µg/mL), penicillin G (10 IU) and vancomycin (30 µg/mL). The major fatty acid is hexadecanoic acid. The genome of strain Marseille-P3109 is 3 149 233 bp long with 68.40% of G+C content. The 16S rRNA gene and genome sequences are available in the EMBL-EBI database under accession numbers LT627670 and OOHN00000000, respectively.

We propose the creation of the new species '*Actinomyces oralis*' sp. nov. (o.ra'lis, N.L. neut. adj. *oralis*, 'from the mouth,' from which the strain was isolated), which was isolated from human sputum. Strain Marseille-P3109^T is the type strain of the new species '*Actinomyces oralis*' (= CSUR P3109 = DSM 103942).

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.nmni.2018.06.004>.

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