Mycobacterium llatzerense sp. nov., a facultatively autotrophic, hydrogen-oxidizing bacterium isolated from haemodialysis water

Margarita Gomila,¹ Antonio Ramirez,² Joan Gascó³ and Jorge Lalucat¹

Correspondence

Jorge Lalucat
jlalucat@uib.es

¹Àrea Microbiologia, Departament de Biologia, Universitat de les Illes Balears, and Institut Mediterrani d'Estudis Avançats (CSIC-UIB), 07122 Palma de Mallorca, Illes Balears, Spain

²Servei de Microbiologia, Hospital Universitari Son Dureta, 07014 Palma de Mallorca, Illes Balears, Spain

³Unitat de Nefrologia (Departament Mèdic), Hospital Son Llàtzer, 07198 Palma de Mallorca, Illes Balears, Spain

Six mycobacterial isolates from haemodialysis water, conforming a homogeneous phylogenetic group in a previous multigenic analysis, were classified taxonomically. Physiological and biochemical characterization, as well as DNA–DNA hybridizations, demonstrated that the isolates were facultatively autotrophic, aerobic and hydrogen-oxidizing bacteria, representing a novel species in the rapid growing *Mycobacterium* group, for which the name *Mycobacterium llatzerense* sp. nov. is proposed. The type strain is MG13^T (=CECT 7273^T=CCUG 54744^T).

Many species of the genus *Mycobacterium* are widely distributed in the environment, and include organisms that might be pathogenic to humans and animals. When isolated from samples taken from patients, these organisms are referred to as non-tuberculous mycobacteria or simply environmental mycobacteria. Environmental mycobacteria are ubiquitous, and are found in many ecosystems, such as water, dust or soil. In hospitals, they occur mainly in aquatic environments and, because of their resistance to many disinfectants, they can survive and might be the cause of nosocomial outbreaks. In addition, they have been implicated increasingly in human diseases, including nosocomial infections, pulmonary and disseminated diseases in immunocompromised patients, and in a variety of other diseases. In the majority of these occurrences, water was proposed as a possible common vehicle of transmission. At the time of writing, the genus *Mycobacterium* comprised 130 species with validly published names (<http://www.bacterio.cict.fr>), 45 of which (35 %) have been described recently, since 2000, in fact. This high increase over the last few years may be due to the introduction of molecular techniques in taxonomy and the identification of isolates from environmental sources and clinical specimens.

Mycobacteria that are able to grow autotrophically with hydrogen have been reported several times in the literature (Eberhardt, 1965; Aragno & Schlegel, 1992) and were

classified as members of the species *Mycobacterium fortuitum* (Lukins and Foster, 1963), *Mycobacterium gordonae* (Bojalil *et al.*, 1962), *Mycobacterium marinum* (Eberhardt, 1965) and *Mycobacterium phlei*, or as other *Mycobacterium* species that are no longer recognized in bacterial taxonomy, namely '*Mycobacterium album*' or '*Mycobacterium citreum*' (Eberhardt, 1965), but have not been classified using modern taxonomical tools. The ability to grow aerobically with carbon monoxide as the only carbon and energy source has also been described recently for many mycobacteria (Park *et al.*, 2003).

In a previous environmental study, 60 isolates from a total of 203 obtained using R2A medium (Reasoner & Geldreich, 1985) from different sampling points of a water distribution system in a haemodialysis unit in the Hospital Son Llàtzer (Mallorca, Spain) were affiliated to the genus *Mycobacterium* (Gomila *et al.*, 2005), by employing partial 16S rRNA gene sequencing. The use of this phylogenetic marker has not been effective in the discrimination of strains at species level, making a phylogenetic multigenic sequence analysis approach necessary, using seven house-keeping genes (16S rRNA gene, ITS1, *gyrB*, *hsp65*, *recA*, *rpoB* and *sodA*). A multilocus sequence typing approach was performed on 20 isolates, leading to the differentiation of five distinct groups (Gomila *et al.*, 2007). On the basis of the genetic analysis, DNA–DNA hybridization and the phenotypic characteristics, including biochemical tests and antimicrobial susceptibility analysis, we concluded that six of the 20 isolates made up a homogeneous group of strains representing a putative novel species. One of these isolates, designated strain MG13^T, was deposited in the Spanish

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene nucleotide sequences determined in this study for the novel strains are AJ746069–AJ746073 and AJ746075 and that for *Mycobacterium mucogenicum* CCUG 47451 is AM902750.

In the multigenic sequence analysis, we demonstrated that the six mycobacterial isolates studied (MG12, MG13^T, MG14, MG15, MG16 and MG18) were closely related phylogenetically to *Mycobacterium aubagnense* CCUG 50186^T, *Mycobacterium mucogenicum* CCUG 47451 and *Mycobacterium phocaicum* CCUG 50185^T at consensus distances around 94.2, 94.5 and 94.8 %, respectively (Gomila *et al.*, 2007). All strains were included in the phenotypic and biochemical analysis. Strains were routinely cultured in R2A medium incubated for 3–4 days at 30 °C.

trophically in the presence of hydrogen and CO₂, but not when a mixture of carbon monoxide and O₂ was given in the gas phase. Additional biochemical tests were performed by inoculating API Coryne and API 20E (bioMérieux) strips according to the manufacturer's instructions, with an incubation time of 3 days at 30 °C. The results are shown in Table 1. Characteristics that differed from those of other closely related species included the ability to hydrolyse Tween 80 and gelatin, nitrate reduction, citrate utilization and hydrolysis of urea. All strains grew well at 22 and 30 °C, but no growth was observed at 37 °C. Pyrrolidonyl arylamidase, α -glucosidase and catalase tests were negative, and alkaline phosphatase was positive. Phenotypically, *M. aubagnense* was the most closely related species.

Antibiotic susceptibility testing was also performed (Table 2). Mycobacterial suspensions of the isolates were prepared by emulsifying colonies grown on R2A plates in 5 ml sterile distilled water, to achieve a density equal to a 1.0 McFarland turbidity standard by visual examination. The suspensions were mixed vigorously on a vortex mixer for 20 s and then inoculated on the entire surface of a Mueller–Hinton agar plate. The minimum inhibitory concentrations (MICs) of amoxicillin/clavulanate, cefotaxime, ceftazidime, ciprofloxacin, clarithromycin, clindamycin, cotrimoxazole, erythromycin, imipenem, linezolid, meropenem, metronidazole, minocycline, penicillin, teicoplanin, tobramycin and vancomycin, were determined by incubation with the respective E-test (AB Biodisk, Sweden) at 30 °C for 3 days. The MIC of the antimicrobial agents tested was determined according to the breakpoints recommended by the NCCLS (NCCLS, 2002, 2003) and

Strains: 1, *M. llutzerense* MG12; 2, *M. llutzerense* MG13^T; 3, *M. llutzerense* MG15; 4, *M. llutzerense* MG16; 5, *M. llutzerense* MG18; 6, *M. llutzerense* MG14; 7, *M. aubagnense* CCUG 50186^T; 8, *M. mucogenicum* CCUG 47451; 9, *M. phocaicum* CCUG 50185^T. +, Positive; −, negative; w, weak growth; ND, no data available. Results given in parentheses were taken from Adékambi *et al.* (2006). All strains are positive for growth at 22 and 30 °C, acetoin production (Voges–Proskauer) and β-glucosidase (aesculin hydrolysis) and negative for growth at 45 °C and in 5% (w/v) NaCl, pigmentation, semiquantitative catalase, β-galactosidase, growth on MacConkey agar without crystal violet, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, indole production, tryptophan deaminase, H₂S production, pyrrolidonyl arylamidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase and fermentation/oxidation of amygdalin, arabinose, glucose, glycogen, inositol, maltose, mannitol, melibiose, ribose, sucrose, sorbitol and xylose.

[illegible]

Table 2. Antimicrobial susceptibility of the novel isolates (*Mycobacterium llatzerense* sp. nov.) and closely related *Mycobacterium* species

Strains: 1, *M. llatzerense* MG12; 2, *M. llatzerense* MG13^T; 3, *M. llatzerense* MG15; 4, *M. llatzerense* MG16; 5, *M. llatzerense* MG18; 6, *M. llatzerense* MG14; 7, *M. aubagnense* CCUG 50186^T; 8, *M. mucogenicum* CCUG 47451; 9, *M. phocaicum* CCUG 50185^T. Values are MIC ($\mu\text{g ml}^{-1}$). AMC, Amoxicillin/clavulanate. Data in parentheses were taken from Adékambi *et al.* (2006).

Antimicrobial	1	2	3	4	5	6	7	8	9
AMC	0.125	0.125	0.125	0.125	0.125	0.125	0.5 (<4)	1	2 (>4)
Amikacin	0.125	0.125	0.125	0.125	0.064	0.125	1 (3)	1.5	1 (1.5)
Cefotaxime	>32	>32	>32	>32	>32	>32	>32 (64)	>32	>32 (32)
Ceftazidime	>256	>256	>256	>256	>256	>256	>256	>256	>256
Ciprofloxacin	<0.004	<0.004	<0.004	<0.004	<0.004	0.012	0.064 (0.125)	1	0.50
Clarithromycin	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	0.064 (0.125)	0.032	0.064
Clindamycin	0.5	4	0.5	1	0.5	4*	1	64	>256
Cotrimoxazole	0.016	0.064	0.004	0.016	0.016	0.064	0.016	0.125	0.5
Erythromycin	0.25	0.25	0.125	0.125	0.25	0.5	0.5 (1)	1	2 (3)
Imipenem	1	0.5	1	1	0.5	1	1 (0.75)	1	0.50
Linezolid	0.25	0.5	0.125	0.125	0.5	0.25	0.125	1	12
Meropenem	1	0.5	1	1	1	1	>32	12	2
Metronidazole	>256	>256	>256	>256	>256	>256	>256	>256	>256
Minocycline	<0.016	0.064	<0.016	<0.016	<0.016	<0.016	32 (1.5)	0.016	128 (0.75)
Penicillin	>32	>32	>32	>32	>32	>32	>32	>32	>32
Teicoplanin	16*	64*	32*	64*	16*	64*	64	256	>256
Tobramycin	0.25	0.5	0.25	0.5	0.5	0.25	2 (<4)	8	4 (>8)
Vancomycin	32*	64*	32*	32*	64*	128*	>256	32	>256

*Indicates the presence of trailing.

those proposed by Brown-Elliott & Wallace (2002). The novel isolates were susceptible to ciprofloxacin, clarithromycin and minocycline, differentiating them from the other species studied. All were resistant to ceftazidime, cefotaxime, metronidazole and penicillin.

DNA of the six phenotypically identical isolates was extracted from colonies grown on R2A medium for 3 or 4 days at 30 °C, as described previously (Gomila *et al.*, 2005). Amplification and sequencing of the complete 16S rRNA genes was performed using primers 16F27, 16F357, 16F610 and 16R1492, according to the procedure described by Gomila *et al.* (2005). The gene sequences were aligned with closely related sequences retrieved from the BLAST nucleotide sequence database. The alignment was done using a hierarchical method for multiple alignments, implemented in the program CLUSTAL_X (Thompson *et al.*, 1997). Sequences that were aligned automatically were checked manually. Evolutionary distances, derived from sequence-pair dissimilarities [Jukes and Cantor correction (Jukes & Cantor, 1969)] were calculated using the program DNADIST included in the Phylogenetic Inference Package [PHYLIP version 3.5c (Felsenstein, 1989)]. A phylogenetic tree was constructed using the neighbour-joining distance method (Fig. 1). Bootstrap analysis (based on 1000 replicates) was done using the PHYLIP package; only values greater than 700 are indicated. Topologies of the trees were visualized with the program TreeView (Page, 1996). The 16S rRNA gene sequences of the novel *Mycobacterium*

strains were identical and had a similarity of 98.7 % (18 bp differences in 1380 bp) with the sequence of *M. aubagnense* CCUG 50186^T, and around 98 % with the other type strains in the same phylogenetic branch: 98.6 % similarity (20 bp differences in 1380 bp) with *M. mucogenicum* CCUG 47451 and 98.2 % (24 bp differences in 1380 bp) with *M. phocaicum* CCUG 50185^T.

A multilocus sequence typing study including ITS1, *gyrB*, *hsp65*, *recA*, *rpoB* and *sodA* genes was undertaken previously (Gomila *et al.*, 2007). The *gyrB* and *recA* genes allowed a better discrimination of the novel isolates from closely related type strains. The *gyrB* gene of the novel strains showed 87 % similarity with *M. aubagnense* CCUG 50186^T, 89 % with *M. phocaicum* CCUG 50185^T and 90 % with *M. mucogenicum* CCUG 47451. *recA* gene similarities were 92.8 % with *M. aubagnense* CCUG 50186^T and 94 % with *M. mucogenicum* CCUG 47451 and *M. phocaicum* CCUG 50185^T. *sodA* gene similarities were 94.7 % with *M. aubagnense* CCUG 50186^T and 97 % with the other two closely related strains, and similarities for the *hsp65* gene were around 95 % between the novel strains and the closely related strains. *rpoB* gene similarities were 95 % with *M. aubagnense* CCUG 50186^T and 96 % with the other two closely related strains, *M. mucogenicum* CCUG 47451 and *M. phocaicum* CCUG 50185^T.

In several bacterial species, a correlation between multigene analysis and levels of DNA–DNA relatedness have been

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of the novel strains and other related, rapidly growing mycobacterial species as determined by comparison of over 1380 aligned bases. The tree was constructed using the neighbour-joining method, following distance analysis calculated by using the method of Jukes & Cantor (1969). The topology of the tree was visualized with the program TreeView (Page, 1996). Bootstrap values greater than 700 (based on 1000 replications) are indicated as percentages at branch points. GenBank accession numbers are given in parentheses. Bar, 0.01 nucleotide substitutions per site.

demonstrated and their study has been encouraged by Stackebrandt *et al.* (2002). Therefore, total DNA was isolated from cells grown in 1 l R2A broth using a method modified according to Marmur (1961). Briefly, to achieve better lysis, pelleted cells were boiled for 20 min and sonicated (10 cycles of 20 s at 22 microns amplitude and cooling for 5 s). DNA–DNA hybridizations were performed in duplicate using a non-radioactive method, as described by Ziemke *et al.* (1998). Reference DNAs of strains *M. aubagnense* CCUG 50186^T, *M. bolletii* CCUG 50184^T and strain MG13^T were double-labelled with DIG-11-dUTP and biotin-16-dUTP

using a Nick Translation kit (Boehringer Mannheim). Labelled DNA was hybridized with DNA of strains MG12, *M. mucogenicum* CCUG 47451 and *M. phocaicum* CCUG 50185^T. The level of relatedness of strains MG13^T and MG12 with other type strains of the genus *Mycobacterium* tested was less than 50 %, in all cases, and was 100 % between strains MG13^T and MG12, indicating that a new genomic group could be delineated in the genus.

Based on the phylogenetic and phenotypic analyses, we conclude that the isolates MG12, MG13^T, MG14, MG15,

MG16 and MG18 represent a novel species in the genus *Mycobacterium*, for which the name *Mycobacterium llatzerense* is proposed.

Description of *Mycobacterium llatzerense* sp. nov.

Mycobacterium llatzerense (l.la.tze.ren'se. N.L. neut. adj. *llatzerense* pertaining to Hospital Son Llàtzer, the hospital where the strains were isolated).

Acid-fast, Gram-positive bacilli, irregular, with frequent V forms during cell division. Aerobic, non-fermentative. Colonies are non-pigmented and grow on R2A medium, 5% sheep blood agar, Middlebrook 7H10 agar and Löwenstein–Jensen slants, in 3–4 days, at temperatures between 22 and 30 °C. No growth is observed at 37 °C or 45 °C, nor with 5% (w/v) NaCl. Negative for catalase activity (catalase at 68 °C and semiquantitative), and positive for hydrolysis of Tween 80 within 5 days. Cannot hydrolyse urea. Negative for citrate utilization, H₂S production, gelatinase, nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, β -glucuronidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase, and positive for alkaline phosphatase, aesculin hydrolysis and acetoin production (Voges–Proskauer). Susceptible to ciprofloxacin, clarithromycin and minocycline and resistant to cefotaxime, ceftazidime, metronidazole and penicillin.

The type strain, MG13^T (=CECT 7273^T=CCUG 54744^T), was isolated from a pure water distribution system of a haemodialysis unit in the Hospital Son Llàtzer, Mallorca, Spain.

Acknowledgements

This work was supported in part by the CICYT (Spain) and FEDER grant REN2002-04035-CO3-01 and by the I Pla Balear de Recerca i Desenvolupament Tecnològic de les Illes Balears. M.G. was the recipient of a predoctoral fellowship from the Spanish Ministerio de Educación y Ciencia. We are grateful to the CCUG for providing type strains of *Mycobacterium*. We also thank Professor Ortwin Meyer from the Bayreuth University and Professor Botho Bowien from the University of Göttingen, for their help in testing autotrophic growth on H₂ and CO.

References

Adékambi, T., Berger, P., Raoult, D. & Drancourt, M. (2006). *rpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. *Int J Syst Evol Microbiol* **56**, 133–143.

Aragno, M. & Schlegel, H. G. (1992). The mesophilic hydrogen-oxidizing (Knallgas) bacteria. In *The Prokaryotes*, 2nd edn, vol. 1,

pp 344–384. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K. H. Schleifer. Berlin: Springer.

Bojalil, L. F., Cerbon, J. & Trujillo, A. (1962). Adansonian classification of mycobacteria. *J Gen Microbiol* **28**, 333–346.

Brown-Elliott, B. A. & Wallace, R. J., Jr (2002). Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev* **15**, 716–746.

Eberhardt, U. (1965). Die Anreicherung von Knallgasbakterien. In *Anreicherungskultur und Mutantenauslese*, pp 155–178. Edited by H. G. Schlegel. Stuttgart: Gustav Fischer (in German).

Felsenstein, J. (1989). PHYLIP – phylogeny inference package (version 3.2). *Cladistics* **5**, 164–166.

Gomila, M., Gascó, J., Busquets, A., Gil, J., Bernabeu, R., Buades, J. M. & Lalucat, J. (2005). Identification of culturable bacteria present in haemodialysis water and fluid. *FEMS Microbiol Ecol* **52**, 101–114.

Gomila, M., Ramirez, A. & Lalucat, J. (2007). Diversity of environmental *Mycobacterium* isolates from hemodialysis water as shown by a multigene sequencing approach. *Appl Environ Microbiol* **73**, 3787–3797.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Lukins, H. B. & Foster, J. W. (1963). Utilization of hydrocarbons and hydrogen by mycobacteria. *Z Allg Mikrobiol* **3**, 251–264.

Lutz, B. (1992). Identification tests for mycobacteria. In *Clinical Microbiology Procedures Handbook*, pp. 3.12.1–3.12.27. Edited by H. D. Isenberg. Washington, DC: American Society for Microbiology.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

NCCLS (2002). Performance standards for antimicrobial susceptibility testing. Twelfth Informational Supplement, M100-S12. Wayne, PA: NCCLS.

NCCLS (2003). Susceptibility testing of *Mycobacteria*, *Nocardia*, and other aerobic actinomycetes. Approved standard M24-A. Wayne, PA: NCCLS.

Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Park, S. W., Hwang, E. H., Park, H., Kim, J. A., Heo, J., Lee, K. H., Song, T., Kim, E., Ro, Y. T. & other authors (2003). Growth of mycobacteria on carbon monoxide and methanol. *J Bacteriol* **185**, 142–147.

Reasoner, D. J. & Geldreich, E. E. (1985). A new medium for the enumeration and subculture of bacteria from potable water. *Appl Environ Microbiol* **49**, 1–7.

Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Ziemke, F., Höfle, M. G., Lalucat, J. & Rosselló-Mora, R. (1998). Reclassification of *Shewanella putrefaciens* Owen's genomic group II as *Shewanella baltica* sp. nov. *Int J Syst Bacteriol* **48**, 179–186.