Mycobacterium sediminis sp. nov. and *Mycobacterium arabiense* sp. nov., two rapidly growing members of the genus *Mycobacterium*

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Two novel isolates of rapidly growing, Gram-stain-positive, non-chromogenic species of the genus *Mycobacterium*, strain YIM M13028^T from a sediment sample collected from the South China Sea (19° 30.261′ N 111° 0.247′ E) at a depth of 42 m and strain YIM 121001^T from a coastal zone sand sample collected in Dubai, United Arab Emirates, were obtained in our laboratory. Their taxonomic positions were determined by a polyphasic approach. Good growth of the two strains was observed at 28 °C and pH 7.0 with 0–2 % NaCl on tryptic soy agar medium. Both strains formed round orange–red colonies, strain YIM M13028^T had a rough surface, while YIM 121001^T was smooth. Cellular fatty acids, whole-cell protein profiles and TLC analysis of their mycolic acids show significant differences from reference stains. Phenotypic characteristics and multilocus sequence analysis (MLSA) of 16S rRNA gene, *hsp65, rpoB* and 16S–23S internal transcribed spacer (ITS) sequences indicated that both strains YIM M13028^T and YIM 121001^T belong to the genus *Mycobacterium*. DNA–DNA hybridization values revealed a low relatedness (<70 %) of the two isolates with the type strains *Mycobacterium neoaurum* DSM 44074^T and *Mycobacterium hodleri* DSM 44183^T. The low DNA–DNA hybridization values (40.4 ± 3.5 %) between strains YIM M13028^T and YIM 121001^T and phenotypic distinctiveness indicated that the two strains were representatives of different novel species of the genus *Mycobacterium*. The names proposed for these novel species are *Mycobacterium sediminis* sp. nov. and *Mycobacterium arabiense* sp. nov., and the type strains are YIM M13028^T (=DSM 45643^T=KCTC 19999^T) and YIM 121001^T (=DSM 45768^T=JCM 18538^T), respectively.

Mycobacterium is the type genus of the family *Mycobacteriaceae* in the suborder *Corynebacterineae*. At the time of writing, there are more than 150 recognized species according to the List of Prokaryotic names with Standing in Nomenclature (LPSN; http://www.bacterio.cict.fr/index.html), which are usually divided into two major groups: the 'slowly growing' and 'rapidly growing' mycobacteria. Many members of the genus have been isolated from clinical specimens and are pathogenic to humans and animals or potentially responsible for disease, while some free-living strains are wildly distributed in the environment and possess other abilities, for example, degradation of polycyclic aromatic hydrocarbons, cholesterol pyrene and fluoranthene (Uhía *et al.*, 2012; Seo *et al.*, 2009; López *et al.*, 2006). Rhodes and colleagues isolated two novel *Mycobacterium* strains from Chesapeake Bay striped bass (Rhodes *et al.*, 2003, 2005), Padgittt & Moshier (1987) isolated one novel strain from a marine sponge. However,

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene, *hsp65*, *rpoB* and 16S–23S ITS gene sequences of strain YIM M13028^T are KC010490, KC010486, KC010488 and KC010492, respectively, and of strain YIM 121001^T are KC010491, KC010487, KC010489 and KC010493, respectively. The GenBank/EMBL/DDBJ accession number for the 16S–23S ITS gene sequence of strain DSM 44183^T is KC010494.

Five supplementary figures and a supplementary table are available with the online version of this paper.

to our knowledge, there has been no novel strain of the genus *Mycobacterium* isolated from marine sediment or coastal zone soil or sand samples reported previously.

Strain YIM M13028^T was isolated from a sediment sample collected from the South China sea (19° 30.261′ N 111° 0.247′ E) at a depth of 42 m, by the serial dilution technique using HV agar medium (1.0 g humic acid; 0.5 g Na_2HPO_4; 1.7 g KCl; 0.05 g $MgSO_4 . 7H_2O$; 0.01 g $FeSO_4 . 7H_2O$; 1 g $CaCl_2$; 12 g agar; B-vitamins; 1 l distilled water; pH 7.2) at the incubation temperature 28 °C for a month. Strain YIM 121001^T was isolated from a sand sample collected from the shore of Dubai, United Arab Emirates (UAE), by the serial dilution technique using YIM 171 medium (10 g glycerol; 1 g asparagines; 1 g $K_2HPO_4 . H_2O$, 0.5 g $MgSO_4 . 7H_2O$, 0.3 g $CaCO_3$, 3.75 mg B-vitamins; 1 ml trace salt; 1 l distilled water; pH 7.2) at the incubation temperature 28 °C for a month. The two isolates were routinely cultivated on TSA at 28 °C and stored as aqueous glycerol suspensions (20 %, v/v) at −70 °C.

Both strains YIM M13028^T and YIM 121001^T grew well on TSA medium and ISP medium 2 without or with NaCl (<2 %). They both formed round, orange–red colonies stably on TSA medium and ISP medium 2, while colonies of strain YIM M13028^T had rough surfaces and those of strain YIM 121001^T had smooth. No diffusible pigment was observed in both media. Gram staining was carried out by using the standard Gram stain and the morphological characteristics were examined using a light microscope (BH-2; Olympus) for two strains, a scanning electron microscope (QUANTA 200; FEI) for YIM M13028^T and a transmission electron microscope (JEM-123; JEOL) for YIM 121001^T after 5 days incubation on TSA with 1.5 % NaCl at 28 °C (Fig. S1 available in IJSEM Online).

Growth with various NaCl concentrations (1, 2, 4, 5, 7 and 8 %, w/v) and at different temperatures (5, 10, 15, 18, 20, 28, 37, 40 and 45 °C), was examined by growing the strains on TSA medium as the basal medium. Growth at different pH values (4.0–10.0, at intervals of 1.0 pH unit) was examined on TSB medium using the buffer system described by Xu *et al.* (2005). Oxidase activity was determined from the oxidation of tetramethyl-*p*-phenylenediamine. Catalase activity was determined using 3 % H_2O_2, and gas production was identified as a positive reaction. Nitrate reduction was determined as described by Lanyi (1987). Gelatin hydrolysis was determined by incubating strains at 28 °C for 2 weeks on peptone–gelatin medium. Urease and hydrolysis of Tweens 20, 40 and 80, were tested as described by Cowan & Steel (1965).

The two Gram-stain-positive strains grew at temperatures between 5 and 37 °C and pH values between 6.0 and 8.0. However, growth of strain YIM M13028^T was very weak at 37 °C. Strain YIM M13028^T tolerated up to 4 % NaCl, while strain YIM 121001^T tolerated up to 5 % NaCl. The other results of physiological and biochemical tests are shown in Table 1 and in the species description.

The biomass used for analyses of cellular fatty acids, cell wall mycolic acids and whole-cell protein profiles was obtained from cultures grown on TSA plates supplemented with 1.5 % (w/v) NaCl for 7 days. For identification of the mycolic acids present in the cell wall, one-dimensional TLC was carried out following the standard procedure described by Minnikin *et al.* (1975). Cellular fatty acids were extracted, methylated and analysed by using the Sherlock Microbial Identification System (MIDI) according to the manufacturer's instructions. Fatty acid methyl esters were analysed by using the Microbial Identification software package (Sherlock Version 6.1; MIDI database TSBA6). One-dimensional SDS-PAGE of whole-cell proteins was performed according to the method of Vandamme *et al.* (1996). The results of TLC and SDS-PAGE profiles show both significant similarity and differences between strains YIM M13028^T, YIM 121001^T and the type strains of two related species, *Mycobacterium neoaurum* DSM 44074^T and *Mycobacterium hodleri* DSM 44183^T (Figs S2 and S3).

Genomic DNA was extracted from the isolates as described previously by Li *et al.* (2007). The DNA G+C contents were determined as described by Mesbah *et al.* (1989). The G+C content of the DNA of strains YIM M13028^T, YIM 121001^T, *Mycobacterium hodleri* DSM 44183^T and *Mycobacterium neoaurum* DSM 44074^T was 64.1 mol%, 62.8 mol%, 63.7 mol% and 63.2 mol%, respectively. The almost-complete 16S rRNA gene sequences (1515 bp and 1535 bp), the entire 16S–23S internal transcribed spacer (ITS) sequences (490 bp and 433 bp), partial *hsp65* (455 bp and 403 bp) and partial *rpoB* (451 bp and 397 bp) sequences for strains YIM M13028^T and YIM 121001^T were determined using methods described by Devulder *et al.* (2005). The 16S rRNA gene sequences obtained in this study were compared with sequences from the EzTaxon-e server using the BLAST program (http://eztaxon-e.ezbiocloud.net/) (Kim *et al.*, 2012), while the *hsp65*, *rpoB* and ITS gene sequences were compared with sequences from the GenBank database using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene, *hsp65* and *rpoB* sequences of strain YIM M13028^T were most similar to those of *Mycobacterium neoaurum* ATCC 25795^T (GenBank accession no. AF480593; 97.8 % similarity across 1456 bp), *Mycobacterium sp.* XN10-17 (EU704110; 95.0 % similarity across 443 bp), *Mycobacterium gilvum* Spyr1 (CP002385; 94.5 % similarity across 439 bp), respectively. The 16S rRNA gene, *hsp65* and *rpoB* sequences of strain YIM 121001^T were most similar to those of *Mycobacterium hodleri* DSM 44183^T (X93184; 98.3 % similarity across 1454 bp), *Mycobacterium sp.* XN10-17 (EU704110; 98.0 % similarity across 404 bp), *Mycobacterium mageritense* (AY147168; 96.0 % similarity across 397 bp), respectively. The similarities of the four gene sequences between strains YIM M13028^T and YIM 121001^T were 98.7 % for the 16S rRNA gene, 93.8 % for *hsp65*, 92.9 % for *rpoB* and 74.8 % for the ITS sequence, respectively.

The ITS sequence of *Mycobacterium hodleri* DSM 44183^T was also tested in this study (456 bp) and showed similarities of 70.8 % with strain YIM M13028^T and

Table 1. Phenotypic and biochemical characteristics of strains YIM M13028^T, YIM 121001^T and their closest phylogenetic neighbours

Taxa: 1, *Mycobacterium sediminis* sp. nov. YIM M13028^T; 2, *Mycobacterium arabiense* sp. nov. YIM 121001^T; 3, *Mycobacterium hodleri* DSM 44183^T; 4, *Mycobacterium neoaurum* DSM 44074^T. All data were obtained in this study. R, Rough; S, smooth; + + +, positive reaction (very strong); + +, positive reaction; +, positive reaction (weak); −, negative reaction; ND, not detected.

Characteristic	1	2	3	4
Colour of colony	Orange–red	Orange–red	Yellow	Golden
Colony surface	R	S	R	S
Temperature for growth (°C) (optimum)	5–37 (25–28)	5–37 (28–37)	5–37 (28)	5–40 (28–37)
Growth at 37 °C	+	+ +	+	+ + +
Growth at 5 °C for 3 weeks	+ + +	+ +	+ +	+
NaCl range for growth (%, w/v) (optimum)	⩽4 (0–2)	⩽5 (0–2)	⩽2 (0–1)	⩽5 (0–1)
pH range for growth (optimum)	6–8	6–8	6–8	6–9
Growth to maturity (days)	5–7	3	3	3
DNA G+C content (mol%)	64.1	62.8	63.7	63.2
Nitrate reduction	−	−	NO_2 (weak)	N_2
Urease	+ +	+ +	−	+ + +
Catalase	+ + +	+ +	+	+ +
Oxidase	−	−	−	−
Hydrolysis of:				
Gelatin	−	−	−	−
Tween 80	+	+ +	+ +	+ +
Tween 40	+ + +	+ +	+ +	+ +
Tween 20	+	+ + +	+	+ +
Fatty acids (%)				
$C_{14:0}$	5.3	4.0	5.3	5.3
$C_{16:0}$	13.6	15.9	16.5	16.4
$C_{17:1}\omega 7c$	22.8	17.5	22.2	23.0
$C_{18:1}\omega 9c$	28.4	31.7	18.3	23.4
$C_{18:1}\omega 7c$ 11-methyl	ND	ND	ND	7.6
$C_{18:0}$ 10-methyl, TBSA	5.6	7.42	11.55	5.1
Summed feature 3*	13.6	15.88	14.7	9.9

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega 7c$ and/or $C_{16:1}\omega 6c$.

64.5 % with strain YIM 121001^T. These three strains located in this clade in the phylogenetic tree all have a longer ITS sequence and share very low similarities and coverage with other mycobacteria. This may support the hypothesis that the usefulness of the ITS sequence for phylogenetic studies of rapidly growing mycobacteria is somewhat limited (Reischl *et al.*, 2006).

To clarify the phylogenetic position of the isolates within the related mycobacteria, multilocus sequence analysis (MLSA) of a concatenated phylogenetic tree using 16S rRNA gene (1410 bp), *hsp65* (377 bp) and *rpoB* (332 bp) sequences was performed as described by Devulder *et al.* (2005). The ITS sequence was not included in the analysis as the reference sequences available are too few to reconstruct reliable trees. The resultant sequence for each gene was aligned with corresponding sequences (retrieved from the GenBank/EMBL/DDBJ database) using CLUSTAL X 1.83. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) tree-making algorithms by using the software package MEGA version 5.0 (Tamura *et al.*, 2011). The topologies of the resultant trees were evaluated by using the bootstrap resampling method of Felsenstein (1985) with 1000 replicates. Phylogenetic analysis of the 16S rRNA gene sequence showed that strains YIM M13028^T and YIM 121001^T were located in a clade adjacent to *Mycobacterium hodleri* DSM 44183^T, with 70 % bootstrap support (Fig. 1), while in the *hsp65* tree YIM M13028^T and YIM 121001^T were located in a clade adjacent to *Mycobacterium nebraskense* DSM 44803^T, *Mycobacterium shinjukuense* GTC 2738^T, *Mycobacterium gordonae* ATCC 14470^T and *Mycobacterium branderi* CIP 104592^T, with a very low bootstrap support (<50 %) (Fig. S4). But in the phylogenetic tree based on *rpoB* sequences, strains YIM M13028^T and YIM 121001^T were not located in the same clade (Fig. S5). However, the concatenated phylogenetic tree using three genes confirmed that strains YIM M13028^T and YIM 121001^T formed a separate clade within the cluster

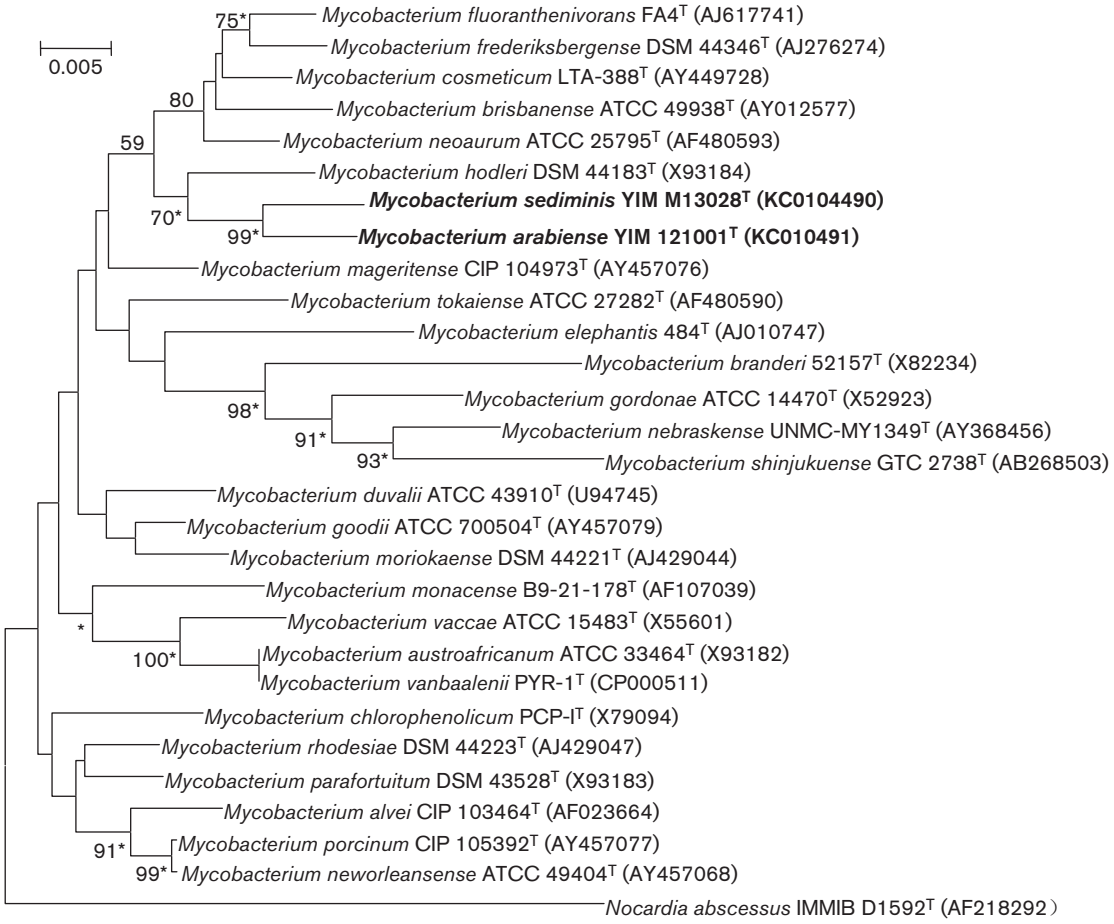

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of strain YIM M13028^T, strain YIM 121001^T and members of the genus *Mycobacterium*. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at the branch points. Asterisks denote nodes that were also recovered using the maximum-likelihood and maximum-parsimony methods. *Nocardia abscessus* IMMIB D1592^T was used as the outgroup. Bar, 0.005 substitutions per nucleotide position.

containing *Mycobacterium hodleri* DSM 44183^T (Fig. 2). This result from phylogenetic analysis suggested that the acquisition of the *rpoB* gene may be via a potential lateral gene transfer (LGT) event for the two isolates, as they had different neighbours distant from each other in the phylogenetic tree for the *rpoB* gene, and no very high similarity sequence was found in the GenBank database, indicating that the event possibly occurred long time ago. LGT events of *rpoB* in Mycobacteria have been reported by other studies (Macheras *et al.*, 2011; Kim *et al.*, 2013).

Given the close phylogenetic position of strains YIM M13028^T and YIM 121001^T to *Mycobacterium neoaurum* DSM 44074^T, *Mycobacterium hodleri* DSM 44183^T, quantitative microplate DNA–DNA hybridizations were carried out under optimal conditions as described by Ezaki *et al.* (1988, 1989). One of the two DNAs for hybridization was labelled while the other was immobilized, and reciprocal experiments were performed. The concentration of the two DNAs was strictly controlled. Six replications for hybridization were performed for each sample and the highest and lowest values for each sample were excluded. The relatedness values are expressed as the mean of the remaining four values and the results of DNA–DNA hybridizations were taken from the two means of relatedness values. Strains YIM M13028^T and YIM 121001^T exhibited <70 % DNA–DNA relatedness with reference strains (Table S1), and DNA–DNA relatedness values between the two strains was only 40.4±3.5 %, all below the suggested threshold for the delineation of species. This result provided further evidence of genetic divergence between strains YIM M13028^T and YIM 121001^T and their closest phylogenetic relationships.

The two novel isolates could be distinguished from each other and from reference strains of species of the genus *Mycobacterium* by several phenotypic characteristics in

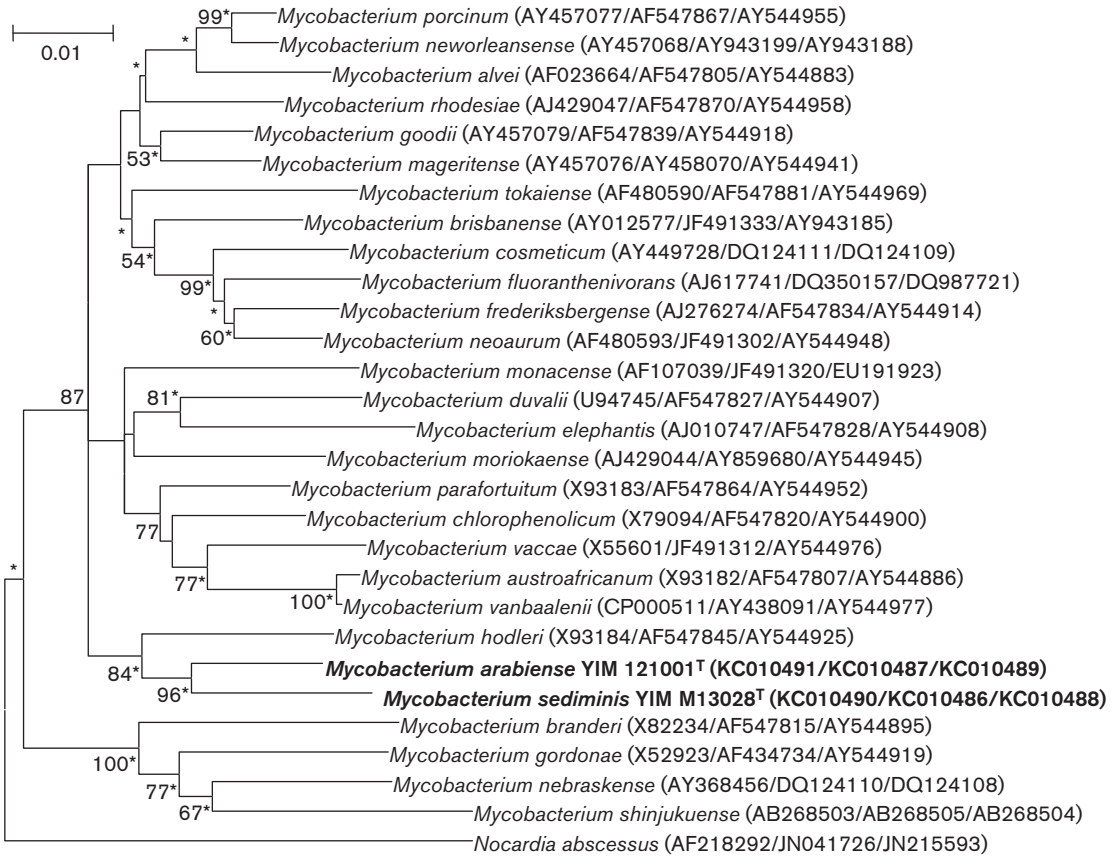

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene, *hsp65* and *rpoB* sequences of strain YIM M13028^T, strain YIM 121001^T and members of the genus *Mycobacterium*. Bootstrap values (expressed as percentages of 1000 replications) >50 % are shown at the branch points. Asterisks denote nodes that were also recovered using the maximum-likelihood and maximum-parsimony methods. *Nocardia abscessus* was used as the outgroup. Bar, 0.01 substitutions per nucleotide position.

addition to phylogenetic evidence (Table 1). On the basis of phenotypic, chemotaxonomic and phylogenetic analysis, strains YIM M13028^T and YIM 121001^T are considered to represent two novel species of the genus *Mycobacterium*, for which the names *Mycobacterium sediminis* sp. nov. for strain YIM M13028^T and *Mycobacterium arabiense* sp. nov. for strain YIM 121001^T are proposed.

Description of *Mycobacterium sediminis* sp. nov.

Mycobacterium sediminis (se.di′mi.nis. L. n. *sedimen*, *-inis* sediment; L. gen. n. *sediminis* of a sediment).

Gram-stain-positive, rod-shaped bacilli. Cording, spores and filaments are not observed. Grows to maturity in 5–7 days at 25–28 °C, pH 7.0. Colonies on 1.5 % NaCl TSA medium are orange–red, rough and non-chromogenic. Growth occurs on TSA medium with ⩽4.0 % NaCl, pH 6.0–8.0, at 5–37 °C. Positive result in tests for hydrolysis of Tweens 20, 40 and 80, catalase and urease. Negative result in tests for nitrate reductase, gelatin hydrolysis, cellulase, amylase and oxidase. Possesses mycolic acid and the major fatty acids (>10 %) are $C_{16:0}$, $C_{17:1}\omega7c$ and $C_{18:1}\omega9c$. Genetically, 16S rRNA, *rpoB*, *hsp65* and 16S–23S ITS gene sequences are unique. Phylogenetic analyses shows that the species is related to *Mycobacterium hodleri* DSM 44183^T and *Mycobacterium arabiense* sp. nov. YIM 121001^T.

The type strain YIM M13028^T (=DSM 45643^T=KCTC 19999^T) was isolated from a sediment sample collected from the South China Sea (19° 30.261′ N 111° 0.247′ E) at a depth of 42 m. The DNA G+C content of the type strain is 64.1 mol%.

Description of *Mycobacterium arabiense* sp. nov.

Mycobacterium arabiense (a.ra.bi.en′se. N.L. neut. adj. *arabiense* of or belonging to Arabia, referring to the isolation of the type strain in Dubai, United Arab Emirates).

Gram-stain-positive, rod-shaped bacilli. Cording, spores and filaments are not observed. Grows to maturity in

3 days at 25–37 °C, pH 7.0. Colonies on 1.5 % NaCl TSA medium are orange–red, smooth and non-chromogenic. Growth occurs on TSA medium with ⩽5.0 % NaCl, pH 6.0–8.0, at 5–37 °C. Positive result in tests for hydrolysis of Tweens 20, 40 and 80, catalase and urease. Negative result in tests for nitrate reductase, gelatin hydrolysis and oxidase. Possesses mycolic acid and the major fatty acids (>10 %) are $C_{16:0}$, $C_{17:1}\omega7c$, $C_{18:1}\omega9c$. Genetically, 16S rRNA, *rpoB*, *hsp65* and 16S–23S ITS gene sequences are unique. Phylogenetic analyses shows that the species is related to *Mycobacterium hodleri* DSM 44183^T and *Mycobacterium sediminis* sp. nov. YIM M13028^T.

The type strain YIM 121001^T (=DSM 45768^T=JCM 18538^T) was isolated from a sand sample collected from coastal zone of Dubai, United Arab Emirates. The DNA G+C content of the type strain is 62.8 mol%.

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