

Transfer of *Rhodococcus aichiensis* Tsukamura 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to the Genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov.

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We investigated the taxonomic status of *Rhodococcus aichiensis* DSM 43978^T (T = type strain) and *Nocardia amarae* DSM 43392^T by using both chemotaxonomic and genetic methods. The occurrence of MK-9(H₂) as the predominant menaquinone, the presence of relatively long-chain mycolic acids (48 to 62 carbon atoms), and the phylogenetic position as determined by a 16S ribosomal DNA sequence analysis provide strong evidence that both *R. aichiensis* and *N. amarae* should be transferred to the genus *Gordona* as *Gordona aichiensis* and *Gordona amarae*, respectively.

The taxonomy of actinomycetes containing mycolic acids (high-molecular-weight α -branched 3-hydroxy fatty acids) has been markedly changed by the use of numerical taxonomic, chemical, and molecular systematic methods (3, 4). Although such investigations have revealed several morphological and chemical markers that distinguish members of this suprageneric group (18, 24–26), which comprises the genera *Corynebacterium*, *Gordona*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, and *Tsukamurella*, there are still a few taxa whose classification is uncertain. Two such taxa, *Nocardia amarae* (16) and *Rhodococcus aichiensis* (27), have been retained in the genera *Nocardia* and *Rhodococcus*, respectively; however, the current affiliations of these species are in some respects contradictory to the diagnostic characteristics of the respective type species. Several analyses of the type strain of *N. amarae* have revealed notable differences between this organism and authentic nocardiae with respect to sensitivity to nocardiphages (28) and lysozyme (5, 16), serological cross-reactions (22), and the presence of MK-9(H₂) as the major menaquinone (5). Furthermore, the results of rRNA-DNA pairing experiments (18) and numerical phenetic analyses (5, 19) have revealed that there is only a loose relationship between *N. amarae* and the genus *Nocardia* sensu stricto. Classification of *R. aichiensis* in the genus *Rhodococcus* is also open to question. The occurrence of MK-9(H₂) as the predominant isoprenolog and mycolic acids having an average chain length of 62 carbon atoms clearly distinguishes *R. aichiensis* from all other rhodococci except *Rhodococcus chubuensis*, which also is considered a species incertae sedis (3). The aims of this study were to clarify the taxonomic positions of *N. amarae* and *R. aichiensis* and to investigate the phylogenetic coherence of the genus *Gordona*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. For chemical and genetic analyses, *R. aichiensis* DSM 43978^T (T = type strain), *N. amarae* DSM 43392^T, and *Gordona sputi* DSM 43986^T were

grown either in shake flasks containing Trypticase soy broth (BBL) or on Trypticase soy broth agar for 4 days at 28°C.

Cell chemistry. An analysis of the isoprenoid quinones, polar lipids, cell wall diamino acids, and sugars of *R. aichiensis* DSM 43978^T was carried out by using previously described methods (10, 14, 15). The fatty acid methyl esters obtained from wet biomass preparations (approximately 40 mg) of *R. aichiensis* DSM 43978^T and *G. sputi* DSM 43896^T were analyzed and identified by gas chromatography, using a model 5898A Microbial Identification System apparatus (Microbial ID, Newark, Del.) as described previously (23).

16S rDNA sequencing. Genomic DNA was isolated and PCR-mediated amplification of the 16S ribosomal DNA (rDNA) was performed as described by Rainey et al. (21). Double-stranded PCR products were sequenced by using a *Taq* Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems) and the protocol recommended by the manufacturer. Sequence reaction mixtures were electrophoresed by using an Applied Biosystems model 373A DNA sequencer. The 16S rDNA sequences were aligned with sequences currently available from the public databases. Evolutionary distances were calculated by the method of Jukes and Cantor (11). A phylogenetic dendrogram was constructed from the distance matrices by using the algorithm of De Soete (1).

Nucleotide sequence accession numbers. The EMBL accession numbers for the 16S rDNA sequences of *N. amarae* DSM 43392^T, *R. aichiensis* DSM 43978^T, *Gordona bronchialis* DSM 43247^T, *Gordona rubropertincta* DSM 43197^T, *Gordona sputi* DSM 43896^T, and *Gordona terrae* DSM 43249^T are X80635, X80633, X79287, X80632, X80634, and X79286, respectively.

RESULTS AND DISCUSSION

The results of this study and previous chemotaxonomic analyses of *N. amarae* DSM 43392^T and *R. aichiensis* DSM 43978^T are shown in Table 1 along with data for other related taxa. The cell walls of *R. aichiensis* DSM 43978^T contained arabinose and galactose as major cell wall sugars, meso-diaminopimelic acid as the only diamino acid, and *N*-glycolated muramic acid moieties of peptidoglycan. The polar lipids included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, and phosphoethanolamine, and MK-9(H₂) was found to be the predom-

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TABLE 1. Chemosystematic characteristics of *N. amarae* (Lechevalier and Lechevalier), *R. aichiensis* (Tsukamura), and related wall chemotype IV taxa containing mycolic acids^a

Taxon	Fatty acid composition ^b	Mycolate size (no. of carbon atoms)	Presence of phosphatidylethanolamine ^c	Major menaquinone(s)	G+C content of DNA (mol%)
<i>R. aichiensis</i>	S, U, T	56–66	+	MK-9(H ₂)	65
<i>N. amarae</i>	S, U, T	46–54	+	MK-9(H ₂)	60–66
<i>Gordona</i> species	S, U, T	48–66	+	MK-9(H ₂)	63–69
<i>Nocardia</i> species	S, U, T	44–60	+	MK-8(H ₄ , ω-cycl)	64–72
<i>Rhodococcus</i> species	S, U, T	34–52	+	MK-8(H ₂)	63–73
<i>Tsukamurella</i> species	S, U, T	64–68	+	MK-9	67–68
<i>Mycobacterium</i> species	S, U, T	60–90	+	MK-9(H ₂)	62–70
<i>Corynebacterium</i> species	S, U ^d	22–36	v ^e	MK-8(H ₂), MK-9(H ₂)	51–67

^a Adapted from the studies of Goodfellow (3, 4) and Stackebrandt et al. (25).

^b S, saturated; U, monounsaturated; T, tuberculostearic acid.

^c +, present; v, variable.

^d Tuberculostearic acid is also present in *Corynebacterium ammoniagenes*, *Corynebacterium bovis*, *Corynebacterium cystitidis*, *Corynebacterium minutissimum*, *Corynebacterium pilosum*, *Corynebacterium urealyticum*, and *Corynebacterium variabilis* (9, 13, 20).

^e Phosphatidylethanolamine is also present in *C. ammoniagenes*, *C. bovis*, *C. cystitidis*, *Corynebacterium pseudodiphtheriticum*, and *C. urealyticum* (9, 20).

inant menaquinone, which is in good agreement with previous results (6). An analysis of the simple fatty acids obtained from whole-cell methanolysates revealed a pattern consisting of straight-chain, saturated and unsaturated fatty acids with significant amounts of tuberculostearic acid (10-methyl octadecanoic acid) (Table 2). The wall chemotype of both *N. amarae* DSM 43392^T and *R. aichiensis* DSM 43978^T is the characteristic wall chemotype IV (sensu Lechevalier and Lechevalier [15]) found in genera containing mycolic acids, but these two strains differ significantly in menaquinone composition [having predominantly MK-9(H₂) and minor amounts of MK-8(H₂)] from the genera to which they are currently assigned, namely, the genera *Nocardia* [MK-8(H₄, ω-cycl)] and *Rhodococcus* [MK-8(H₂)]. Furthermore, the sizes of the mycolic acids of *R. aichiensis* DSM 43978^T and *N. amarae* DSM 43392^T are similar to the sizes of the mycolic acids found in members of the genus *Gordona* (3, 29) (Table 1), although pyrolytic cleavage of methyl mycolates of *N. amarae* DSM 43392^T releases unusual monounsaturated esters (5, 16) that are unique to this species within the family *Nocardiaceae*. Nevertheless, as shown in Table 1, the combined chemotaxonomic characteristics of *R. aichiensis* DSM 43978^T and *N. amarae* DSM 43392^T suggest that these organisms are closely related to the genus *Gordona* (25). Therefore, almost complete 16S rDNA sequences (>1,500 nucleotides) were determined for *N. amarae* DSM 43392^T, *R. aichiensis* DSM 43978^T, *G. bronchialis* DSM 43247^T,

G. rubropertincta DSM 43197^T, *G. sputi* DSM 43896^T, and *G. terrae* DSM 43249^T. The phylogenetic dendrogram (in Fig. 1) shows the relationships of *R. aichiensis* DSM 43978^T and *N. amarae* DSM 43392^T to some members of related genera for which sequence data are available. The results of a comparison of sequence data for all *Gordona* species and related organisms are shown in Table 3; the genus *Gordona* was found to be phylogenetically coherent and was clearly distinct from other actinomycete genera (Fig. 1). In addition, both *R. aichiensis* DSM 43978^T and *N. amarae* DSM 43392^T were found to cluster with strains belonging to the genus *Gordona*. This close relationship was also observed in the rDNA similarity values shown in Table 3. The levels of similarity between *N. amarae* DSM 43392^T and members of the genus *Gordona* ranged from 96.9 to 97.8%, while the levels of similarity to members of other genera were less than 94.4%. In the case of *R. aichiensis* DSM 43978^T an extremely high level of similarity (99.7%) to *G. sputi* DSM 43896^T was observed, although the level of

TABLE 2. Fatty acid compositions of *R. aichiensis* and *G. sputi*

Fatty acid	% in:	
	<i>R. aichiensis</i>	<i>G. sputi</i>
10:0	1.18	0.91
i-3-OH-11:0	1.72	1.53
14:0	1.64	2.56
15:0	1.45	2.36
16:1(ω6)	18.05	— ^a
16:0	28.92	43.19
17:1(ω8)	1.68	0.85
17:0	1.56	1.51
10-Me-17:0	0.87	—
18:1(ω9)	17.51	27.96
18:0	—	1.94
TBSA (10-Me-18:0) ^b	24.06	16.51

^a —, not detected.

^b TBSA, tuberculostearic acid.

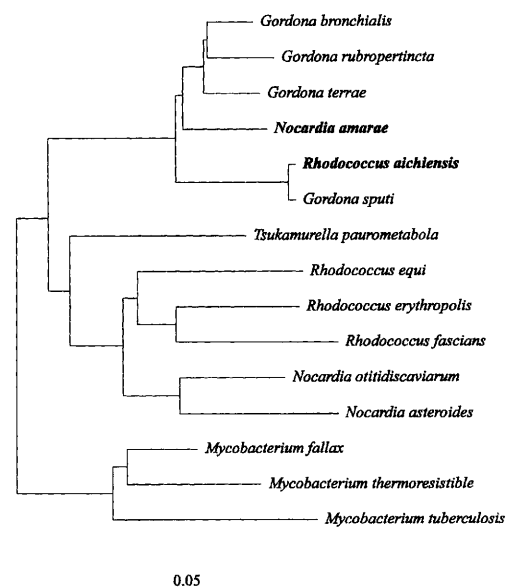


FIG. 1. Phylogenetic dendrogram indicating the relationships of *R. aichiensis* and *N. amarae* to members of the genus *Gordona* and related taxa. Bar = 5 substitutions per 100 nucleotides.

TABLE 3. 16S rDNA similarity values for organisms used in this study

Species	% 16S rDNA similarity with:													
	<i>Gordona bronchialis</i>	<i>Gordona rubropertincta</i>	<i>Gordona terrae</i>	<i>Gordona sputi</i>	<i>Rhodococcus aichiensis</i>	<i>Rhodococcus erythropolis</i>	<i>Rhodococcus equi</i>	<i>Rhodococcus fascians</i>	<i>Nocardia amarae</i>	<i>Nocardia asteroides</i>	<i>Nocardia otitidiscaviarum</i>	<i>Tsukamurella paurometabola</i>	<i>Mycobacterium fallax</i>	<i>Mycobacterium tuberculosis</i>
<i>Gordona bronchialis</i>														
<i>Gordona rubropertincta</i>	98.3													
<i>Gordona terrae</i>	98.3	98.1												
<i>Gordona sputi</i>	97.2	96.4	96.6											
<i>Rhodococcus aichiensis</i>	97.3	96.7	96.7	99.7										
<i>Rhodococcus erythropolis</i>	93.5	93.3	93.4	93.0	92.9									
<i>Rhodococcus equi</i>	93.7	93.8	93.9	92.5	92.6	95.2								
<i>Rhodococcus fascians</i>	92.9	93.0	93.2	91.9	92.0	95.8	94.7							
<i>Nocardia amarae</i>	97.5	97.4	97.8	96.9	97.0	93.4	93.3	92.6						
<i>Nocardia asteroides</i>	93.0	93.0	93.0	92.2	92.3	94.2	94.4	93.4	92.8					
<i>Nocardia otitidiscaviarum</i>	93.7	93.6	93.8	92.4	92.5	94.7	95.6	94.7	92.9	96.1				
<i>Tsukamurella paurometabola</i>	93.6	93.5	93.5	93.7	93.6	94.6	94.0	93.7	94.4	93.6	93.9			
<i>Mycobacterium fallax</i>	93.7	93.2	93.7	93.7	94.0	93.4	93.1	93.1	93.7	93.2	94.0	94.8		
<i>Mycobacterium tuberculosis</i>	92.8	91.8	92.6	91.6	91.7	91.3	91.3	92.0	92.1	91.0	92.8	92.5	96.1	
<i>Mycobacterium thermoresistibile</i>	93.0	92.3	93.0	93.6	93.5	92.5	92.1	91.7	93.3	92.1	92.9	93.5	97.1	94.6

DNA-DNA relatedness between these species was found to be only 38 and 40% when the level was determined by the nitrocellulose filter and spectrophotometric methods, respectively (13, 30). *R. aichiensis* DSM 43978^T and *G. sputi* DSM 43896^T can be differentiated not only by DNA relatedness data but also by fatty acid data (Table 2) and phenotypic properties (Table 4). Obvious discrepancies between DNA-DNA hybridization results and levels of 16S rRNA sequence similarity have also been reported previously (2), thus strongly emphasizing the fact that a polyphasic approach is highly recommended for the establishment of new genospecies.

In conclusion, the combined chemosystematic and phylogenetic data indicate that *N. amarae* DSM 43392^T and *R. aichiensis* DSM 43978^T are closely related to the genus *Gor-*

dona. On the basis of the data presented here we propose that *N. amarae* DSM 43392^T and *R. aichiensis* DSM 43978^T should be transferred to the genus *Gordona* as *Gordona amarae* and *Gordona aichiensis*, respectively. The species descriptions are emended accordingly below to incorporate the data obtained in this study.

Description of *Gordona amarae* (Lechevalier and Lechevalier) comb. nov. The species description below is based on information obtained from several sources, including references 5, 16, and 17 and our own observations.

Convolutated tannish to white colonies with filamentous margins are formed on various media. There is sparse aerial mycelium which is not visible macroscopically. The substrate mycelium consists of moderately branching vegetative hyphae

TABLE 4. Diagnostic characteristics of *Gordona* species

Characteristic	<i>G. aichiensis</i>	<i>G. amarae</i>	<i>G. bronchialis</i>	<i>G. rubropertincta</i>	<i>G. sputi</i>	<i>G. terrae</i>
Colony color white to tan ^a	- ^b	+	-	-	-	-
Cleavage of the following 7-amino-4-methylcoumarin substrates ^c :						
D-Alanine-amino-4-methylcoumarin	+	ND	-	+	-	+
Isoleucine-7-amino-4-methylcoumarin	+	ND	-	-	+	-
Pyroglutamate-7-amino-4-methylcoumarin	-	ND	-	-	-	+
Valine-7-amino-4-methylcoumarin	+	ND	-	+	+	+
Growth on the following sole carbon sources ^d :						
D-Galactose (1.0%, wt/vol)	+	-	-	+	+	+
meso-Inositol (1.0%, wt/vol)	-	+	+	-	-	-
Raffinose (1.0%, wt/vol)	-	+ ^e	-	-	+	+
L-Rhamnose (1.0%, wt/vol)	-	+	-	-	-	+
Citric acid (Na salt) (0.1%, wt/vol)	+	-	-	+	-	+
Mycolic acids ^f :						
Avg carbon no.	62	50-52	64	58-59	61-62	58
FAME released upon pyrolysis of MAME ^g	16:0	16:1, 18:1	18:0	16:0, 18:0	16:0, 18:0	16:0, 18:0
16:1 fatty acid present ^h	+ ^h	+	+	+	- ^h	+

^a Data from references 5 and 16.^b -, ≥90% of the strains are negative; +, ≥90% of the strains are positive; ND, not determined.^c Data from references 5, 7, and 8.^d Data from references 5, 7, 8, and 16.^e The concentration used to determine raffinose utilization was 0.2% (wt/vol) (12).^f Data from references 5, 13, and 29 and this study.^g FAME, fatty acid methyl esters; MAME, mycolic acid methyl esters.^h Data for *G. sputi* DSM 43896^T and *G. aichiensis* DSM 43978^T.

that grow into the agar medium and do not fragment in undisturbed cultures. Secondary branching is rare. Filaments are gram positive and partially acid fast. Hydrolyzes esculin, Tween 20, Tween 40, Tween 60, and urea but not casein, elastin, gelatin, hypoxanthine, adenine, tyrosine, or xanthine.

Catalase positive and *p*-nitrophenoloxidase and *m*-nitrophenoloxidase negative. Reduces nitrate to nitrite.

Grows on ethanol, fructose, glycerol, maltose, *D*-mannose, *D*-mannitol, *L*-rhamnose, salicin, sucrose, trehalose, adipate, pimelic acid, sebacic acid, acetate, fumarate, gluconate, lactate, malate, propionate, pyruvate, and succinate as sole carbon sources, but not on ribitol, *L*-arabinose, cellobiose, lactose, melezitose, raffinose, *D*-glucitol, *D*-xylose, testosterone, *L*-tyrosine, acetamide, benzamide, benzoate, citrate, *p*-cresol, *m*-hydroxybenzoate, oxalate, and tartrate. Does not utilize serine and trimethylenediamine as sole carbon and nitrogen sources.

Acid is produced from fructose, glucose, glycerol, inositol, maltose, *D*-mannitol, *D*-mannose, *L*-rhamnose, salicin, sucrose, and trehalose but not from ribitol, *L*-arabinose, cellobiose, galactitol, *D*-galactose, lactose, melibiose, α -methyl-*D*-glucoside, raffinose, *D*-glucitol, *D*-xylose, or β -methyl-*D*-xyloside.

Susceptible to lysozyme broth, penicillin (10 IU), phenol (0.1%, wt/vol), phenyl ethanol (0.3%, wt/vol), and sodium chloride (5 and 7%, wt/vol) but not crystal violet (0.0001 and 0.001%, wt/vol), phenol (0.01%, wt/vol) or sodium azide (0.01%, wt/vol).

Grows at 23 to 37°C, but not at 10 or 40°C.

The cell wall chemotype is wall chemotype IV, with *meso*-diaminopimelic acid as the only diamino acid and arabinose and galactose as major cell wall sugars. The predominant isoprenolog is MK-9(H₂). The polar lipids include phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides. Contains major amounts of straight-chain, saturated and unsaturated fatty acids and tuberculostearic acid (2% 14:0, 8% 16:1, 43% 16:0, 21% 18:1, 18% 18:0, 8% 10-Me-18:0). Mycolic acids with 46 to 54 carbons and up to three double bonds are present. Upon pyrolysis gas chromatography of mycolates 16:0, 18:0, 16:1, and 18:1 fatty acid esters are released.

The G+C content of the DNA is 60 to 66 mol%.

The type strain is SE6 (= DSM 43392), as described by Lechevalier and Lechevalier (16).

Description of *Gordona aichiensis* (Tsukamura) comb. nov.

The species description below is based on our data and additional information obtained from several sources, including references 3, 27, 29, and 30.

Gram-positive, nonmotile short rods that grow amycelially and have a rod-coccus cycle. Rough pinkish or orange colonies are formed on egg media. Hydrolyzes Tween 80 and reduces nitrate to nitrite. Acetamidase, urease, nicotinamidase, pyrazinamidase, and valine arylamidase positive, but allantoinase, benzamidase, isonicotinamidase, salicynamidase, succinamidase, and valine arylamidase negative. Acid phosphatase and α -glucosidase positive, but arylsulfatase, α -esterase, β -esterase, and β -galactosidase negative.

Uses glutamate, acetamide, and monoethanolamine as sole carbon and nitrogen sources, but does not use serine, glucosamine, benzamide, and trimethylene diamine.

Utilizes acetate, citrate, succinate, malate, pyruvate, fumarate, *p*-hydroxybenzoate, pimelate, ethanol, 1-propanol, 1-butanol, isobutanol, butane-2,3-diol, *D*-glucose, *D*-fructose, lactose, sucrose, *D*-mannose, and trehalose as sole carbon sources, but does not utilize benzoate, citraconate, *D*-mandelate, malonate, glutamate, *L*-arabinose, *D*-galactose, *L*-rhamnose, *D*-xylose, inositol, *D*-mannitol, *D*-glucitol, propylene glycol, 1,3-, 1,4-, and 2,3-butylene glycols, andosterone, and testosterone.

Grows at 28, 37, and 42°C, but not at 45°C.

Resistant to picric acid (0.2%, wt/vol), sodium nitrite (0.1%, wt/vol), and sodium salicylate (0.1%, wt/vol), but not mitomycin C (5 μ g/ml) and 5-fluorouracil (20 μ g/ml).

The cell wall chemotype is wall chemotype IV, with *meso*-diaminopimelic acid as the only diamino acid and arabinose and galactose as major cell wall sugars. The predominant isoprenolog is MK-9(H₂); minor amounts of MK-8(H₂) are also present. The polar lipids include phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, and phosphatidylinositol mannosides. Contains major amounts of straight-chain, saturated and unsaturated fatty acids and tuberculostearic acid. Mycolic acids with 56 to 66 carbons and up to six double bonds are present. Upon pyrolysis gas chromatography of mycolates 16:0 and 18:0 fatty acid esters are liberated.

The G+C content of the DNA is 65 mol%.

Isolated from human sputum.

The type strain is E9028 (= DSM 43978), as described by Tsukamura (27).

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