International Journal of Systematic Bacteriology (1998), **48**, 907–912 Printed in Great Britain

Gordonia rhizosphera sp. nov. isolated from the mangrove rhizosphere

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The taxonomic position of bacterial strain 141^T, isolated from the mangrove rhizosphere, has been clarified by phenotypic, chemotaxonomic and phylogenetic studies. The strain possesses wall chemotype IV, MK-9(H_2) as the predominant menaquinone, relatively long-chain mycolic acids (56–64 carbon atoms) and straight-chain saturated and monounsaturated fatty acids with a small amount of tuberculostearic acid. The G C content of the DNA is 66·8 mol%. Similarity values for genes encoding 16S rRNA indicated that strain 141^T represents a new species within the genus *Gordonia* for which the name *Gordonia rhizosphera* sp. nov. is proposed. The type strain of *G. rhizosphera* is 141^T (= IFO 16068^T).

Keywords: *Gordonia rhizosphera* sp. nov., mangrove rhizosphere

INTRODUCTION

Mangrove forests in Japan are mainly distributed in the Okinawa prefecture where complex environments have formed under the influence of tidal ebb and flow, the influx of fresh water, and high temperature and humidity in the subtropical and tropical climates. The soils in such environments are muddy and are reported to be anoxic, low in nutrients and to have higher concentrations of heavy metals and higher salinity than terrestrial soils (Ito & Nakagiri, 1997; Wakushima *et al.*, 1994).

Recently, Hatano (1997) examined actinomycete populations in rhizosphere soils of six kinds of mangrove forest on the Iriomote and Ishigaki islands in Japan and suggested that the mangrove rhizosphere is a good source for isolating new and diverse actinomycetes.

In the course of an ecological study of actinomycetes in mangrove rhizospheres, six colonies of similar appearance were isolated from the surface of fine roots (two colonies) and soil (four colonies) in the rhizosphere of *Bruguiera gymnorrhiza* Lamk. in the Shiira River estuary, Iriomote Island. Strain 141^T used in this study is representative of these isolates. This organism is a rod-shaped bacterium with all of the characteristic chemotaxonomic markers of the genus *Gordonia* (Stackebrandt *et al.*, 1988), including wall chemotype IV (*meso*-diaminopimelic acid, arabinose and galactose) (Lechevalier & Lechevalier, 1970), an MK-9(H_2)-type menaquinone and mycolic acids.

In this study we describe the morphological, physiological and genetic characteristics of strain 141^T (= IFO 16068^T) and propose that this strain represents a new species within the genus *Gordonia*, *Gordonia rhizosphera* sp. nov. This is the first report of the appearance of *Gordonia* species in the mangrove rhizosphere.

METHODS

Bacterial strain and cultivation. Samples were collected in late October 1995 from the rhizosphere of *Bruguiera gymnorrhiza* Lamk. in the Shiira River estuary, Iriomote Island, Japan. Strain 141^T (= IFO 16068^T) was isolated from soil and the surface of fine roots by the method described by Hayakawa & Nonomura (1987, 1989). *Gordonia aichiensis* IFO 16046^T (Klatte *et al.*, 1994), *Gordonia amarae* IFO 15530^T (Klatte *et al.*, 1994; Ruimy *et al.*, 1994), *Gordonia bronchialis* IFO 16047^T (Stackebrandt *et al.*, 1988), *Gordonia rubropertincta* IFO 16048^T (Stackebrandt *et al.*, 1988), *Gordonia sputi* IFO 16049^T (Riegel *et al.*, 1994; Stackebrandt *et al.*, 1988) and *Gordonia terrae* IFO 16050^T (Stackebrandt *et al.*, 1988) were used for comparison of physiological properties and for DNA–DNA hybridization tests. Each strain was cultivated aerobically at 28 °C in a peptone/yeast extract medium (PY medium) containing 1% peptone, 0·2% yeast extract, 0·2% NaCl and 0·2% D-glucose (pH 7·2). Cells used for biochemical tests were harvested by centrifugation during the stationary phase, washed with water and lyophilized.

Morphological and physiological property tests. Cell morphology was determined by phase-contrast microscopy following growth on PY agar. Motility was determined by

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Abbreviation: TBSA, tuberculostearic acid.

The DDBJ accession number for the 16S rDNA sequence of strain 141^T is AB004729.

the hanging drop method. Assimilation of organic acids was tested in a medium containing 0·5 % organic acid (sodium salt), 0·1 % NH_4NO_3, 0·1 % KH_2PO_4, 0·05 % $MgSO_4 . 7H_2O$ and 0·02 % KCl (pH 7·2). Acid production from carbohydrates was studied in a medium containing 1 % peptone, 0·5 % NaCl, 0·003 % bromocresol purple and 0·5 % carbohydrate (pH 7·2) (Cowan, 1974).

Cell wall analysis. Cell walls were prepared from about 500 mg (dry wt) bacterial cells as described by Schleifer & Kandler (1972). Amino acids in the acid hydrolysate of the cell walls were identified by HPLC as described by Yokota *et al.* (1993). Cell wall sugars were analysed as described by Mikami & Ishida (1983). The glycolyl test was performed by the method of Uchida & Aida (1977).

Cellular fatty acid analysis. Fatty acids were extracted from dried cells, purified and analysed by GC-MS with a GCMS-QP5000 spectrometer (Shimadzu) combined with a CLASS-5000 MS Workstation computer system. GLC analyses were performed with a GC-17A gas chromatograph (Shimadzu). A BPX70 capillary column (SGE) containing 70 % cyanopropyl-equivalent modified siloxane (50 m × 0·25 mm) was used at 80 °C for 2 min, 80–150 °C at 15 °C min^{-1}, 150–250 °C at 8 °C min^{-1} and then at 250 °C for 5 min with helium as carrier gas (1·4 ml min^{-1}).

Mycolic acid analysis. Mycolic acids were analysed by the method of Yano *et al.* (1978). The dried cells were hydrolysed with 25 % KOH/methanol for 16–18 h at 80 °C and mycolic acids were extracted with n-hexane after acidification to pH 2 with 6 M HCl. Trimethylsilyl (TMS) derivatives of methyl mycolates were analysed by GC (HP5890 series II; Hewlett-Packard) equipped with a flame ionization detector on a capillary column (15 m × 0·2 mm) coated with dimethyl silicone (SPB-1; Supelco) with helium as carrier gas (1 ml min^{-1}). The oven temperature was programmed from 300 to 350 °C at 4 °C min^{-1}, holding the final temperature for 60 min. TMS derivatives of methyl mycolates were identified by capillary GC-MS. GC-MS was performed on a JEOL SX102A mass spectrometer combined with an electron impact (EI) ion source.

DNA base composition. DNA was obtained by the method of Saito & Miura (1963). The G+C content of the DNA was determined by the method of Mesbah *et al.* (1989) after treatment with P1 nuclease and alkaline phosphatase, and by HPLC by using a model LC-6AD instrument (Shimadzu) equipped with a Cosmosil 5C18-AR column (150 × 4·6 mm; Nacalai Tesque) (Tamaoka & Komagata, 1984).

DNA–DNA hybridization. DNA–DNA hybridization was carried out fluorometrically in microdilution wells by using biotinylated DNA (Ezaki *et al.*, 1989).

Phylogenetic analysis. 16S rRNA-specific DNA was amplified by PCR (Hiraishi, 1992; Hiraishi *et al.*, 1994) and sequenced directly with the Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit with 7-deaza-dGTP (Amersham) and a Pharmacia Model ALF DNA Sequencer following the manufacturers' instructions. Nucleotide substitution rates (K_{nuc}; Kimura, 1980) were calculated by using the CLUSTAL V software package (Higgins *et al.*, 1992) and phylogenetic trees were constructed by the neighbour-joining method (Saitou & Nei, 1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Nucleotide sequence accession numbers. The DDBJ, GenBank and EMBL accession numbers for the 16S rDNA sequences used for comparison are as follows: *Arthrobacter globiformis* DSM 20124^T, M23411; *Brevibacterium linens* DSM 20425^T, X77451; *Corynebacterium amycolatum* NCTC 3224^T, X84244; *Corynebacterium bovis* NCTC 3224^T, X84444; *Corynebacterium diphtheriae* NCTC 11397^T, X84248; *Corynebacterium glutamicum* NCIB 10025^T, X84257; *Dietzia maris* DSM 43672^T, X79290; *Gordonia aichiensis* DSM 43978^T, X80633; *Gordonia amarae* DSM 43392^T, X80635; *Gordonia bronchialis* DSM 43247^T, X79287; *Gordonia hirsuta* DSM 44140^T, X93485; *Gordonia hydrophobica* DSM 44015^T, X87340; *Gordonia rubropertincta* DSM 43197^T, X80632; *Gordonia sputi* DSM 43896^T, X80634; *Gordonia terrae* DSM 43249^T, X79286; *Kineococcus aurantiacus* IFO 15268^T, AB007420; *Mycobacterium bovis* ATCC 12910^T, X55589; *Mycobacterium intracellulare* ATCC 15985, X52927; *Nocardia asteroides* ATCC 3306, X57949; *Rhodococcus fascians* DSM 43188, X53204; *Rhodococcus erythropolis* DSM 43066^T, X79289; *Rhodococcus rhodochrous* DSM 43241^T, X79288; *Skermania piniformis* (Chun *et al.*, 1997) IFO 15059^T, Z35435; *Terrabacter tumescens* NCIB 8914^T, X53215; and *Tsukamurella paurometabola* DSM 20162^T, X53206.

RESULTS

Population in mangrove rhizosphere

Six colonies were isolated from the rhizosphere soils of six types of mangrove forest on Iriomote and Ishigaki islands, Japan: two colonies from the surface of fine roots of *Bruguiera gymnorrhiza* Lamk. and four colonies from rhizosphere soils. All colonies were pink to orange with irregular margins and flat, rough surfaces on HV agar (Hayakawa & Nonomura, 1987) and PY agar. The actinomycete population of the colonies was about 5 % (6/117 colonies).

Chemotaxonomic characteristics

The results of this study are shown in Tables 1 and 2 along with data for related taxa. The cell wall of strain 141^T contained arabinose and galactose (molar ratio about 1:1) as major cell wall sugars and *meso*-diaminopimelic acid as the only diamino acid, properties characteristic of wall chemotype IV *sensu* Lechevalier & Lechevalier (1970). *N*-Glycolated muramic acid moieties of peptidoglycan were present. MK-9(H_2) was found to be the predominant menaquinone. Whole-organism fatty acids were mainly straight-chain, saturated and monounsaturated (27 % $C_{16:0}$, 13 % $C_{17:0}$, 27 % $C_{18:1}$) with 3 % tuberculostearic acid (10-methyl $C_{18:0}$, TBSA). As shown in Table 2, the amount of TBSA in strain 141^T was smaller than that in other species of the genus *Gordonia*, but it did not change with culture conditions (data not shown). The mycolic acids of strain 141^T, as determined from trimethylsilylated derivatives by high-temperature GC, were similar in size as described for members of the genus *Gordonia*, having an overall range of 56–64 carbon atoms (7 % C_{58}, 5 % C_{59}, 26 % C_{60}, 10 % C_{61}, 34 % C_{62}, 6 % C_{64} and traces of C_{56}, C_{57} and C_{63}) with a mean chain length of between 60 and 62 carbon atoms. The mycolic acids were mainly composed of C_{42-46}-dienoic or -trienoic *β*-units and C_{16-18}-saturated

Table 1. Differential characteristics of *G. rhizosphera* and related taxa containing mycolic acids

Data are from this and other studies (Chun *et al*., 1997; Collins & Cummins, 1986; Goodfellow, 1992; Rainey *et al*., 1995).

Taxon	**Aerial hyphae**	**Predominant menaquinone**	**G+C content (mol %)**	**Fatty acid composition***	**No. of carbon atoms in mycolic acids**	**Acyl group of muramic acid**
Gordonia rhizosphera	−	MK-9(H_2)	66·8	S, U, TBSA	56–64	*N*-Glycolated
Corynebacterium	−	MK-8(H_2) or MK-9(H_2)	51–67	S, U, (TBSA)†	22–36	*N*-Acetylated
Dietzia	−	MK-8(H_2)	73	S, U, TBSA	34–38	*N*-Acetylated
Gordonia	−	MK-9(H_2)	63–69	S, U, TBSA	48–66	*N*-Glycolated
Mycobacterium	−	MK-9(H_2)	61–71	S, U, TBSA	60–90	*N*-Glycolated
Nocardia	+	MK-8(H_4, ω-cycl)	64–72	S, U, TBSA	44–60	*N*-Glycolated
Rhodococcus	−	MK-8(H_2)	63–73	S, U, TBSA	34–52	*N*-Glycolated
Skermania	+	MK-8(H_4, ω-cycl)	73·5	S, U, TBSA	58–64	*N*-Glycolated
Tsukamurella	−	MK-9	67–68	S, U, TBSA	64–68	*N*-Glycolated

* S, Saturated; U, monounsaturated.

† TBSA is present in *Corynebacterium ammoniagenes*, *Corynebacterium bovis*, *Corynebacterium cystitidis*, *Corynebacterium minutissimum*, *Corynebacterium pilosum*, *Corynebacterium urealyticum* and *Corynebacterium variabilis* (Herrera-Alcaraz *et al*., 1990; Pitcher *et al*., 1992).

Table 2. Cellular fatty acids of *G. rhizosphera* and type strains of *Gordonia* species

Strain	**Cellular fatty acid composition (%)**								
	n-14:0	**n-15:0**	**n-16:1**	**n-16:0**	**n-17:1**	**n-17:0**	**n-18:1**	**n-18:0**	**TBSA**
G. rhizosphera IFO 16068^{T}	3	4	4	27	7	13	27	11	3
G. aichiensis IFO 16046^{T}	8	7	8	31		3	5	2	25
G. amarae IFO 15530^{T}			9	36			15	20	20
G. bronchialis IFO 16047^{T}	9	5	4	47		14	6	6	9
G. hirsuta DSM 44140^{T}*	2		17†	30	1‡	1	30§	3	17
G. hydrophobica DSM 44015^{T}*	1	4	13	27	4‡	4	14§		17
G. rubropertincta IFO 16048^{T}			15	37			19	9	19
G. sputi IFO 16049^{T}	2	2		44	2	2	29		15
G. terrae IFO 16050^{T}	4	1	10	32			16	19	18

* Data from Klatte *et al*. (1994, 1996).

† Total amount of n-16:1*cis*9 and n-16:1*cis*10.

‡ n-17:1*cis*9.

§ n-18:1*cis*9.

α-units. The G+C content of the DNA was 66·8 mol %.

Physiological characteristics

Strain 141^{T} was a Gram-positive, non-motile, strictly aerobic and rod-shaped bacterium. Pink to orange colonies with irregular margins and flat, rough surfaces were formed on various media. Mycelium was not formed. The strain was catalase-positive and oxidase-negative. Cells were 0·2–0·4 μm wide and 0·4–1·0 μm long. L-Alanine, citrate and gluconate were utilized, but the following carbon substrates were not used or used weakly: galactose, inositol, rhamnose, ribose, sucrose, aspartate, benzoate, propionate, succinate, leucine, proline, L-valine, acetamide and *N*-acetylglucosamine. Acids were not produced from galactose, inositol, sorbitol, sucrose or trehalose, but were produced weakly from rhamnose. Table 3 shows some of the diagnostic and differentiating characteristics of strain 141^{T} and other species of the genus *Gordonia*.

Analysis of 16S rDNA

The nucleotide sequence of 16S rDNA, enzymically amplified from strain 141^{T}, was determined by direct automated sequencing. A total of 1475 nt were sequenced and the primary structure was aligned with

Table 3. Diagnostic and differentiating characteristics of *G. rhizosphera* and other species of the genus *Gordonia*

Data are from other studies (Bendinger *et al.*, 1995; Klatte *et al.*, 1994, 1996; Riegel *et al.*, 1994; Ruimy *et al.*, 1994; Stackebrandt *et al.*, 1988).

Characteristic	*G. rhizosphera*	*G. aichiensis*	*G. amarae*	*G. bronchialis*	*G. hirsuta*	*G. hydrophobica*	*G. rubropertincta*	*G. sputi*	*G. terrae*
Colour of colonies	Pink to orange	Pinkish to orange	Tannish to white	Pink to red	White to light yellow	Tannish to white	Pink to red	Salmon	Pink to orange
Assimilation of:*									
Galactose	−	+	−	−	ND	+	+	+	+
Inositol	−	−	−	+	+	−	ND	−	−
Rhamnose	+w	−	−	v	−	−	v	−	v
Ribose	−	−	+	−	−	−	+	−	−
Sucrose	−	−	+	+	−	+	+	−	+
Aspartate	−	+	−	−	−	−	−	−	−
Benzoate	−	−	−	−	−	−	+	−	+
Citrate	+	+	+	−	−	+	−	+	+
Gluconate	+	−	−	+	−	−	+	−	−
Succinate	−	−	−	+	−	+	−	+	−
L-Alanine	+	+	−	+	−	−	+	−	−
Leucine	+w	−	−	+	+	−	−	−	−
Proline	+w	−	+	+	−	+	−	−	−
L-Valine	+w	−	+	+	−	+	−	−	−
Acetamide	−	−	−	v	−	+	−	−	−
Acid produced from:*									
Rhamnose	+w	ND	ND	+	ND	ND	ND	ND	−
Trehalose	−	ND	ND	+	ND	ND	ND	ND	+
Mean no. of carbon atoms in mycolic acids	60–62	62	50–52	64	60–64	58–60	58–59	61–62	58
Presence of 16:1 fatty acid	+	+	+	+	+	+	+	−	+

* +, Positive reaction; −, negative reaction; +w, weakly positive reaction; v, variable reaction; ND, not determined.

those of 25 representative members of wall chemotype IV taxa, containing mycolic acids, and other related organisms. Sequence similarity calculations were based on 1077 nt, because only partial sequences were available in some of the reference strains. A phylogenetic tree (Fig. 1) was constructed on the basis of the distance matrix data. It showed that strain 141^T formed a coherent cluster with species of the genus *Gordonia* and represents a distinct lineage within the genus. The closest related species was *G. bronchialis* and the level of 16S rDNA sequence similarity between strain 141^T and *G. bronchialis* was 99·2 % (data not shown).

DNA–DNA hybridization

The levels of DNA–DNA relatedness between strain 141^T and *G. bronchialis* IFO 16047^T and between these strains and other species of the genus *Gordonia* were 29–31 % and 7–29 %, respectively (Table 4).

DISCUSSION

From the results of this work, it is evident that strain 141^T represents a new *Gordonia* species. It has all of the characteristic chemotaxonomic markers of this genus, including an MK-9(H_2)-type menaquinone, mycolic acids and straight-chain saturated and unsaturated fatty acids together with TBSA, and it can be differentiated from all previously described *Gordonia* species on the basis of physiological properties and fatty acid profiles, as well as DNA–DNA hybridization data. We propose that strain 141^T (= IFO 16068^T) should be classified as a new species of the genus *Gordonia*, *Gordonia rhizosphera* sp. nov.

Eight species have hitherto been accommodated within the genus *Gordonia*. They are widely distributed in various environments, such as human sputum (Klatte *et al.*, 1994; Riegel *et al.*, 1994; Stackebrandt *et al.*, 1988), activated-sludge sewage-treatment plants (Lemmer & Kroppenstedt, 1984), fibre bark compost of a large-scale biofilter used for biological odour abatement of biofilters for waste gas treatment (Bendinger *et al.*, 1995; Klatte *et al.*, 1996) and soil (Klatte *et al.*, 1994; Stackebrandt *et al.*, 1988). *G. rhizosphera* was isolated from the surface of fine roots or soil in the mangrove rhizosphere. This is the first report of the appearance of *Gordonia* species in the mangrove rhizosphere, where complex environments are formed involving muddy, anoxic conditions and lower nutrient levels than terrestrial soils (Wakushima *et al.*, 1994).

Description of *Gordonia rhizosphera* sp. nov.

Gordonia rhizosphera (rhi.zo′spher.a. L. fem. m. *rhizo* root; Gr. adj. *sphera* sphere; L. fem. adj. *rhizosphera* within the sphere of the root).

Cells are Gram-positive, non-motile rods, 0·2–0·4 μm wide and 0·4–1·0 μm long. Rough, pink to orange colonies are formed on PY agar or HV agar and no aerial mycelia or diffusible pigments are produced. Catalase-positive and oxidase-negative. L-Alanine, citrate and gluconate are utilized, but the following

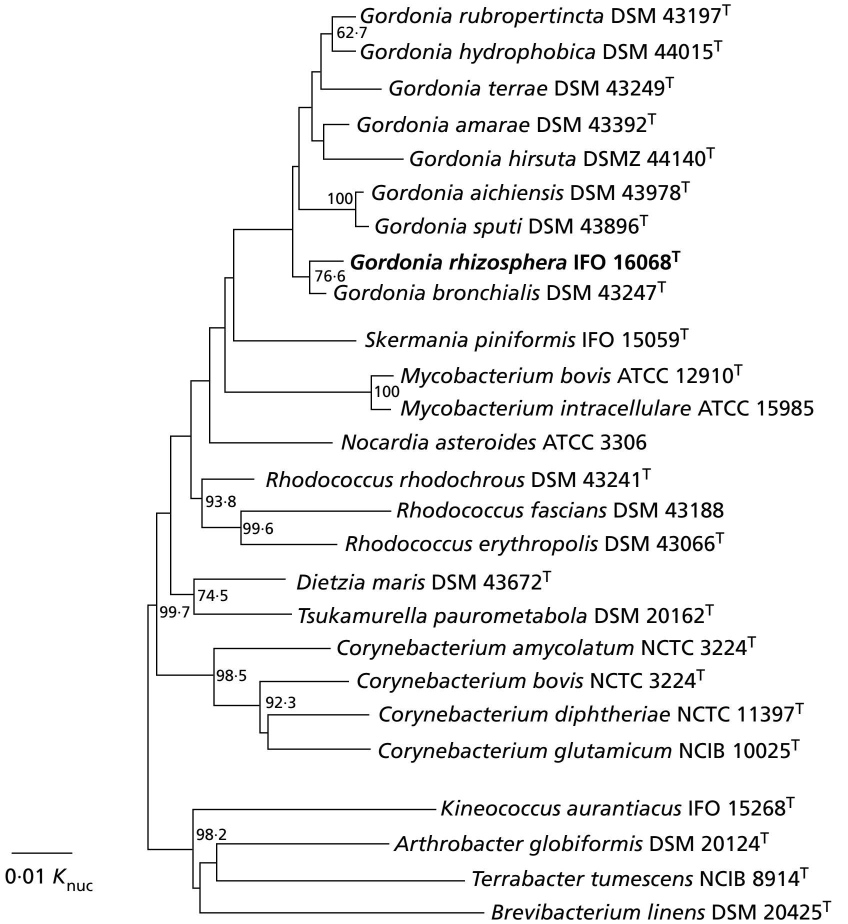

Fig. 1. Unrooted phylogenetic tree displaying the relationship of *G. rhizosphera* strain 141^T (= IFO 16068^T) to reference organisms. The numerals indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches (Felsenstein, 1985).

Table 4. DNA–DNA homology values

Species	IFO no.	Percentage reassociation with:	
		IFO 16068^T	IFO 16047^T
G. rhizosphera	16068^T	100	30
G. aichiensis	16046^T	30	28
G. amarae	15530^T	10	23
G. bronchialis	16047^T	31	100
G. rubropertincta	16048^T	11	10
G. sputi	16049^T	10	12
G. terrae	16050^T	13	7

carbon substrates are not used or used weakly: galactose, inositol, rhamnose, ribose, sucrose, aspartate, benzoate, propionate, succinate, leucine, proline, L-valine, acetamide and *N*-acetylglucosamine. Acids are not produced from galactose, inositol, sorbitol, sucrose or trehalose, but are produced weakly from rhamnose. Cell wall is of chemotype IV *sensu* Lechevalier & Lechevalier (1970), containing *meso*-diaminopimelic acid, arabinose and galactose. *N*-Glycolated muramic acid moieties of peptidoglycan are present. Predominant isoprenoid quinone is menaquinone MK-9(H_2). Mycolic acids with 56–64 carbon atoms are present. Major amounts of straight-chain, saturated and monounsaturated fatty acids and TBSA are found. G+C content of the DNA is 66·8 mol%. Habitat is mangrove rhizosphere. Type strain is strain 141^T (= IFO 16068^T).

ACKNOWLEDGEMENTS

We thank Ikuya Yano and Yukiko Nishiuchi, Osaka City University Medical School, for help in analysing mycolic acids and valuable suggestions. This work was supported by Fujiwara Natural History Foundation.

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