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Jamal Saad, Michael Phelippeau, May Khoder, Marc Lévy, Didier Musso, et al.. “*Mycobacterium mephinesia*”, a *Mycobacterium terrae* complex species of clinical interest isolated in French Polynesia. *Scientific Reports*, 2019, 9 (1), 10.1038/s41598-019-47674-8 . hal-02481898

HAL Id: hal-02481898

<https://amu.hal.science/hal-02481898>

Submitted on 28 Sep 2023

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"*Mycobacterium mephinesia*", a *Mycobacterium terrae* complex species of clinical interest isolated in French Polynesia

Jamal Saad¹, Michael Phelippeau², May Khoder¹, Marc Lévy³, Didier Musso⁴ & Michel Drancourt¹

A 59-year-old tobacco smoker male with chronic bronchitis living in Taravao, French Polynesia, Pacific, presented with a two-year growing nodule in the middle lobe of the right lung. A guided bronchoalveolar lavage inoculated onto Löwenstein-Jensen medium yielded colonies of a rapidly-growing non-chromogenic mycobacterium designed as isolate P7213. The isolate could not be identified using routine matrix-assisted laser desorption ionization-time of flight-mass spectrometry and phenotypic and probe-hybridization techniques and yielded 100% and 97% sequence similarity with the respective 16S rRNA and *rpoB* gene sequences of *Mycobacterium virginense* in the *Mycobacterium terrae* complex. Electron microscopy showed a 1.15 µm long and 0.38 µm large bacillus which was *in vitro* susceptible to rifampicin, rifabutin, ethambutol, isoniazid, doxycycline and kanamycin. Its 4,511,948-bp draft genome exhibited a 67.6% G + C content with 4,153 coding-protein genes and 87 predicted RNA genes. Genome sequence-derived DNA-DNA hybridization, OrthoANI and pangenome analysis confirmed isolate P7213 was representative of a new species in the *M. terrae* complex. We named this species "*Mycobacterium mephinesia*".

The International Working Group on Mycobacterial Taxonomy delineated the *Mycobacterium terrae* complex in 1998¹. The *M. terrae* complex initially consisted of two species *Mycobacterium terrae* and *Mycobacterium nonchromogenicum*^{1,2}. *M. nonchromogenicum* had been described in 1965 by Tsukamura³, while *M. terrae* had been described one year later by Wayne^{4,5}. This complex has expanded over the years and currently includes a total of fifteen species with *Mycobacterium virginense* being the last one to be assigned to the complex⁶. Members of the *M. terrae* complex have been implicated as responsible for tenosynovitis and osteomyelitis after Hirata and Tomiyama published in 1976 the first report of tenosynovitis caused by the *M. terrae* complex⁶.

In February 2017, a non-tuberculous mycobacterium (NTM) P7213 was isolated from the sputum collected in a 59-year-old male patient living in Taravao (17°44'S, 149°18'W), French Polynesia, a remote French overseas territory in the South Pacific area with a population of 280,000 inhabitants distributed in 72 inhabited islands grouped in 5 archipelagoes. Phenotypic and genetic investigations herein reported based on 16S rRNA and *rpoB* gene sequencing, DNA-DNA hybridization⁷ and OrthoANI⁸ revealed that strain P7213 was representative of a NTM species in the *M. terrae* complex.

In this study, we report the clinical features of the patient as well as the phenotypic and genotypic characterization of isolate P7213 indicating that this isolate is a new species in the *M. terrae* complex.

Clinical Case

Case report. In February 2017, a 59-year-old, thirty-year tobacco-smoker patient consulted a general physician for chronic cough and sputum. At the time of referral to a pulmonologist in June 2017, chest computed tomography (CT) scanner disclosed a 24 × 25 mm nodular opacity in the middle lobe of the right lung. Careful

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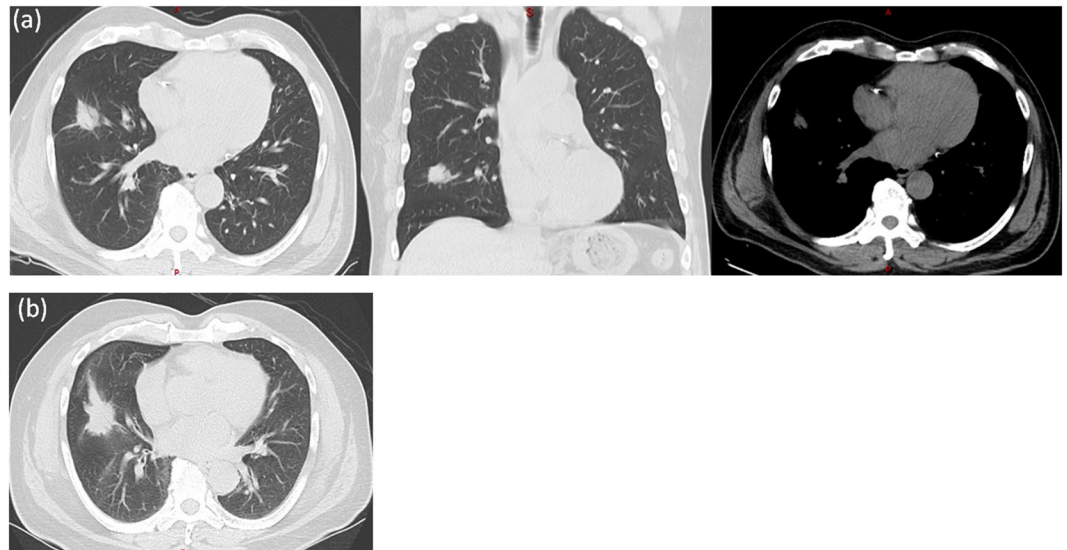


Figure 1. Evolution of a nodular, centimetric alveolar and speculated opacity in the right lung, middle lobe on a computed tomography scanner, (a) May 2017, (b) July 2018.

examination of previous chest radiographs performed between September 2015 and June 2016 revealed that this opacity had slowly increased over 21 months (Fig. 1a). The patient denied any thoracic pain, weight loss (83 kg, stable) nor fatigue, but reported dyspnea (modified Medical Research Council (mMRC) scale 1/4), cough and difficult to produce sputum. His treatment included metformin 850 mg twice daily for diabetes mellitus and daily febuxostat 80 mg for chronic hyperuricemia and perindopril 2.5 mg for hypertension. Biological tests showed normal hemoglobin rate (147 g/L), normal white cell count (6.7 G/L: neutrophils = 3.4 G/L, eosinophils = 0.36 G/L, basophils = 0.02 G/L, lymphocytes = 2.3 G/L and monocytes = 0.5 G/L), high fasting glucose = 1.26 g/L, normal HbA1C = 6.1%, creatinine = 9 mg/L and renal clearance = 91 mL/min, C-reactive protein < 5 mg/L and normal level for liver enzymes. Total cholesterol and triglycerides were elevated at 2.16 g/L and 5.51 g/L, respectively. Spirometry revealed an obstructive ventilatory disorder characterized by a forced expiratory volume in one second (FEV1) of 2.35 L (76%) and 2.39 L (79%) after 400 µg salbutamol bronchial challenge and a forced vital capacity (FVC) of 3.19 L (84%) resulting in a FEV1/FVC ratio of 73%, corresponding to a COPD pattern. A bronchial endoscopy with bronchoalveolar lavage (BAL) in the right middle lobe performed on June 20th 2017 revealed no mucosal abnormality. The BAL was addressed for microbiological analysis, including direct examination (Ziehl-Neelsen and auramine staining) and culture (broth and Löwenstein Jensen solid medium). Meanwhile, the patient refused a scanned-guided transthoracic biopsy proposed for pathological examination. Another chest CT-scan performed in July 2018 revealed that the nodule's size had increased to 35 mm of diameter (Fig. 1b), and a scanned-guided transthoracic biopsy proposed for pathological examination failed unfortunately. Another BAL performed in the same middle lobe, on September 2018, did not reveal any pathological cell, nor acid-fast bacilli using Ziehl-Neelsen staining and direct examination. Mycobacterial culture remained sterile after a 45-days incubation period.

Microbial Investigations

First line microbial investigations. This study has been performed in accordance with relevant guidance and regulations and was approved by the IHU Méditerranée Infection, Ethics Committee Approval n°2016-025, Marseille, France. Collection of sputum was part of the patients' routine care activity. After being informed, the patient agreed to participate and signed an informed anonymised consent.

Direct microscopic examination of BAL after auramine and Ziehl-Neelsen stainings revealed no acid-fast bacilli and routine bacteriological cultures remained sterile. Six days after inoculation of the BAL on Löwenstein-Jensen culture medium (Biorad, Marnes-la-Coquette, France), twenty colonies of a non-chromogenic isolate P7213 could not be identified using probe-hybridization (GenoType Mycobacterium CM ver 2.0 + AS ver 1.0, Hain Lifescience, Nehren, Germany). As no such organisms had been isolated in the laboratory in months preceding and following this isolation, this isolate was not regarded as a mere in-laboratory contaminant and was then referred to the Mycobacterium reference laboratory of Institut Hospitalo-Universitaire, Marseilles, France in August 2017, for further identification and characterization. There, the isolate was deposited in the public collection Collection de Souches de l'Unité des Rickettsies under number CSUR P7213. The isolate could not be identified using routine matrix-assisted laser desorption ionization-time of flight-mass spectrometry and phenotypic and probe-hybridization techniques. According to 16S rRNA and *rpoB* gene sequencing, the isolate P7213 was found to display 100% and 97% sequence similarity with 16S rRNA and *rpoB* gene respectively with *Mycobacterium virginense* (NZ_PUEV00000000.1) (Fig. 2), a newly described species of the *Mycobacterium terrae* complex⁶. These values being equal of cut-off value 97% previously proposed to delineate different species among the genus *Mycobacterium*⁹, enforced the suggestion that isolate P7213 was representative of a new species

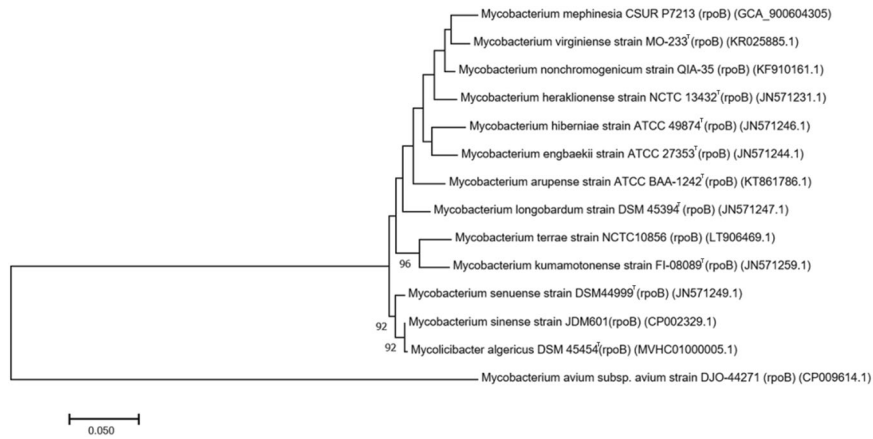


Figure 2. Phylogenetic tree based on the *rpoB* gene sequence indicating the phylogenetic position of isolate P7213 relative to the other species of the *M. terrae* complex. Sequences were aligned using Muscle v3.8.31 implemented on MEGA7 software. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. Phylogenetic inferences obtained using the Maximum Likelihood method with 1,000 bootstrap replicates.

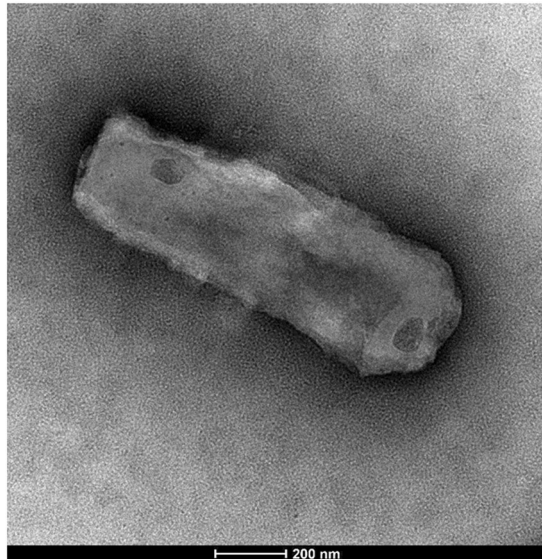


Figure 3. Transmission electron microscopy of *M. mephinesia* strain P7213. The scale bar represents 200 nm.

in the *M. terrae* complex. Isolate P7213 was therefore thoroughly analyzed to confirm it as representative of a hitherto undescribed *Mycobacterium* species.

Phenotypic characterization. Isolate P7213 displayed smooth and non-pigmented colonies growing at a temperature range of 25 °C to 37 °C after 7-day incubation on Middlebrook 7H10 agar medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD, USA). The observation of colonies by electron microscopy showed rod-shaped bacilli measuring 1.15 μm long and 0.38 μm large (Fig. 3). However, isolate P7213 reproducible matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) profile did not match any of the profiles entered in the Bruker database (Supplementary Fig. S1). Currently, Bruker database does not contain the spectrum of *M. virginense*. *In vitro* susceptibility testing was performed using broth microdilution method with MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), the MIC values found for isolate P7213 are: rifampicin [minimal inhibitory concentration (MIC) < 8.2 μg/mL], rifabutin (MIC < 8.2 μg/mL), ethambutol (MIC = 4 μg/mL), isoniazid (MIC = 2.3 μg/mL), doxycycline (MIC = 8.8 μg/mL) and kanamycin (MIC = 8 μg/mL), trimethoprim (MIC = 25 μg/mL) and sulfamethoxazole (MIC = 29 μg/mL) (Table 1). Using Biolog[®] Phenotype MicroArray testing (Hayward, California, United States of America) as previously described¹⁰, isolate P7213 grew under 15 inhibitory chemical conditions including fusidic acid, troleandomycin, minocycline, lincomycin, guanidine HCl, niaproof 4, aztreonam, vancomycin, nalidixic acid, tetrazolium violet, tetrazolium blue, potassium tellurite, sodium butyrate

Antibiotic	MIC (µg/mL)
Ethambutol	4
Isoniazid	2.3
Rifabutin	<8.4
Rifampicin	<8.2
Doxycycline	8.8
Kanamycin	8
Sulfamethoxazole	25
Trimethoprim	29

Table 1. Minimum inhibitory concentration of selected antibiotics against *M. mephnesia* strain P7213.

		<i>M. mephnesia</i>	<i>M. kumamotonense</i>	<i>M. nonchromogenicum</i>	<i>M. terrae</i>	<i>M. hiberniae</i>
Growth at	30	Grows	Grows	Grows	Grows	Grows
	37	Grows	Grows	Nd	Nd	Grows
	42	Not Grows	Grows	Grows	Not Grows	Not Grows
Pigmentation		Non-pigmented	Non-pigmented	Non-pigmented	Non-pigmented	Rose-pink-pigmented
Leucine arylamidase	+	+	–	–	–	+
Cystine arylamidase	+	+	–	–	–	–
Phosphatase acid	+	+	+	+	+	+
β-galactosidase	–	–	+	+	+	+
β-glucosidase	+	–	+	–	–	–
Reduction of nitrates	–	+	+	+	+	+
Pyrazinamidase	–	Nd	+	+	+	+
β-galactosidase	–	–	+	+	+	+
Urease	+	Nd	–	+	+	–
Reference		6,22	2,6	6,22–24	22	

Table 2. Phenotypic characteristics of *M. mephnesia* P7213 and related mycobacteria in the *M. terrae* complex.

and sodium bromate (Supplementary Table S1). Using the API ZYM strip (bioMérieux, Craponne, France) as previously described^{10,11}, isolate P7213 was shown to metabolize four carbon sources including D-fructose 6-PO₄, α-keto-glutaric acid, glucuronamide and acetoacetic acid. In addition, isolate P7213 positively reacted to phosphatase alkaline, esterase (C4), esterase lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase; and negative to lipase (C 14), D-chymotrypsine, D-galactosidase, β-galactosidase, β-glucuronidase, D-glucosidase, D-mannosidase and D-fucosidase. Furthermore, the API-CORYNE strip (bioMérieux) method indicated that P7213 was positive for alkaline phosphatase, urease and hydrolysis gelatine. The comparison with closely related taxa showed that strain P7213 differed in a combination of positive cystine arylamidase and positive β-glucosidase activities (Table 2). All identified mycolic acids are listed in Supplementary Table S2 and showed a measured mass accuracy below 5 ppm error). The mass spectrometry analysis of *Mycobacterium tuberculosis* H37Rv used as a positive control showed the previously well described mycolic acid pattern¹², including α- (C_{74–86}), methoxy- (C_{83–92}) and keto- (C_{84–89}) forms (Fig. 4). Isolate P7213 showed a completely different mycolic acid pattern, composed of α- (C_{59–83}), dicarboxy/wax- (C_{57–84}) and keto/epoxy/ω-1- (C_{81–84}) forms. The most abundant structures were dicarboxy/wax-, representing 95% of the detected peak areas. Shorter carbon backbones were described for this isolate, in particular for α- and dicarboxy/wax- forms. No methoxy- or α'- mycolic acids were detected. All measured ion collision cross section (CCS) values were consistent with the mycolic acids estimated CCS (below 2% deviation) and confirmed the identification results above. Indeed, experimental CCS values showed a linear correlation with the number of carbons. The corresponding equation calculated from different *Mycobacterium* samples was “CCS = 3.4 C + 133.4”, with “C” the number of carbons.

Genomic characterization. Full genome sequencing of isolate P7213 yielded 134 scaffolds corresponding to 4,511,948-pb with a 67.6% GC content (EBI accession number GCA_900604305). Within the complex *M. terrae*, the genome size of P7213 is larger than that of *M. hiberniae* (4.20 Mb) and *M. minnesotense* (4.05) and smaller than that of *M. nonchromogenicum*, *M. heraklionense*, *M. virginense*, *M. longobardum*, *M. terrae*, *M. arupense*, *M. hiberniae*, *M. engbaekii* and “*M. sinense*”. Moreover, the GC % content of isolate P7213 is lower than that of *M. hiberniae*, *M. kumamotonense*, *M. algericum*, *M. engbaekii*, *M. terrae*, *M. longobardum*, *M. heraklionense*, *M. nonchromogenicum*, *M. senuense* and “*M. sinense*” and higher than that of *M. virginense*, *M. icosiummassiliensis*, *M. arupense* and *M. minnesotense* (Table 3). Isolate P7213 genome is predicted to encode 4,240 genes including 4,153 protein-coding genes and 87 RNAs including 53 tRNA, 5 rRNA, 1 tmRNA and 28 miscellaneous other RNA (misc_RNA). A total of 36 proteins were detected to be associated with virulence including a type VII secretion system protein EsxG, iron-regulated transporter and lysine-N-oxygenase. A total of 2,819 (66.4%) genes were

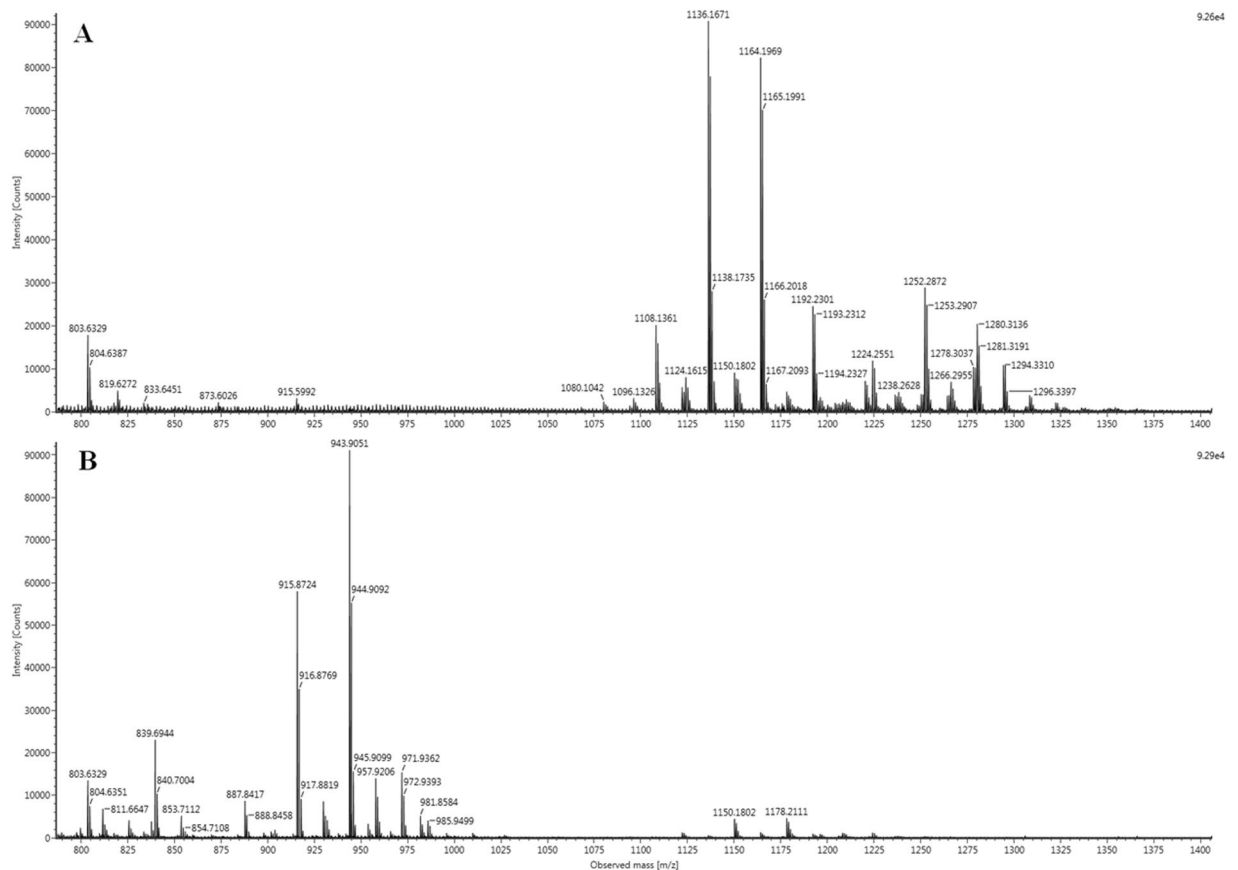


Figure 4. ESI-IMS-MS spectra of the $[M-H]^-$ mycolic acid ions. (A) *Mycobacterium tuberculosis* H37RV (control), (B) *Mycobacterium mephinesia* (P7213).

Type strains	Accession number	Size (Mb)	GC %	No. of contigs	Gene content
<i>M. mephinesia</i>	GCA_900604305	4.30	67.6	134	4240
<i>M. heraklionense</i>	NZ_LDPO01000001.1	4.98	67.9	75	4798
<i>M. arupense</i>	NZ_LASW02000001.1	4.31	67.3	173	4230
<i>M. hiberniae</i>	NZ_LQOZ01000036.1	4.20	68.5	48	4078
<i>M. longobardum</i>	NZ_LQPG01000007.1	4.66	67.9	59	4660
<i>M. terrae</i>	NZ_LT906469.1	4.38	68.4	1	4240
<i>M. engbaekii</i>	NZ_LQOT01000069.1	4.38	68.5	79	4192
<i>M. virginiae</i>	NZ_PUEV00000000.1	4.70	67.2	128	4675
<i>M. nonchromogenicum</i>	NZ_LQPI00000000.1	4.32	67.8	91	4154
<i>M. algericum</i>	NZ_MVHC00000000.1	4.48	68.3	107	4126
<i>M. icosiummassiliensis</i>	NZ_FJVP00000000.1	4.69	66.8	79	4758
<i>M. kumamotoense</i>	NZ_MVHU00000000.1	4.67	68.1	128	4602
<i>M. senuense</i>	NZ_LQPS00000000.1	4.39	68.7	54	4271
<i>M. tuberculosis</i> H37Rv	NC_000962.3	4.26	65.6	1	4125
« <i>M. sinense</i> »	NC_015576.1	4.49	68.4	1	4405
<i>M. mimnesotense</i>	NZ_MVHZ00000000.1	4.05	67.1	60	3911

Table 3. Genomic comparison (sequence size, GC %, number of contigs and gene content) of *M. mephinesia* strain P7213 with the other species in the *M. terrae* complex.

assigned with putative clusters of orthologous groups (COG) function or nr blast. The remaining 1.421 genes (33.5%) encode for hypothetical proteins without COG assignment. The distribution of genes into COGs functional categories are detailed in Supplementary Table S3. Within the complex *M. terrae*, isolate P7213 genome exhibits an average nucleotide identity (ANI) of 91.6% with *M. virginiae*, 89.9% with *M. icosiummassiliensis*,

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>M. nonchromogenicum</i>	100	88.8	88.54	85.09	88.22	85.67	85.69	85.17	83.51	83.18	88.63	83.86	83.28	83.39	85.08
<i>M. virginiense</i>		100	91.65	85.8	90.34	85.87	85.84	86.31	84.18	83.86	90.55	84.11	83.78	83.72	85.62
<i>M. mephinesia</i>			100	86.13	89.89	85.56	85.58	85.94	84.05	83.68	89.96	84.22	83.67	83.8	85.74
<i>M. arupense</i>				100	86.13	84.25	84.19	84.51	82.84	82.42	85.71	82.69	82.46	82.52	90.6
<i>M. heraklionense</i>					100	86.38	86.3	86.88	84.76	84.38	90.25	84.71	84.42	84.52	86.18
<i>M. engbaekii</i>						100	95.13	84.7	83.26	85.26	86.26	85.3	85.15	82.96	84.22
<i>M. hiberniae</i>							100	84.7	83.18	82.96	85.81	83.15	82.9	82.88	84.11
<i>M. longobardum</i>								100	85.52	85.26	86.26	85.3	85.3	85.24	84.07
<i>M. terrae</i>									100	86.75	84.44	90.7	86.75	86.85	82.65
<i>M. algericum</i>										100	84.06	86.54	92.5	97.69	82.32
<i>M. icosiumassiliensis</i>											100	84.41	83.96	84.07	85.79
<i>M. kumamotonense</i>												100	86.41	86.58	82.44
<i>M. senuense</i>													100	92.35	82.26
« <i>M. sinense</i> »														100	82.33
<i>M. minnesotensis</i>															100

Table 4. OrthoANI values calculated from Orthologous Average Nucleotide Identity tool version of complex *M. terrae*.

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>M. mephinesia</i>	100	44.5 ± 5.2	39.1 ± 5	30.2 ± 4.8	29.4 ± 4.9	31.1 ± 4.9	27 ± 4.8	30.9 ± 4.8	35.4 ± 4.8	27.4 ± 4.9	39.4 ± 5	26.4 ± 4.8	26.5 ± 4.9	26.6 ± 4.9	29.5 ± 4.8
<i>M. virginiense</i>		100	43.9 ± 4.8	33.4 ± 4.9	29.9 ± 4.8	30.1 ± 4.8	27.3 ± 4.8	29.7 ± 4.8	36.4 ± 4.9	26.2 ± 4.8	44.1 ± 5.1	26.4 ± 5.9	26.1 ± 4.8	26.8 ± 4.8	29.7 ± 4.9
<i>M. heraklionense</i>			100	30.8 ± 4.8	30.9 ± 4.9	22.8 ± 4.9	28.1 ± 4.9	30.9 ± 4.8	35 ± 4.8	28.6 ± 4.9	40.9 ± 5	28.2 ± 4.9	28.4 ± 4.9	27.5 ± 4.8	30.4 ± 4.9
<i>M. arupense</i>				100	27.1 ± 4.8	28 ± 4.8	25.5 ± 4.8	27.3 ± 4.9	30.8 ± 4.8	26.3 ± 4.8	30.4 ± 4.9	25.3 ± 4.8	25.2 ± 4.8	25.2 ± 4.9	41.2 ± 5
<i>M. hiberniae</i>					100	25.2 ± 4.8	25.2 ± 4.8	60.6 ± 4.8	28.7 ± 4.8	25.4 ± 4.8	29.3 ± 4.9	25.3 ± 4.8	25.2 ± 4.8	25.4 ± 4.9	27 ± 4.8
<i>M. longobardum</i>						100	27.7 ± 4.8	27.9 ± 4.8	28.6 ± 4.8	27 ± 4.8	31.7 ± 5	27.7 ± 5.9	27.5 ± 4.9	29 ± 4.9	27.3 ± 4.8
<i>M. terrae</i>							100	26 ± 4.8	26.2 ± 4.9	41.3 ± 5	27.8 ± 4.9	31.6 ± 4.9	31.5 ± 5	31.7 ± 4.9	25.1 ± 4.8
<i>M. engbaekii</i>								100	28.8 ± 4.8	25.7 ± 4.8	31.7 ± 4.9	25.5 ± 4.8	25.9 ± 4.8	26.1 ± 4.8	27.1 ± 4.9
<i>M. nonchromogenicum</i>									100	21.8 ± 4.7	35.8 ± 4.9	26 ± 4.8	21.2 ± 4.7	26 ± 5	28.5 ± 4.9
<i>M. kumamotonense</i>										100	27.9 ± 4.9	31.3 ± 4.9	30.7 ± 4.9	31 ± 4.9	25.3 ± 4.8
<i>M. icosiumassiliensis</i>											100	27.2 ± 4.9	27.1 ± 4.9	27.2 ± 4.8	30.1 ± 4.9
<i>M. algericum</i>												100	47.9 ± 5.2	80.7 ± 5.6	24.08 ± 4.8
<i>M. senuense</i>													100	47 ± 5.2	24.7 ± 4.8
« <i>M. sinense</i> »														100	24.8 ± 4.8
<i>M. minnesotensis</i>															100

Table 5. Comparison of *M. mephinesia* P7213 with related mycobacteria species in *M. terrae* complex using GGDC, formula 2 (DDH estimates based on identities/HSP length).

89.8% with *M. heraklionense*, 88.5% with *M. nonchromogenicum*, 86.1% with *M. arupense*, 85.9% with *M. longobardum*, 85.7 with *M. minnesotense*, 85.5% with *M. engbaekii* and *M. hiberniae*, 84.2% with *M. kumamotonense*, 84% with *M. terrae*, 83.8 with “*M. sinense*” and 83.6% with *M. senuense* and *M. algericum* (Table 4). Within the complex *M. terrae*, *in silico* DNA-DNA hybridization (DDH) analysis yielded 44.5 ± 5.2% identity with *M. virginiense*, 39.4 ± 5% with *M. icosiumassiliensis*, 39.1 ± 5% with *M. heraklionense*, 35.4 ± 4.8% with *M. nonchromogenicum*, 31.1 ± 4.9% with *M. longobardum*, 30.9 ± 4.8% with *M. engbaekii*, 30.2 ± 4.8% with *M. arupense*, 29.5 ± 4.8% with *M. minnesotense*, 29.4 ± 4.9% with *M. hiberniae*, 27.4 ± 4.9% with *M. kumamotonense*, 27 ± 4.8% with *M. terrae*, 26.6 ± 4.9% with “*M. sinense*”, 26.5 ± 4.9% with *M. senuense* and 26.4 ± 4.8% with *M. algericum* (Table 5). As a supplementary analysis, we made DDH and Ortho average nucleotide identity (ANI) values between all the members of the *M. terrae* complex in order to redefine the position of isolate P7213 within this complex. For the DDH analysis the minimum value found was of 21.2% between *M. nonchromogenicum* and *M. senuense* while the maximum was 80.7% between “*M. sinense*” and *M. algericum*. Concerning OrthoANI analysis, the minimum value found was of 82.42% between *M. algericum* and *M. arupense* and the maximum value was of 97.69% between *M. algericum* and “*M. sinense*”. The pangenome of this complex contained 41,095 genes including 278 conserved genes, 3,583 shell genes shared by several species and 37,234 cloud genes unique to one species. Pangenome study showed divergence between all the members of this complex: we found one large cluster comprising of seven species: isolate P7213, *M. virginiense*, *M. icosiumassiliensis*, *M. heraklionense*, *M. nonchromogenicum*, *M. arupense* and *M. minnesotense*; two small clusters each comprising of two species; and four unclustered species (Fig. 5).

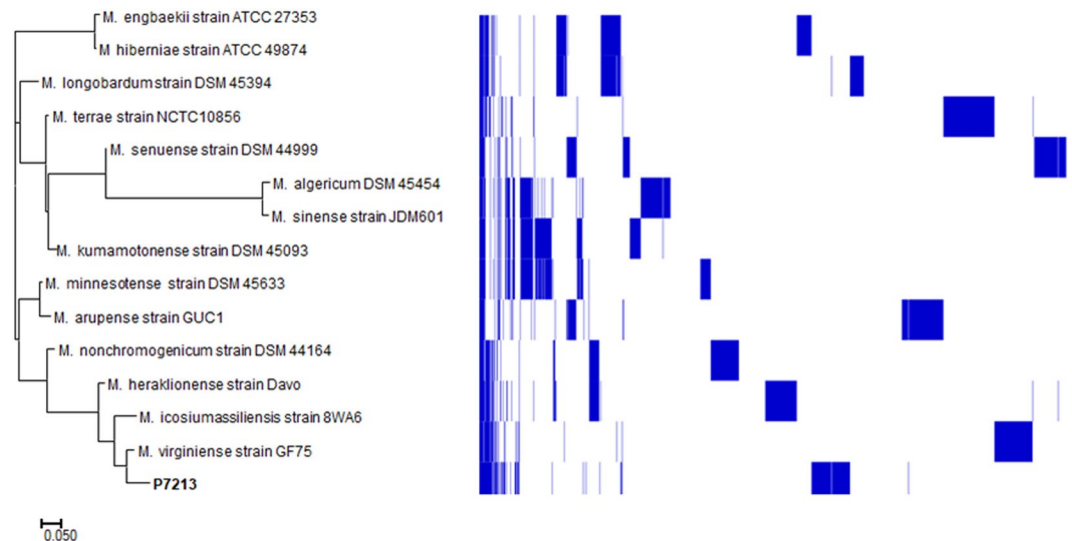


Figure 5. Pangenome tree of isolate P7213 with all members of *Mycobacterium terrae* complex.

Whole genome sequence-based fishing in the databases. Fishing NCBI database using the P7213 strain *hsp65* partial sequence as probe, we observed 99.05%, 99.05%, 99.25%, 97.24% and 97.01% sequence similarity with *M. terrae* strain variant MS699 isolated in Canada, *Mycobacterium* sp. FI-11038 isolated from Italy, *Mycobacterium* sp. FI-7105 isolated from Italy, *M. engbaekii* strain FI-06247 isolated from Italy and *Mycobacterium virginense*, respectively. Moreover, phylogenetic tree based on *hsp65* gene with 50 Max target sequences showed that strain P7213 was away to *M. virginense* but grouped with *M. sp.* FI-11038, *M. terrae* strain variant MS699 and *Mycobacterium* sp. FI-7105 (Supplementary Fig. S2). While the complete *rpoB* sequence (extracted using Prokka software) showed 97%, 94% and 94% sequence similarity with *M. virginense*, *M. terrae* strain NCTC10856 unknown origin and “*M. sinense*” strain JDM601 isolated in China, respectively. But, blastn against NCBI database with 724-bp partial *rpoB* gene (extracted by Blastn using reference partial *rpoB*) of *M. mephinesia* P7213 showed 99%, 99%, 99% and 97% with *Mycobacterium* sp. MO-4693 isolated from USA, *Mycobacterium* sp. FI-7105, *Mycobacterium* sp. FI-11038 and *M. virginense* respectively.

Discussion

Tuberculosis due to *M. tuberculosis* is highly endemic in French Polynesia, the incidence of NTM infections is unknown¹³. In the clinical case herein reported, the large opacity found on chest CT-scanner and the isolation of a NTM from a bronchoalveolar lavage was considered as clinically relevant according the American Thoracic Society criteria for NTM lung infection¹⁴: compatible clinical presentation and symptoms (cough, sputum and dyspnea), one NTM isolate from a BAL and radiological patterns that are compatible with NTM lung infection. However, pathological analysis is currently lacking to definitely assess the infectious origin of this lung nodule. The patient did not manage to perform other sputum specimens and is still in good health except for chronic cough 21 months after first consultation. Chest malignancy was exploded based on the patient good and persistent general condition, the slowly increasing nodule size, the lack of other metastatic-like nodules in chest CT-scanner.

The BAL culture yielded colonies that remained unidentified using a commercial hybridization test, as it has been previously reported in the case of unfrequently encountered species¹⁵. This isolate was then identified as a potential new species of *Mycobacterium* according to *rpoB* gene sequencing with cut-off less than or equal 97%¹⁶. The new species herein described was named *Mycobacterium mephinesia* according to the name of our research unit (MEPHI) and the name of island (French Polynesia) where the P7213 strain was isolated for the first time. Because of the potential role of this new species *mycobacterium* in the clinical situation of the patient could not be derived from the sole isolate here reported, antibiotic susceptibility profile here reported was performed to contribute to the phenotypic characterization of the isolate and not to guide in anyway antibiotic treatment. Interestingly, this potential new species has been found in French Polynesia where a previous investigation using the same molecular tools, yielded another new *Mycobacterium* species, *Mycobacterium massilipolynesiensis*, in the *Mycobacterium simiae* complex¹⁷. In French Polynesia also, genotyping techniques allowed the discovery of another nontuberculous *Mycobacterium* species and new *Mycobacterium tuberculosis* lineages¹⁸. Looking for microbes in some remote areas where appropriate identification methods are not available yet, is a source of discovery of new microbial species.

Genome-based analyses anchored isolate P7213 within the *M. terrae* complex, most closely related to *M. virginense* which has been isolated in Virginia, USA⁶. This complex has expanded over the years to include *M. nonchromogenicum*³ isolated in Japan, *M. terrae*⁴, *Mycobacterium engbaekii*¹⁹, *Mycobacterium hiberniae*²⁰ isolated in Ireland, *Mycobacterium arupense*²¹ isolated in the USA, *Mycobacterium kumamotoense*²² isolated in Japan, *Mycobacterium senuense*²³ isolated in Korea, *Mycobacterium algericum*²⁴ isolated in Algeria, “*Mycobacterium sinense*” *M. sp.* JDM601²⁵ isolated in China, *Mycobacterium longobardum*²⁶ isolated in Italy, *Mycobacterium*

Species	Isolation source	Clinical presentation	Isolation site	Growth	Ref
<i>M. nonchromogenicum</i>	Sputum	Pulmonary infection	Japan	SGM	2,3
<i>M. heraklionense</i>	Sputum	Lung disease	United States	RGM	6
<i>M. arupense</i>	Sputum	Bronchiectasis	United States	RGM	6
<i>M. hiberniae</i>	Soil, sphagnum vegetation, true moss	Nd	Ireland	SGM	20
<i>M. engbaekii</i>	Water	Nd	Denmark	RGM	26
<i>M. longobardum</i>	Sputum	Nd	Italy	RGM	26
<i>M. virginiae</i>	Human	Tenosynovitis, osteomyelitis	Virginia, USA	SGM	6
<i>M. algericum</i>	Goat	Pulmonary lesion	Algeria	RGM	24
<i>M. icosiummassiliensis</i>	Lake	Nd	Algeria	RGM	29
<i>M. kumamotoense</i>	Sputum	Pulmonary disease	Japan	SGM	22
« <i>M. sinense</i> »	Sputum	Tuberculosis-like disease	China	SGM	25
<i>M. minnesotense</i>	Sphagnum peat bogs	Nd	Northern Minnesota, USA	IG	27
<i>M. senuense</i>	Sputum	Pulmonary infection	South Korea	SGM	23

Table 6. Synopsis of the *M. terrae* complex species. SGM: Slowly Growing Mycobacteria, RGM: Rapid Growing Mycobacteria IG: Intermediat Growing ND: No Data.

heraklionense which ten isolates have been made in Greece, seven in Italy and six in India²⁶, *Mycobacterium minnesotense*²⁷ isolated in northern Minnesota, USA., *Mycobacterium paraterrae*²⁸ isolated in Korea, *M. icosiummassiliensis*²⁹ that we isolated in Algeria and *M. virginiae*^{6,30} isolated in Virginia, USA (Table 6). DDH and OrthoANI values were very low between several members of this rapidly expanding *M. terrae* complex. Moreover, comparison of the core and accessory genes of the different species currently assigned to the *M. terrae* complex indicated that so-called *M. terrae* complex is heterogeneous, comprising of three clusters grouping a total of 11 species in addition to four unclustered species. In particular, *M. terrae* and *M. nonchromogenicum* which have historically founded the *M. terrae* complex are scattered apart with *M. terrae* remaining apart from any other species in the complex. Exploiting P7213 whole genome sequence to probe the large nucleotide database NCBI using *rpoB* and *hsp65* partial gene sequences, we detected three additional strains previously isolated from Italy and one from Canada, as belonging to the same new species as P7213, expanding the probable geographical repartition of this new species.

In order to clarify the on-going taxonomy of this group of microorganisms, we propose to name *Mycobacterium mephinesia* complex the largest cluster including *M. mephinesia*, *M. virginiae*, *M. icosiummassiliensis*, *M. heraklionense* and *M. nonchromogenicum* and characterized by DHH values higher than 30 and OrthoANI values higher than 85 among members of this cluster.

Materials and Methods

Phenotypic characterization. Standard method was used for biochemical tests³¹. In addition, the broth microdilution method with MTT test^{32,33} was used for determining the MIC of different antimycobacterial agents.

Transmission electron microscopy. Transmission electron microscopy (Morgani 268D; Philips, Eindhoven, The Netherlands) was used to measure the size of isolate P7213 after negative staining at an operating voltage of 60 kV, as previously described³⁴. For that, a total of seven mycobacteria was measured.

Biolog phenotype microarray. Isolate P7213 was cultured on Middlebrook 7H10 agar medium at 37°C supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) (Becton Dickinson, Sparks, MD, USA) for one weeks. Colonies were then suspended in IF-A (Biolog inoculating fluid recommended for strongly reducing and capsule producing bacteria, including mycobacteria) and adjusted between 90% and 98% transmittance using a turbidimeter (Biolog Inc). In each case we launched two GEN α -MicroPlate and then incubated in the OmniLog PM System (Biolog Inc.) at 37°C for 7 days.

Lipid extraction and Ion mobility-mass spectrometry analysis of mycolic acids. Isolate P7213 and *Mycobacterium tuberculosis* H37Rv (control) were cultured as detailed above. Mycolic acids were prepared as detailed previously with modifications^{35,36}. At least five inoculation loops were collected from a culture plate and transferred into 2 mL of potassium hydroxide 9 M. Mycolic acids were hydrolysed at 100°C during two hours. Free mycolic acids were then extracted with 2 mL of chloroform a low pH by adding 3 mL of 6 N hydrochloric acid to the aqueous phase. The organic layer was collected and dried at 40°C under a stream of nitrogen. Free mycolic acids were then dissolved in 100 μ L of a methanol-chloroform mixture (50:50, v/v) and subjected to electrospray-mass spectrometry analysis after a 1,000-fold dilution in methanol. Samples were analyzed in the Sensitivity Negative ionization mode using a Vion IMS QToF ion-mobility time-of-flight high resolution mass spectrometer (Waters, Guyancourt, France). Samples were infused at 15 μ L/min, after washing the fluidics with chloroform/methanol (20:80%; v/v). Ionization parameters were set as follow: capillary voltage 3 kV, cone voltage 50 V, source and desolvation temperatures 120/650°C. Mass calibration was adjusted automatically during analysis using a Leucine Enkephalin solution at 50 pg/ μ L (554.2620 m/z). Ions were monitored from 500 to 2000 m/z for 2 minutes using a High Definition MS (E) acquisition method. This method enabled the survey of ion mobility

drift times and alternate parent/fragments times of flight. Mass spectra were automatically combined by UNIFI (version 1.9.3, Waters), then monoisotopic peak areas and collision cross section (CCS) values were collected for each detected ion component. Mycolic acids were targeted according to $[M-H]^-$ ions of previously detailed structures^{37,38} with parent mass errors below 5 ppm and CCS values between 300 and 500 Å². Here, *keto/epoxy/ω-1 and dicarboxyl/wax ester* mycolic acid subclasses could not be distinguished from each other because of same chemical formula.

MALDI-TOF-MS. One colony was picked on a Middlebrook 7H10 solid-medium and put directly on the MALDI-TOF plate. Next, 1.5 μL of a matrix solution (saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) (Bruker Daltonics) was used to cover the sample. Then, when the matrix is dry, the plate was loaded into the Microflex LT (Bruker Daltonics) mass spectrometer. The spectra were recorded according to the parameters as previously described³⁹. All signals with resolution ≥ 400 were automatically acquired using AutoXecute acquisition control in flexControl software version 3.0 and the identifications were obtained by MALDI Biotyper software version 3.0 with the Mycobacteria Library v2.0 database.

Phylogenetic analyses. We found the phylogenetic position of isolate P7213 in *Mycobacterium terrae* complex based on the 16S rRNA and *rpob* gene sequence. A phylogenetic tree was created using the Maximum Likelihood method and Kimura 2-parameter model within the MEGA 7 software⁴⁰. Sequences were aligned by Muscle v3.8.31 with default parameters and the statistical support for internal branches of the trees was evaluated by bootstrapping with 1,000 iterations.

DNA preparation and genome sequencing. Total DNA of isolate P7213 was extracted by using InstaGen matrix (Biorad, France). 200 μL of InstaGen matrix was added to the pellet of isolate P7213 and incubated at 56 °C for 30 min. One spatula of glass beads was added, and the preparation was vortexed at high speed for 10 seconds and incubated at 100 °C for 10 min. Next, a mechanical treatment by FastPrep BIO 101 instrument (Qbiogene Strasbourg, France) was used at maximum speed (6.5 m/sec) for 45 seconds. Finally, the tube was centrifuged for 15 min and the supernatant DNA was collected. Extracted DNA was quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA) and 0.2 μg/μL of DNA was sequenced by Illumina MiSeq runs (Illumina Inc., San Diego, USA). The DNA was fragmented and amplified by limited PCR (12 cycles), introducing dual-index barcodes and sequencing adapters. After purification on AMPure XP beads (Beckman Coulter Inc, Fullerton, CA, USA), the libraries were normalized and pooled for sequencing on the MiSeq. Paired-end sequencing and automated cluster generation with dual indexed 2 × 250-bp reads were performed during a 40-hour run. Total information of 8.2 Gb was obtained from a 1,207 k/mm² cluster density with a cluster passing quality control filters of 89.3% (10,507.2 passed filtered reads).

Genome characterization and genome comparisons. The genome was assembled by A5 pipeline, organized by mauve alignment and annotated by Prokka as described previously⁴¹. The virulence factor was determined by ABRICATE. Emboss GC% was used for detection of the presence of plasmid. Furthermore, the Argannot database⁴² was used for required antibiotic resistance genes using the BLAST search in Bio-Edit interface under moderately stringent conditions (e-value of 10⁻⁵). The putative ARGs were further verified through a web-enabled NCBI GenBank BLAST search. In addition, to estimate the similarity between the compared genome, we used Genome to Genome Distance Calculator (GGDC) available in the web server (<http://ggdc.dsmz.de>), formula 2 was recommended into account to interpret the results of the draft genome analyzed. The mean levels of relatedness between isolate P7213 genome sequence and the other members of the *M. terrae* complex we measured using OrthoAni (Orthologous Average Nucleotide Identity) (<https://www.ezbiocloud.net/tools/orthoani>). We measured the DDH and OrthoANI values between all the members of *M. terrae* complex. Moreover, Roary pangenome pipeline in Galaxy software (<https://usegalaxy.org.au/>) was used to release the pangenomic analysis of the *M. terrae* complex members.

Whole genome sequence-based fishing in the databases. Using the *rpob* gene and *hsp65* gene sequences of P7213 strain as probes, we probed the nucleotide sequence database NCBI for similar sequences (97% *rpob* sequence similarity and 97% *hsp65* sequence similarity) potentially indicative of other strains belonging to the same species as P7213, previously unidentified or misidentified.

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Acknowledgements

This work was supported by the French Government under the « Investissements d'avenir » (Investments for the Future) program managed by the Agence Nationale de la Recherche (ANR, fr: National Agency for Research), (reference: Méditerranée Infection 10-IAHU-03). This work was supported by Région Provence Alpes Côte d'Azur and European funding FEDER PRIMI. Jamal Saad benefits from a Foundation Méditerranée Infection PhD grant. The authors also acknowledge Nicolas Armstrong for the technical assistance in HPLC experiments.

Author Contributions

J.S. performed phenotype and genome characterization, drafted the MS. M.P. took care of the patient, drafted the MS. M.K. performed genome analyses, drafted the MS. M.L. isolated the strain, performed phenotype and drafted the MS. D.M. interpreted data and drafted the MS. M.D. conceived the study, interpreted data and drafted the MS.

Additional Information

Supplementary information accompanies this paper at <https://doi.org/10.1038/s41598-019-47674-8>.

Competing Interests: The authors declare no competing interests.

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