Mycobacterium canariasense sp. nov.

M. Soledad Jiménez,¹ M. Isolina Campos-Herrero,² Diana García,² Marina Luquin,³ Laura Herrera¹ and María J. García⁴

Correspondence

M. Soledad Jiménez
msjimenez@isciii.es

¹Laboratorio de Micobacterias, Servicio de Bacteriología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

²Servicio de Microbiología, Hospital de Gran Canaria Dr Negrin, Las Palmas de Gran Canaria, Spain

³Unitat de Microbiologia, Departament de Genetica i de Microbiologia, Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain

⁴Departamento de Medicina Preventiva, Facultad de Medicina, Universidad Autónoma de Madrid, Madrid, Spain

A novel rapidly growing, non-pigmented mycobacterium was isolated from blood samples obtained from 17 patients with febrile syndrome. Bacterial growth occurred at 30 and 37 °C on Löwenstein–Jensen medium and also on MacConkey agar without crystal violet. Strains contained α - and α' -mycolates in their cell wall. Sequence analysis of the *hsp65* and 16S rRNA genes identified the isolates as rapidly growing mycobacteria. Sequences of both genes were unique within the mycobacteria. DNA–DNA hybridization showed that the isolates had less than 15% reassociation with 13 other recognized rapidly growing mycobacteria. The name *Mycobacterium canariasense* sp. nov. is proposed for this novel opportunistic pathogen, which is most closely related to *Mycobacterium diernhoferi*. The type strain is 502329^T (= CIP 107998^T = CCUG 47953^T).

Rapidly growing mycobacteria are ubiquitous environmental bacteria commonly found in water and soil (Brown-Elliott & Wallace, 2002). Members of this group, especially the three major species *Mycobacterium fortuitum*, *Mycobacterium chelonae* and *Mycobacterium abscessus*, have been reported as aetiological agents of a variety of infections, including bacteraemia and disseminated disease in patients with long-term venous catheters (Raad *et al.*, 1991). They have also been involved in nosocomial outbreaks or pseudo-outbreaks related to contamination of hospital water supplies and reagents (Ashford *et al.*, 1997; Chadha *et al.*, 1998; LaBombardi *et al.*, 2002). In this study, a novel mycobacterium involved in nosocomial infection is described; the name proposed for this species is *Mycobacterium canariasense* sp. nov.

During the period January 2000 to September 2002, a mycobacterium that could not be identified by conventional

procedures was isolated from blood specimens of 17 patients with suspected nosocomial acquisition. Patients were admitted to a tertiary care hospital in the Canary Islands, Spain. Most of them ($n=15$) had a malignant disease and all of them carried at that time a central venous catheter. Mycobacteria were considered to be the cause of the febrile syndrome in 12 cases. Ten patients were treated with specific antibiotic therapy and the central catheter was removed in nine of them. Catheters were left in two patients, who later relapsed. The same mycobacterium grew in their subsequent blood cultures. Most of the patients recovered after treatment. However, two patients died as a consequence of their disease status. All bacterial isolates had homogeneous biochemical characteristics and antimicrobial susceptibility patterns. The first isolate and four other randomly selected strains were sent to the reference Laboratory of Mycobacteria (Centro Nacional de Microbiología, Madrid, Spain) for identification. These five isolates were examined for several phenotypic and genotypic characteristics and results were compared with those displayed by other rapidly growing mycobacterial species.

Acid–alcohol-fastness, colony morphology and pigment production, as well as the ability to grow at various temperatures (22–45 °C), on Löwenstein–Jensen medium (LJ), in the presence of 5% NaCl and on MacConkey agar without crystal violet (Kubica & David, 1980) were examined. A total of 12 biochemical tests was performed

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *hsp65* genes of *Mycobacterium canariasense* strain 502329^T are AY255478 and AY255477, respectively.

Some characteristics of strain 502329^T (Table A), TLC of methyl mycolates of *M. canariasense* strains and related *Mycobacterium* species (Fig. A), GC analysis of fatty acid methyl esters of strain 502329^T (Fig. B), hybridization dot-blot assays (Fig. C) and DNA–DNA relatedness between strain 502329^T and other *Mycobacterium* species (Table B) are available as supplementary material in IJSEM Online.

(Marks & Trollope, 1960), including the use of single carbon sources and acid formation (Silcox *et al.*, 1981; Tsukamura, 1967). Hydrolysis of seven different amides was also tested (Vestal, 1975). Sensitivities to eight different antimycobacterial drugs were tested by the proportion method in LJ (Canetti *et al.*, 1963). E-test strips on Mueller–Hinton medium were used to determine sensitivity to 13 further antibiotics.

Bacterial cells were partially acid-fast rods that grew on LJ, initially as non-pigmented colonies, but later developing a more yellowish, smooth, moist and shiny appearance. Growth on LJ occurred after 2–3 days at 30 and 37 °C; growth did not occur at the other temperatures tested. The main phenotypic characteristics of isolates are indicated in Table 1 (see supplementary material in IJSEM Online for a complete set of data). All isolates of *M. canariensis* exhibited identical characteristics. The novel species could be differentiated from the *M. chelonae* group by single carbon source testing, from *M. fortuitum* and *M. diernhoferi* by the nitrate reduction test and from *Mycobacterium*

frederiksborgense and other related species by pigmentation development (Table 1).

Fatty and mycolic acids were liberated from each strain sample by saponification. These compounds were then extracted with diethyl ether and treated with diazomethane to obtain the methyl esters (Daffé *et al.*, 1983). Mycolic acid methyl esters were studied by analytical one-dimensional TLC using silica gel 60 TLC plates (Merck) as described previously (Luquín *et al.*, 1991a). Fatty acid methyl esters and methyl mycolate cleavage products were determined by GC-MS (Luquín *et al.*, 1991b).

TLC analysis of the mycolic acid methyl esters revealed that the five *M. canariensis* strains contained α - and α' -mycolates (see supplementary material available in IJSEM Online). To date, this mycolate pattern has only been described in *M. chelonae* and *M. abscessus* (see Table 1) (Hinrikson & Pfyffer, 1994) and species within the genus *Tsukamurella* (Hamid *et al.*, 1993). This mycolate pattern allows *M. canariensis* to be differentiated from related

Table 1. Phenotypic characteristics of *M. canariensis* and other rapidly growing mycobacteria

Species: 1, *M. canariensis*; 2, *M. abscessus*; 3, *M. chelonae*; 4, *M. fortuitum*; 5, *M. mucogenicum*; 6, *M. senegalense*; 7, *M. immunogenum*; 8, *M. neoaurum*; 9, *M. diernhoferi*; 10, *M. frederiksborgense*. –, Less than 15% of isolates are positive; +, more than 85% of isolates are positive; numbers in parentheses indicate percentages of positive strains; ND, not determined. Biochemical data corresponding to mycobacteria other than *M. canariensis* were taken from Schröder *et al.* (1997), Springer *et al.* (1995), Wayne & Kubica (1986), Willumsen *et al.* (2001) and Wilson *et al.* (2001).

Characteristic	1	2	3	4	5	6	7	8	9	10
Pigmentation	–	–	–	–	–	–	–	+	–	+
MacConkey agar*	+	+	+	+	ND	ND	+	–	ND	–
Growth on 5% NaCl	–	+	–	+	–	+	–	+	+	ND
Nitrate reductase	–	–	(17)	+	(45)	–	–	(80)	+	+
Tween hydrolysis	+	–	–	–	ND	ND	ND	(60)	–	+
Arylsulfatase (3 days)	+	+	+	+	(84)	+	+	–	–	ND
Iron uptake	–	–	–	+	–	–	–	+	ND	ND
Use as sole carbon source:										
Citrate	–	–	+	–	+	+	–	+	ND	ND
D-Mannitol	+	–	–	–	+	+	–	+	+	ND
<i>i</i> -myo-Inositol	+	–	–	–	–	ND	–	+	+	ND
Acid production from:										
Arabinose	+	–	–	–	ND	ND	ND	(80)	+	ND
Xylose	+	–	–	–	ND	ND	ND	(80)	+	+
Dulcitol	–	–	–	–	ND	ND	ND	–	ND	ND
Allantoinase	+	–	–	+	ND	+	ND	+	+	ND
Benzamidase	–	–	–	–	ND	+	ND	–	–	ND
iso-Nicotinamidase	–	–	–	–	ND	+	ND	–	–	ND
Succinidamidase	–	–	–	–	ND	ND	ND	–	–	–
Mycolic acid type†	I, II	I, II	I, II	I, II‡, V, VII‡	I, VI	I, II‡, V, VII‡	ND	I, IV, VI	I, IV, VI	I, IV, VI

*MacConkey agar without crystal violet.

†Mycolic acid type: I, α -mycolate; II, α' -mycolate; III, methoxy-mycolate; IV, keto-mycolate; V, epoxy-mycolate; VI, was esters; VII, ω -1 methoxy-mycolate.

‡May not be present in certain strains.

species such as *Mycobacterium mucogenicum* and *M. diernhoferi* (see Table 1; Muñoz *et al.*, 1997). GC-MS analysis of *M. canariasense* strains indicated the presence of fatty acid methyl esters with 14–24 carbon atoms, of which hexadecanoate ($C_{16:0}$) and octadecenoate ($C_{18:1}$) were the most prominent. All strains also showed appreciable amounts of tuberculostearate. The primary methyl ester derived from thermal cleavage of methyl mycolates was tetracosanoate ($C_{24:0}$; see supplementary material available in IJSEM Online), which allows *M. canariasense* to be differentiated from *Tsukamurella*. Species of this genus released C_{20} and C_{22} esters from pyrolysis of mycolates, but not C_{24} (Goodfellow *et al.*, 1978). Unlike *M. canariasense*, strains of *M. diernhoferi*, *M. frederiksbergense*, *M. mucogenicum* and *Mycobacterium neoaurum* revealed secondary alcohols by GC-MS analysis; thus, the absence of these compounds in *M. canariasense* strains enables them to be clearly differentiated from these closely related species.

PCR was performed on DNA isolated from bacterial LJ cultures using the boiling method and centrifugation. A 439 bp region of the *hsp65* gene was subjected to PCR amplification followed by restriction analysis using the primers and conditions described by Telenti *et al.* (1993). Amplicons were also sequenced in triplicate according to Ringuet *et al.* (1999) using the BigDye Terminator sequencing kit and the ABI Prism 3700 automated sequencer (Applied Biosystems). In addition, 1514 bp of the 16S rRNA gene were sequenced from PCR amplicons produced as described by Springer *et al.* (1995). Sequences of *hsp65* and 16S rRNA genes were aligned against previously described sequences from rapidly growing mycobacterial species, using EDITSEQ and MEGALIGN software (Lasergene MegAlign; DNASTar) (Altschul *et al.*, 1997). Sequences were clustered using CLUSTAL W and weightings were used to construct a phylogenetic dendrogram (Kimura, 1980).

The *hsp65* gene of *M. canariasense* strains contained a PCR-RFLP pattern that differed from those published

previously or compiled in the PRASite database (<http://www.hospvd.ch:8005>). Patterns consisted of two fragments of 325 and 130 bp by *Bst*EII digestion and three fragments of 140, 90 and 80 bp by *Hae*III digestion. The sequence of the *M. canariasense* hypervariable region (Ringuet *et al.*, 1999) was significantly different from those of other closely related rapidly growing species. Phylogenetic analysis of 441 bp of this gene demonstrated that *M. diernhoferi* and *M. mucogenicum* are the closest non-pigmented relatives (94.3 and 93.5 % similarity, respectively), whereas *M. neoaurum* and *M. frederiksbergense* were the most closely related pigmented species (94.4 and 93.6 % similarity, respectively) (Fig. 1).

Sequences of the 16S rRNA gene were also identical in all five novel isolates studied. A detailed comparison of the 16S rRNA gene sequence with sequences of other rapidly growing mycobacterial species demonstrated 100 % similarity in the genus-specific regions, but revealed a significant number of differences in helix 18 and the species-specific regions (namely hypervariable regions B and A, respectively) (Kirschner *et al.*, 1993). The complete sequence of the 16S rRNA gene of *M. canariasense* shows substantial differences when compared with those corresponding to other rapidly growing mycobacteria. Fig. 2 shows a comparison of the *M. canariasense* 16S rRNA gene sequence with 10 other closely related mycobacteria; *Tsukamurella paurometabola* was used as an outgroup. The dendrogram shows that *M. diernhoferi* is the closest non-pigmented relative (99.2 % similarity) to *M. canariasense*, followed by *M. mucogenicum* (97.0 %) and, as seen previously with the *hsp65* gene, *M. neoaurum* was the most closely related pigmented rapidly growing mycobacteria (98.7 %). The similarity between *M. canariasense* and *T. paurometabola* was 91.2 %.

Total genomic DNA was purified from liquid bacterial cultures, as described previously (Domenech *et al.*, 1994). DNA–DNA hybridization experiments were performed on membrane filters, using a novel dot-blot-based procedure. Genomic DNA from *M. canariasense* strain 502329^T

Fig. 1. Phylogenetic position of *M. canariasense* among other closely related, rapidly growing mycobacterial species as determined by *hsp65* gene sequences using the CLUSTAL W method with weightings. *M. tuberculosis* was used as outgroup. GenBank accession numbers are given in parentheses.

Fig. 2. Phylogenetic position of *M. canariensis* among other related rapidly growing mycobacterial species as determined by comparison of the 16S rRNA gene sequences. The tree was inferred using the CLUSTAL W method with weightings and rooted using *T. paurometabola* as outgroup. GenBank accession numbers are given in parentheses.

(300 ng) was labelled *in vitro* using the Megaprime DNA labelling system (Amersham) and 25 μ Ci (925 kBq) [α - 32 P]dCTP (Amersham). Portions (500 ng) of each unlabelled DNA were dot-blotted and bound to nylon membrane filters (Hybond-N+; Amersham). Hybridization and washes were carried out as described previously (Brown *et al.*, 1999), except that ECL buffer (Amersham) was used both for pre-hybridization and hybridization steps. The relative binding ratios (expressed as percentages) for each species were calculated from the counts of homologous bound DNA, as measured using an Instant Imager counter (Izasa Instruments). In addition, the blots were stripped and rehybridized with a PCR amplicon from the 16S rRNA gene as the new probe, as described by Domenech *et al.* (1997). This procedure has several advantages compared to previous methods (Keswani & Whitman, 2001), mainly because the amount of DNA fixed in each dot can be determined by measuring the amount of radioactivity when using the 16S rRNA gene as the second probe. Due to the high sequence conservation within mycobacterial species inherent within this probe (Menendez *et al.*, 2002), the radioactivity level can be considered to be proportional to the amount of target molecules on the filter. The number of copies of the 16S rRNA gene was already known for the mycobacterial species included in this work (Menendez *et al.*, 2002; M. J. Garcia, unpublished results). The levels of DNA–DNA hybridization between *M. canariensis* strains were greater than 90 %. On the other hand, the level of reassociation between the *M. canariensis* type strain and 13 other related rapidly growing species corresponded to less than 15 %. These results strongly suggest that our isolates represent a novel species (Lan & Reeves, 2001). Results of DNA–DNA hybridization analysis are available as supplementary material in IJSEM Online.

RFLP analysis of the 16S rRNA gene was also performed to complement DNA–DNA hybridization data (Domenech *et al.*, 1994, 1997; Brown *et al.*, 1999). A previously described experimental procedure (Domenech *et al.*, 1997) was used,

with the exception that ECL buffer (Amersham) was used for both the pre-hybridization and hybridization steps. Fig. 3 shows the RFLP patterns from *Bam*HI-digested

Fig. 3. RFLP patterns of 16S rRNA genes from *M. canariensis* strains and other rapidly growing mycobacterial species. Patterns were obtained using *Bam*HI as the restriction enzyme. Lanes: 1, *M. canariensis* 502329^T; 2, *M. canariensis* 513578; 3, *M. canariensis* 517062; 4, *M. canariensis* 552822; 5, *M. abscessus* ATCC 19977^T; 6, *M. fortuitum* subsp. *fortuitum* ATCC 6841^T; 7, *Mycobacterium goodii* ATCC 700504^T; 8, *Mycobacterium mageritense* ATCC 700351^T; 9, *M. mucogenicum* ATCC 49650^T; 10, *Mycobacterium porcinum* CIP 141460001=CIP 105392^T; 11, *Mycobacterium smegmatis* CIP 141330010=CIP 104444^T; 12, *Mycobacterium wolinskyi* ATCC 700009; 13, *N. asteroides* ATCC 3308. Fragment sizes (in kbp) are indicated on the left.

genomic DNAs from *M. canariasense* strains, eight other rapidly growing mycobacterial species and *Nocardia asteroides*. Patterns of different species were different, but the patterns of all four *M. canariasense* strains were identical. All species tested, with the exception of *M. abscessus*, produced a pattern with two bands, indicating the presence of two copies of the 16S rRNA gene. These results indicate that *M. canariasense* belongs to the II-s mycobacterial class, i.e. species with two *rrn* operons per genome and short helix 18 in the 16S rRNA gene coding region (Menendez *et al.*, 2002).

On the basis of biochemical characteristics, mycolic and fatty acid patterns, PCR-RFLP of the *hsp65* gene, sequences of conserved genes and DNA–DNA hybridization, *M. canariasense* is proposed as a novel rapidly growing mycobacterium, most closely related to *M. diernhoferi* and *M. chelonae*.

Description of *Mycobacterium canariasense* sp. nov.

Mycobacterium canariasense (ca.na.ri.a.sen'se. L. gen. adj. *canariasense* referring to the Latin adjective of the Spanish islands where all strains were isolated).

Cells are partially acid-fast rods. Visible growth appears in 2–3 days as smooth, moist, shiny, non-pigmented colonies on Löwenstein–Jensen medium. Growth occurs at 30 and 37 °C, but not at 22, 42 or 45 °C. Grows on MacConkey agar without crystal violet, but does not grow in the presence of 5 % NaCl. Positive for arylsulfatase activity (3 days) and Tween 80 hydrolysis. Produces a low level of heat-stable catalase and is negative for reduction of nitrates. These characteristics allow this novel species to be differentiated from other closely related species such as *M. diernhoferi* and *M. mucogenicum*. The inability to utilize citrate as a single carbon source allows differentiation from *M. chelonae*. Lipid composition of the cell wall is characterized by the presence of α - and α' -mycolates, similar to *M. chelonae* and *M. abscessus*. Results of DNA analyses such as PCR-RFLP of the *hsp65* gene, sequences of conserved genes and DNA–DNA hybridization define *M. canariasense* as a distinct genomic mycobacterial species most closely related to *M. diernhoferi* and *M. mucogenicum*. The 16S rRNA and *hsp65* gene sequences of *M. canariasense* are unique. This species belongs to the II-s mycobacterial class according to the classification of Menendez *et al.* (2002).

The type strain is 502329^T (=CIP 107998^T=CCUG 47953^T).

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