

Mycobacterium saopaulense sp. nov., a rapidly growing mycobacterium closely related to members of the *Mycobacterium chelonae*–*Mycobacterium abscessus* group

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Five isolates of non-pigmented, rapidly growing mycobacteria were isolated from three patients and, in an earlier study, from zebrafish. Phenotypic and molecular tests confirmed that these isolates belong to the *Mycobacterium chelonae*–*Mycobacterium abscessus* group, but they could not be confidently assigned to any known species of this group. Phenotypic analysis and biochemical tests were not helpful for distinguishing these isolates from other members of the *M. chelonae*–*M. abscessus* group. The isolates presented higher drug resistance in comparison with other members of the group, showing susceptibility only to clarithromycin. The five isolates showed a unique PCR restriction analysis pattern of the *hsp65* gene, 100 % similarity in 16S rRNA gene and *hsp65* sequences and 1–2 nt differences in *rpoB* and internal transcribed spacer (ITS) sequences. Phylogenetic analysis of a concatenated dataset including 16S rRNA gene, *hsp65*, and *rpoB* sequences from type strains of more closely related species placed the five isolates together, as a distinct lineage from previously described species, suggesting a sister relationship to a group consisting of *M. chelonae*, *Mycobacterium salmoniphilum*, *Mycobacterium franklinii* and *Mycobacterium immunogenum*. DNA–DNA hybridization values >70 % confirmed that the five isolates belong to the same species, while values <70 % between one of the isolates and the type strains of *M. chelonae* and *M. abscessus* confirmed that the isolates belong to a distinct species. The polyphasic characterization of these isolates, supported by DNA–DNA hybridization results, demonstrated that they share characteristics with *M. chelonae*–*M. abscessus* members, but constitute a different species, for which the name *Mycobacterium saopaulense* sp. nov. is proposed. The type strain is EPM 10906^T (=CCUG 66554^T=LMG 28586^T=INCQS 0733^T).

Abbreviations: DDH, DNA–DNA hybridization; ITS, internal transcribed spacer; PFGE, pulsed-field gel electrophoresis; PRA, PCR restriction analyses.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2 are KM973037, KM973036, KM973038, DQ866774 and DQ866775, respectively; for the *hsp65* sequence are KM973026, KM973025, KM973027, DQ866786 and DQ866787, respectively; for the *rpoB* sequence are KM973029, KM973028, KM973030, KM973031 and KM973032, respectively; and for the internal transcribed spacer (ITS) sequence are KM973034, KM973033, KM973035, DQ866774 and DQ866775, respectively.

Three supplementary tables and a supplementary figure are available with the online Supplementary Material.

Nontuberculous mycobacteria are ubiquitous environmental organisms and several species can cause opportunistic infections in humans, in particular the members of the *Mycobacterium chelonae*–*Mycobacterium abscessus* group. This group comprises closely related, rapidly growing mycobacteria that can cause a broad spectrum of infections mainly affecting lung, skin and soft tissue (Simmon *et al.*, 2011; Wallace *et al.*, 1983). The ubiquitous distribution of these organisms facilitates the contamination of medical equipment and solutions that, associated with the growing number of therapeutic interventions, generate nosocomial infections and outbreaks representing a serious public health problem in some settings (Leão *et al.*, 2010; Tortoli, 2009).

Until recently, the *M. chelonae*–*M. abscessus* group was composed of *M. chelonae*, *M. abscessus* (Kusunoki & Ezaki, 1992), *Mycobacterium immunogenum* (Wilson *et al.*, 2001), *Mycobacterium massiliense* (Adékambi *et al.*, 2004, 2006b), *Mycobacterium bolletii* (Adékambi *et al.*, 2006a) and *Mycobacterium salmoniphilum* (Whipps *et al.*, 2007). Taxonomic changes have been proposed and *M. abscessus*, *M. massiliense* and *M. bolletii* have been assigned to a single species (*M. abscessus*) with two subspecies, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii*, the latter including those isolates previously identified as *M. massiliense* and *M. bolletii* (Leão *et al.*, 2011; Leão *et al.*, 2009). Two novel members of this group have been recently described, *Mycobacterium franklinii* (Nogueira *et al.*, 2015; Simmon *et al.*, 2011) and '*Mycobacterium fukienense*' (Zhang *et al.*, 2013).

The aim of the present study was to define the taxonomic position of five mycobacterial isolates without conclusive species assignments (isolates EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2), which share a PCR restriction analyses (PRA) profile of the *hsp65* gene not present in the PRASITE database (<http://app.chuv.ch/prasite/index.html>). Our data indicate that these five isolates belong to a single taxon and represent a novel species of the *M. chelonae*–*M. abscessus* group.

The first two isolates (EPM 10906^T and EPM 10695) were obtained in 1999 from corneal specimens of two patients with infectious crystalline keratopathy after LASIK surgery (laser-assisted *in situ* keratomileusis) performed in the same ophthalmological clinic, in São Paulo city (Brazil). These isolates were first misidentified as *M. chelonae* (Alvarenga *et al.*, 2002) and subsequently as *M. abscessus* (Sampaio *et al.*, 2006) on the basis of PRA of the *hsp65* gene. Typing of these isolates by pulsed-field gel electrophoresis (PFGE) using a previously described protocol (Matsumoto *et al.*, 2011), revealed that they share indistinguishable patterns and thus might belong to a single strain (Fig. 1). The third isolate (IAL 3785) was obtained in 2007 from a cervical abscess in the city of Ribeirão Preto, São Paulo (Brazil). The other two isolates (JAN1 and JAN2) were isolated from zebrafish (*Danio rerio*) (Kent *et al.*, 2004) and initially categorized as *M. chelonae*. Using greater

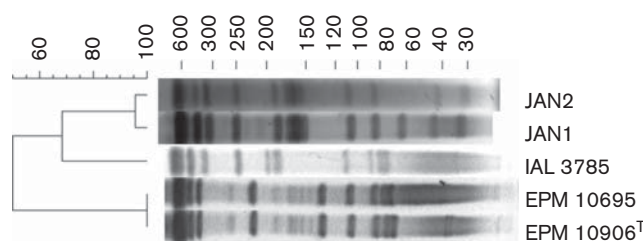


Fig. 1. Pulsed-field gel electrophoresis (PFGE) patterns of *DraI* digested DNA of the five isolates studied in this work. PFGE images were analysed with the BioNumerics program v. 7.1 (Applied Maths). The band-based Dice unweighted-pair group method using average linkages was used to prepare a dendrogram of PFGE profiles, based on 1.5 % optimization and position tolerance.

taxon sampling in a later phylogenetic analysis, these strains were recognized as phylogenetically distinct (Whipps *et al.*, 2007), but they were not described as a novel species at the time. Isolates JAN1 and JAN2 share highly similar PFGE patterns, differing only in a single band, and were isolated from zebrafish at the same research facility about two months apart in 2003 (Kent *et al.*, 2004); considering this, they could represent a single strain.

We investigated the classification of these five isolates, comprising three different strains, using a polyphasic approach that included microscopic and macroscopic morphological examination, cultural and biochemical tests, drug susceptibility testing, HPLC analysis of cell-wall mycolic acids, PRA, sequencing of three housekeeping genes and DNA–DNA hybridization. The results were compared with those displayed by *M. abscessus* subsp. *abscessus* ATCC 19977^T, *M. abscessus* subsp. *bolletii* CCUG 50184^T, *M. chelonae* ATCC 35752^T, *M. immunogenum* ATCC 700505^T, *M. salmoniphilum* ATCC 13758^T and *M. franklinii* DSM 45524^T.

Cultures were grown on solid media [Löwenstein-Jensen (LJ), Middlebrook 7H10 supplemented with OADC (oleic acid, albumin, glucose and catalase) and Luria–Bertani agar], and in liquid Muller-Hinton medium or Luria–Bertani broth with 1 % Tween 80 at 28–30 °C for 5 days. Microscopic examination of colony smears by Ziehl–Neelsen staining confirmed that the isolates were acid-fast bacilli. Analysis of pigment production, single-source carbon utilization (mannitol, inositol and citrate), growth at 26 °C and 37 °C, and tolerance to 5 % NaCl, 0.2 % picric acid, *para*-nitrobenzoic acid (PNB) and nitrite were performed on 7H10-OADC and LJ. Nitrate reduction, Tween 80 hydrolysis and arylsulfatase production were also examined. All tests were performed as described in standard protocols for the biochemical identification of mycobacteria (Kent & Kubica, 1985; Leão *et al.*, 2004; Tsukamura, 1984). The five isolates exhibited indistinguishable phenotypic and biochemical characteristics, which are listed in Table S1 (available in the online

Table 1. Antimicrobial susceptibility results for isolates and type strains included in this study

Isolates/strains: 1, EPM 10695; 2, EPM 10906^T; 3, IAL 3785; 4, JAN1; 5, JAN2; 6, *M. abscessus* subsp. *abscessus* ATCC 19977^T; 7, *M. abscessus* subsp. *bolletii* CCUG 50184^T; 8, *M. chelonae* ATCC 35752^T; 9, *M. immunogenum* ATCC 700505^T; 10, *M. salmoniphilum* ATCC 13758^T; 11, *M. franklinii* DSM 45524^T. R, Resistant; I, intermediate; S, susceptible – criteria for rapidly growing mycobacteria established by CLSI (2011); ND, not determined.

Drug	MIC ($\mu\text{g ml}^{-1}$)										
	1	2	3	4	5	6	7	8	9	10	11
Amikacin	32 I	128 R	32 I	32 I	32 I	8 S	16 S	8 S	≤ 4 S	16 S	≤ 4 S
Ciprofloxacin	2 I	2 I	4 R	4 R	4 R	4 R	8 R	0.5 S	1 S	1 S	0.5 S
Clarithromycin											
3 days	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S
14 days	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	≤ 0.5 S	> 64 R	> 64 R	≤ 0.5 S	≤ 0.5 S	ND	≤ 0.5 S
Doxycycline	> 32 R	> 32 R	> 32 R	> 32 R	> 32 R	> 32 R	> 32 R	4 I	> 32 R	> 32 R	≤ 0.25 S
Cefoxitin	512 R	> 512 R	512 R	> 512 R	512 R	64 I	32 I	512 R	256 R	512 R	16 S
Tobramycin	16 R	32 R	16 R	8 R	16 R	8 R	16 R	2 S	4 I	4 I	2 S
Minocycline	32 R	16 R	4 I	16 R	8 R	8 R	16 R	2 I	8 R	2 I	≤ 0.25 S
Moxifloxacin	2 I	8 R	4 R	4 R	2 I	8 R	8 R	≤ 0.25 S	1 S	2 I	1 S

Supplementary Material). These cultural and biochemical tests were not helpful for distinguishing these isolates from other members of the *M. chelonae*–*M. abscessus* group (Table S1).

Antimicrobial drug-susceptibility testing was performed using a microdilution method in cation-supplemented Mueller–Hinton broth, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2011) for rapidly growing mycobacteria. Amikacin, cefoxitin, ciprofloxacin, clarithromycin, doxycycline, minocycline, moxifloxacin and tobramycin were tested. The five isolates were more drug resistant than the other members of the *M. chelonae*–*M. abscessus* group, showing susceptibility only to clarithromycin at 3 and 14 days incubation. The isolates were resistant to doxycycline, cefoxitin and tobramycin, and resistant or intermediate to amikacin, ciprofloxacin, minocycline and moxifloxacin (Table 1). These results are consistent with susceptibility testing previously conducted for strain JAN1 (Chang & Whipps, 2015). The drug resistance profile of the novel species described in this study highlights the importance of its correct identification for patient management.

For HPLC analysis of cell-wall mycolic acids, two strains of the panel characterized here were selected (the proposed type strain EPM 10906^T and JAN1) and three reference strains belonging to the closely related *M. chelonae*–*M. abscessus* group (*M. abscessus* subsp. *abscessus* ATCC 19977^T, *Mycobacterium massiliense* CCUG 48898^T and *M. abscessus* subsp. *bolletii* CCUG 50184^T). The cells of these strains, grown in culture on Middlebrook 7H10 agar, were saponified, extracted and derivatized as recommended by the Sherlock Mycobacteria Identification System (SMIS; MIDI) and separated using a gradient of methanol and 2-propanol. All the strains analysed produced nearly identical HPLC patterns characterized by

two late emerging clusters of peaks (Fig. 2). The Sherlock software (version Myco 1.0) identified all the strains as *M. chelonae*–*M. abscessus* with very high similarity indexes

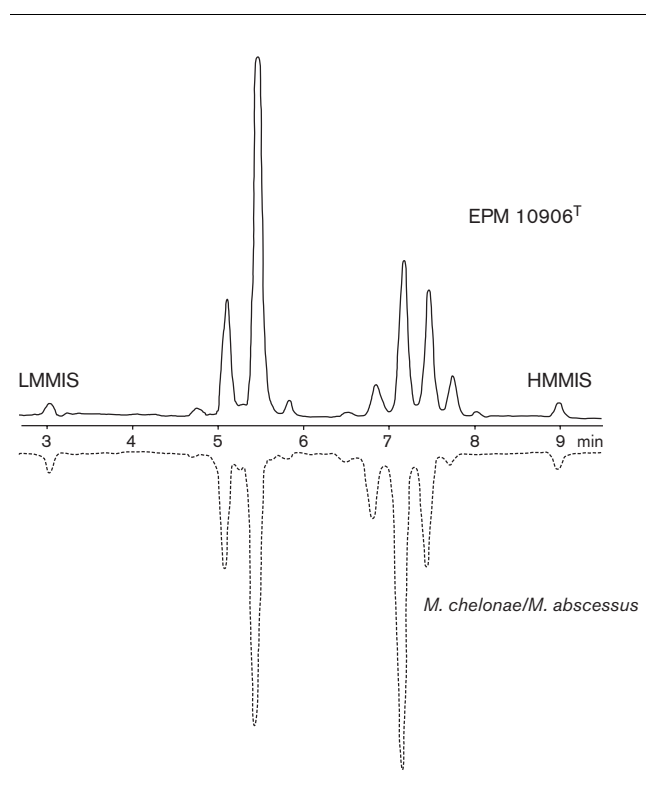


Fig. 2. Representative mycolic acid pattern of isolate EPM 10906^T paired with the reference profile of *Mycobacterium chelonae*–*Mycobacterium abscessus* (Sherlock database). LMMIS, Low molecular mass internal standard; HMMIS, high molecular mass internal standard.

(range 0.802–0.899). The low discriminatory power of HPLC analysis in differentiating most rapidly growing mycobacterial species (Tortoli, 2003) is therefore confirmed for the proposed novel species, with this approach being unsuitable to go further in the assignation to the *M. chelonae*–*M. abscessus* group.

GenoType *Mycobacterium* (Hain Lifescience), a commercial DNA strip assay for mycobacteria identification, was performed according to the manufacturer's instructions. Using the GenoType CM strip, the isolates showed the *M. chelonae* profile (hybridization with probes 5 and 10). GenoType AS only identified the isolates at the level of *Mycobacterium* species.

For molecular identification, PRA of the *hsp65* gene and of the ITS, and partial sequencing of the small subunit (16S) rRNA gene, *rpoB*, *hsp65* and ITS were performed. The DNA was prepared by boiling one loop full of bacteria for 10 min in 300 µl TET (10 mM Tris, 1 mM EDTA, 1 % Triton X-100; pH 8.0) followed by centrifugation at 14 000 g for 2 min. For PRA-*hsp65*, a 441 bp fragment of the *hsp65* gene was amplified using primers Tb11 and Tb12 (Table S2), and the amplicon was digested in two separate tubes with BstEII and HaeIII restriction enzymes (Telenti *et al.*, 1993). For PRA-ITS, amplicons generated with primers Sp1 and Sp2 (Table S2) were digested with *TaqI* restriction enzyme (Roth *et al.*, 2000). PRA-*hsp65* and PRA-ITS digestion products were visualized in 3 % agarose gels stained with ethidium bromide after electrophoresis, using the 50 bp ladder as the molecular size standard. The restriction fragment sizes were estimated using the BioNumerics program version 7.1 (Applied Maths) and compared to the patterns included in the PRASITE database for PRA-*hsp65* and published by Roth *et al.* (2000) for PRA-ITS. The five isolates showed identical PRA-*hsp65* patterns – BstEII [bp] (235, 210) and HaeIII [bp] (145, 60, 50), which differs from patterns of other members of the *M. chelonae*–*M. abscessus* group. This profile was not registered in the PRASITE database. The isolates showed the same PRA-ITS pattern – *TaqI* [bp] (225, 30), which is common to *M. abscessus* and *M. franklinii*. Therefore, only PRA-*hsp65* was useful for differentiation of this novel species.

Primers used for PCR amplification and partial sequencing of the 16S rRNA gene, *hsp65*, *rpoB* and ITS sequences are listed in Table S2. PCR products were purified using a QIAquick PCR purification kit (Qiagen). Dideoxy sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3100 DNA Analyser (Applied Biosystems). Using CLUSTAL w multiple alignment (BioEdit version 7.2.5), the obtained sequences of isolates EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2 were aligned and the percentages of sequence similarity were calculated after alignment. The five isolates shared 100 % similarity in the partial *hsp65* and 16S rRNA gene sequences and had 12 nt differences in the partial *rpoB* and ITS sequences. The high-16S rRNA

gene sequence similarity to *M. chelonae* had been previously recognized by Kent *et al.* (2004) who assigned the two isolates investigated (JAN1 and JAN2) to such species. Greater differences in other genes (*rpoB*, *hsp65*, ITS) and in the PRA-*hsp65* pattern subsequently revealed that these isolates are distinct (Sampaio *et al.*, 2006; Whipps *et al.*, 2007), and this is supported by the data presented here.

Previous phylogenetic analyses of individual genes (Whipps *et al.*, 2007) had shown sister relationships of JAN1 and JAN2 isolates to different species. In the tree from *hsp65* sequences, these isolates were sister to *M. abscessus*. In the tree from *rpoB* sequences, they showed a sister relationship to the entire *M. chelonae*–*M. abscessus* group. In the tree from ITS sequences, a sister relationship between the novel species and *M. abscessus* emerged. Analysis of 16S rRNA gene sequences alone revealed that sequences from the five isolates, *M. abscessus* and *M. chelonae* were intermixed (Fig. S1). Thus, the 16S rRNA gene alone was not sufficient to resolve these relationships and other genes yielded conflicting results. For this study, we chose to analyse a concatenated dataset using 16S rRNA gene, *hsp65* and *rpoB* sequences from type strains of related species of the genus *Mycobacterium* (Table S3). Sequences were aligned with CLUSTAL X version 1.8 (Thompson *et al.*, 1997) with default parameters (penalty for gap opening=10, gap extension=0.2). A maximum-likelihood analysis was performed using PhyML (Guindon & Gascuel, 2003) and bootstrap confidence values were calculated with 100 replicates. The resulting tree was edited and annotated in Adobe Illustrator (Adobe Systems). The ITS region was excluded from the final analysis because equivalent sites were difficult to confidently identify from outgroup taxa; however, when ITS data are included (unaligned data treated as missing), the tree topology was unaltered (data not shown). In either analysis, the five isolates showed a sister relationship to a clade made up of four species (*M. chelonae*, *M. salmoniphilum*, *M. immunogenum* and *M. franklinii*) (Fig. 3). These species, plus the five isolates, are sister to *M. abscessus*. These concatenated data present the phylogenetic hypothesis that the five isolates are sister to *M. chelonae* versus *M. abscessus* as suggested by individual gene trees from *hsp65* and ITS sequences (Whipps *et al.*, 2007). Regardless of the precise sister relationship, all analyses support inclusion in the *M. chelonae*–*M. abscessus* group, and a phylogenetic position for the five isolates that is distinct from all other nominal species.

For DNA–DNA hybridization (DDH), high-molecular-mass DNA was prepared from 2 g cell mass using the protocol described by Pitcher *et al.* (1989) with modifications. Bacterial cells were centrifuged, inactivated at 90 °C for 30 min and resuspended in 3 ml lysis buffer containing ml⁻¹: 200 µg RNase, 25 mg fresh lysozyme and 100U mutanolysine. The suspensions were incubated overnight at 37 °C and the DNA was extracted with chloroform/isomyl alcohol, treated with RNase and precipitated with ethanol as described by Marmur (1961). DDH was performed as described by Ezaki *et al.* (1989), using

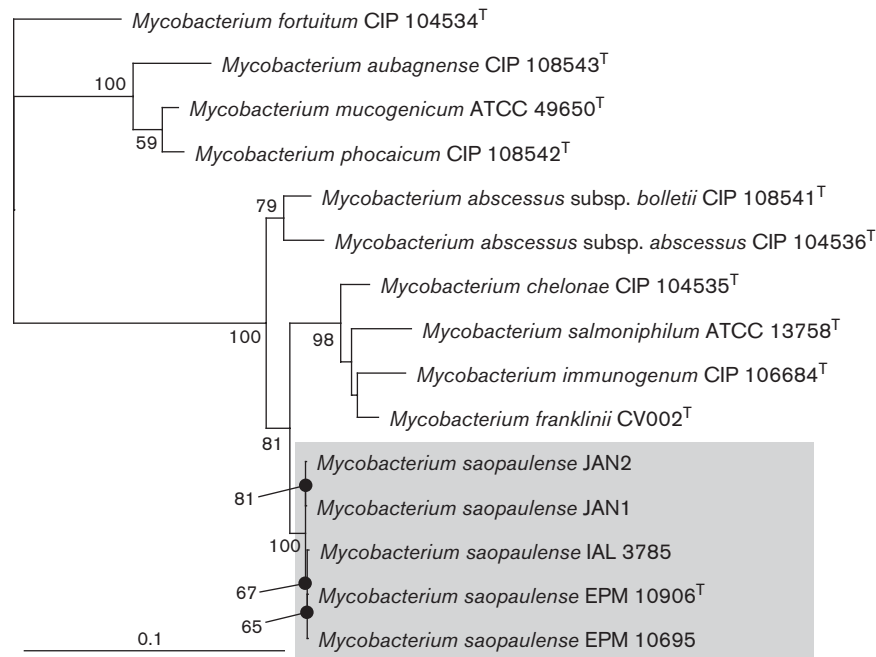


Fig. 3. Estimate of phylogeny of *Mycobacterium saopaulense* sp. nov. and other closely related rapid growers based on maximum-likelihood analysis of concatenated dataset of 16S rRNA gene, *hsp65* and *rpoB* sequences. Bootstrap support values >50 % are shown at nodes. GenBank accession numbers of the 16S rRNA gene, *hsp65* and *rpoB* sequences are shown in Table S3.

photobiotin-labelled probes in microplate wells. Fluorescence was measured in a HTS7000 Bio Assay Reader (Perkin-Elmer). DNA–DNA hybridization values are presented as means of reciprocal experiments, performed in quadruplicate hybridization reactions. The DNA G+C content, estimated as described by Mesbah & Whitman (1989), was 64.6 mol% (EPM 10906^T), 64.8 mol% (EPM 10695), 64.7 mol% (IAL 3785), 64.5 mol% (JAN1) and 64.7 mol% (JAN2). These values were used for calculation of the 50 °C hybridization temperature, and are consistent with the DNA G+C contents of the genus *Mycobacterium*, between 59 mol% and 66 mol% (Devulder *et al.*, 2005). DDH experiments performed with the five isolates yielded

hybridization values above 70 % (data not shown), confirming that they belong to the same species. Isolate EPM 10906^T was selected to perform DDH reciprocal experiments with the type strains of the *M. chelonae*–*M. abscessus* group. All values were below 70 %, confirming that EPM 10906^T and the other four isolates belong to a distinct species of the *M. chelonae*–*M. abscessus* group (Table 2).

In conclusion, phenotypic and genotypic tests indicated that isolates EPM 10906^T, EPM 10695, IAL 3785, JAN1 and JAN2 belong to the *M. chelonae*–*M. abscessus* group. In addition, several results clearly indicated that these isolates form a uniform group separated from the other members of

Table 2. DNA–DNA hybridization values of isolate EPM 10906^T against the type strains of members of the *M. chelonae*–*M. abscessus* group

Reciprocal experiments were performed in quadruplicate hybridization reactions.

Strain	Mean DNA–DNA relatedness ±SD (%)	Reciprocal values
<i>M. abscessus</i> subsp. <i>abscessus</i> ATCC 19977 ^T	41.5 ± 2.5	39;44
<i>M. abscessus</i> subsp. <i>bolletii</i> CCUG 50184 ^T	40 ± 1	39;41
<i>M. chelonae</i> ATCC 35752 ^T	47 ± 5	42;52
<i>M. immunogenum</i> ATCC 700505 ^T	40 ± 7	33;47
<i>M. salmoniphilum</i> ATCC 13758 ^T	44.5 ± 1.5	43;46
<i>M. franklinii</i> DSM 45524 ^T	51 ± 4	47;55

M. chelonae–*M. abscessus* group. Therefore, we propose to classify these isolates as a novel species of the genus *Mycobacterium* in the *M. chelonae*–*M. abscessus* group, with the name *Mycobacterium saopaulense* sp. nov.

Description of *Mycobacterium saopaulense* sp. nov.

Mycobacterium saopaulense (sa.o.paul.en'se. N.L. neut. adj. *saopaulense* of or pertaining to the Brazilian state of São Paulo, where the first strains were isolated).

Cells are acid-fast bacilli and visible growth on solid media requires 3–5 days at 28 °C. Colonies are non-pigmented and smooth. After some days, the medium often acquires a brown colour. Growth occurs in the presence of 5 % NaCl, picric acid, *para*-nitrobenzoic acid (PNB) and nitrite. Growth is observed in the presence of citrate as a single source of carbon, but not in the presence of mannitol or inositol. Negative reactions are observed for nitrate reduction and Tween 80 hydrolysis. Conventional biochemical testing cannot distinguish this species from other members of the *M. chelonae*–*M. abscessus* group. The antimicrobial pattern is characterized by susceptibility to clarithromycin and resistance to doxycycline, tobramycin and cefoxitin. Variable results, intermediate or resistant, were obtained with amikacin, ciprofloxacin, minocycline and moxifloxacin. The mycolic acid profile is similar to that of *M. chelonae*–*M. abscessus* by HPLC analysis. Genotype CM shares the same profile as *M. chelonae*. The partial sequencing of *rpoB* and *hsp65* genes can distinguish *Mycobacterium saopaulense* from other members of *M. chelonae*–*M. abscessus* group. The PRA-*hsp65* pattern that characterizes this species is BstEII [bp] (235, 210) and *Hae*III [bp] (145, 60, 50).

The type strain is EPM 10906^T (=CCUG 66554^T=LMG 28586^T=INCQS 0733^T). The DNA G+C content of the type strain is 64.6 mol%.

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