Polyphasic Evidence for the Transfer of *Rhodococcus roseus* to *Rhodococcus rhodochrous*

FREDERICK A. RAINEY, JUTTA BURGHARDT, REINER KROPPENSTEDT,
STEPHAN KLATTE, AND ERKO STACKEBRANDT*

German Collection of Microorganisms and Cell Cultures GmbH,
38124 Braunschweig, Germany

Investigation of fatty acid and mycolic acid properties of certain members of the genera *Nocardia* and *Rhodococcus* indicated identical spectra for certain pairs of organisms. Binary 16S rDNA sequence homology for the pairs *Rhodococcus rhodochrous* and *R. roseus*, *Nocardia calcaraea* and *R. erythropolis*, and *N. restricta* and *R. equi* ranged between 99.2 and 99.9% for each of these pairs of species. Corresponding DNA-DNA similarity values, obtained by the spectrophotometric method, were significantly higher than 70%. Because certain chemotaxonomic properties and the majority of published phenotypic characters support the high degree of relatedness, we propose to transfer *R. roseus* to *R. rhodochrous*. The transfer of *N. calcaraea* to *R. erythropolis*, and the transfer of *Nocardia restricta* to *Rhodococcus equi* (M. Goodfellow, p. 1472–1481, in P. H. A. Sneath et al., ed., *Bergey's Manual of Systematic Bacteriology*, vol. 2, 1986) is confirmed.

The unambiguous assignment of actinomycete species to the genera *Rhodococcus* and *Nocardia* constitutes a problem, because virtually no single characteristic of diagnostic value, such as phage susceptibility (19), cell wall chemistry, fatty acid and phospholipid composition, overall size of mycolic acids, and DNA G+C content, has been found to clearly distinguish between members of the two genera (6). Today, the morphological life cycle and the presence of a cyclic menaquinone of the MK-8(H₄) type (1, 9, 15) are the main features that distinguish members of *Nocardia* from those of *Rhodococcus*. Recent applications of 16S rDNA sequence analysis, DNA hybridization, and chemotaxonomic analysis to strains of both genera have led to taxonomic rearrangements that excluded certain species from the genus *Rhodococcus* (2, 8, 13, 21, 23) or showed them to be synonyms (12). In this communication we present further evidence that the present classification of certain additional *Nocardia* and *Rhodococcus* species is not supported by their phylogenetic and phenotypic relationships.

MATERIALS AND METHODS

Bacterial strains and cultivation. The following strains were investigated for fatty acid and mycolic acid pattern analysis, DNA reassociation, and 16S rDNA analysis (16S rDNA accession numbers in brackets): *Nocardia restricta* DSM 43199^T (X80613), *Rhodococcus rhodochrous* DSM 43241^T (X79288), *R. equi* DSM 20307^T (X80614), *R. erythropolis* DSM 43066^T (X79289), *R. roseus* DSM 43274^T (X80624), and *R. ruber* DSM 43338^T (X80625). Strains were grown on TSB agar (3% [wt/vol] Trypticase soy broth [BBL], 1.5% [wt/vol] Bacto Agar [Difco]) for 4 days at 28°C. For DNA hybridization experiments, strains were grown in Trypticase soy broth for 4 days at 28°C on a rotary shaker, checked for purity, inactivated by the addition of 1% (vol/vol) formaldehyde, harvested by centrifugation, and washed twice with distilled water.

16S rDNA sequencing. Extraction of genomic DNA and amplification of the 16S rDNA were performed as described previously (20). PCR products were sequenced directly with a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) as specified by the manufacturer. The sequence reactions were electrophoresed in an Applied Biosystems 373A DNA Sequencer. New sequences were manually aligned with published sequences from *Corynebacte-*

rium, *Mycobacterium*, *Gordona*, *Rhodococcus*, and *Nocardia* species included in the Ribosome Database Project (17) and from our own entries. Direct pairwise sequence homologies were calculated and are presented as percentage similarity values.

DNA isolation and characterization. DNA isolation and DNA-DNA hybridization (4, 10) and determination of renaturation rates (3, 11) were performed by previously described methods.

Lipid and mycolic acid analysis. Whole-cell fatty acids and free mycolic acids were analyzed as described previously (8, 14, 18).

RESULTS AND DISCUSSION

16S rDNA analysis of certain pairs of type strains of *Nocardia* and *Rhodococcus* species yielded almost identical results (Table 1). Even at this high level of sequence homology, DNA reassociation values may drop below the level of 70% similarity recommended for identification of separate species (25); therefore, DNA hybridization was carried out for these pairs of strains. In each case, values ranged above 78% (Table 1), which can be taken as clear evidence that these strains should be united as a single species. The differentiation of authentic *Rhodococcus* species by the DNA reassociation approach is possible as demonstrated by the binary value of 39 and 44% for the pair *R. rhodochrous* and *R. ruber* and the pair *R. roseus* and *R. ruber*, respectively. The corresponding 16S rDNA homology values are about 2.5% lower than those observed between pairs of organisms considered

TABLE 1. Comparison of binary 16S rDNA homology values and DNA reassociation values and taxonomic conclusion based on the genotypic and phenotypic characterization of the strains

Pair of organisms	16S rDNA homology (%)	DNA reassociation values (%)	Taxonomic conclusion based on priority
<i>N. calcaraea</i> , <i>R. erythropolis</i>	99.5	93	<i>R. erythropolis</i>
<i>N. restricta</i> , <i>R. equi</i>	99.9	96	<i>R. equi</i>
<i>R. rhodochrous</i> , <i>R. roseus</i>	99.2	78, 82 ^a	<i>R. rhodochrous</i>
<i>R. rhodochrous</i> , <i>R. ruber</i>	97.5	39	NC ^b
<i>R. roseus</i> , <i>R. ruber</i>	97.4	44	NC

^a Two independent determinations.

^b NC, no charge.

* Corresponding author. Mailing address: German Collection of Microorganisms and Cell Cultures GmbH, Mascheroder Weg 1b, 38124 Braunschweig, Germany. Phone: 49 531 2616 352. Fax: 49 531 2616 418. Electronic mail address: stackebrandt@venus.gbf-braunschweig.d400.de.

TABLE 2. Comparison of mycolic acid chain length and principal menaquinone of closely related *Nocardia* and *Rhodococcus* species

DSM no.	Name	Mycolic acid chain length ^a	Principal menaquinone
DSM 43757 ^T	<i>N. asteroides</i>	C ₅₃ -C ₅₉	MK-8 (H _{4cycl})
DSM 43199 ^T	<i>N. restricta</i>	C ₃₀ -C ₃₆	MK-8 (H ₂)
DSM 20307 ^T	<i>R. equi</i>	C ₃₀ -C ₃₆	MK-8 (H ₂)
DSM 43188 ^T	<i>N. calcarrea</i>	C ₃₄ -C ₄₀	MK-8 (H ₂)
DSM 43066 ^T	<i>R. erythropolis</i>	C ₃₄ -C ₄₀	MK-8 (H ₂)
DSM 43241 ^T	<i>R. rhodochrous</i>	C ₃₈ -C ₄₆	MK-8 (H ₂)
DSM 43274 ^T	<i>R. roseus</i>	C ₃₈ -C ₄₆	MS-8 (H ₂)
DSM 43338 ^T	<i>R. ruber</i>	C ₄₂ -C ₄₈	MK-8 (H ₂)

^a Number of carbon atoms in mycolic acid.

to belong to the same species (Table 1). In addition, results of chemotaxonomic analysis of mycolic acids (Table 2), principal menaquinones (Table 2), and fatty acids (Table 3) agree with the conclusions of the genotypic analyses. Pairs of genomically highly related organisms exhibited identical chain length of mycolic acids and almost identical fatty acid spectra. The close overall similarity between the pair *N. calcarrea* and *R. erythropolis* and between the pair *N. restricta* and *R. equi* has already been reported by Goodfellow (5), who described *N. calcarrea* and *N. restricta* as synonyms of *R. erythropolis* and *R. equi*, respectively. Results of phylogenetic and additional chemotaxonomic studies fully confirm these conclusions.

Thus, on the basis of rule 42 of the *International Code of Nomenclature of Bacteria* (16), according to which the oldest legitimate epithet must be retained following the union of taxa of equal rank, we propose to transfer as synonyms *Rhodococcus roseus* to *Rhodococcus rhodochrous*.

The species *R. roseus* (24) was revived for two strains of a taxon that was not included in the 1980 edition of the *Approved Lists of Bacterial Names* (22). The justification for the distinct status of *R. roseus* was based on a numerical phenetic analysis. Its relatedness to *R. rhodochrous* (7) was mentioned, but differences between the two taxa in the number of double bonds in mycolic acids and in certain pheno-

typic characteristics, such as resistance to 250 µg of NH₂OH · HCl per ml, tolerance to 1% Tween 80, presence of acetamidase, utilization of mannitol as a carbon source, and use of nicotinamide as a nitrogen source, were believed to justify the description of a new species. DNA reassociation values of about 80% indeed indicate that certain genomic and hence certain phenotypic differences are expected to exist between the type strains of the two species. However, we consider the physiological differences not to be significant, especially as only two strains of *R. roseus* had been included in the numerical phenetic analysis and the possible influence of plasmid-encoded physiological characteristics has not been investigated.

The following is an emended description of *R. rhodochrous*, based on the descriptions of *R. rhodochrous* (5) and *R. roseus* (24) and data presented in this study. Because Tsukamura et al. (24) provide comparative data on strains of both species, the emendation is based mainly on their study. Only properties that show either 0 to 100% positive reactions are listed. Reactions in which strains of the species differ are listed in reference 24.

Slightly acid-fast rods. Some strains undergo an elementary branching-rod-coccus growth cycle. Temporary mycelium may be formed. Rough, orange or pinkish to red colonies are formed in egg media, on Saunton's agar, and on modified Saunton's agar medium (glutamate substituted for asparagine). Growth occurs at 28 and 40°C but not at 45°C.

Resistant to hydroxylamine hydrochloride (500 µg/ml) and *para*-nitrobenzoic acid (0.5 mg/ml); most strains are resistant to ethambutol (5 µg/ml). Tolerant to 0.1% salicylate and 0.2% picric acid; most strains are tolerant to 0.2% nitrite. Sensitive to rifampin (25 µg/ml).

Allantoinase and nicotinamidase negative. α-Esterase, β-esterase, and β-galactosidase negative. Nitrate is reduced to nitrite.

Glutamate is used as an N and C source, but benzamide and monoethanolamine are not. Glucose, sucrose, mannitol, ethanol, glucitol, *n*-propanol, and *n*-butanol are used as sole sources of carbon, but arabinose, galactose, inositol, and rhamnose are not.

Isolated from soil.

The mol% G+C of the DNA is 67 to 70 (*T_m*).

Type strain: DSM 43241 (ATCC 13808).

TABLE 3. Comparison of fatty acid profiles of closely related *Nocardia* and *Rhodococcus* species

DSM no.	Name	Fatty acid content (% of total fatty acids methyl esters) ^a												
		14:0	15:0	16:1 <i>cis</i> 9	16:1 <i>cis</i> 10	16:0	16:0 10Me	17:1 <i>cis</i> 9	17:0	17:0 10Me	18:1 <i>cis</i> 9	18:0	18:0 10Me	19:1 <i>cis</i> 9
DSM 43757 ^T	<i>N. asteroides</i>	1	1	5	15	22		2	6		17	4	27	
DSM 43199 ^T	<i>N. restricta</i>	8	3	5	12	25	4	3	3	2	13	1	17	1
DSM 20307 ^T	<i>R. equi</i>	10	2	6	14	30	7	2	2	2	8	1	12	2
DSM 43188 ^T	<i>N. calcarrea</i>	5	4	3	17	27	1	3	3	5	12	1	16	2
DSM 43066 ^T	<i>R. erythropolis</i>	6	5	2	15	25	3	3	2	4	10		18	
DSM 43241 ^T	<i>R. rhodochrous</i>	2	1	6	18	29	7	1	1	2	8	1	22	
DSM 43274 ^T	<i>R. roseus</i>	1	2	4	15	30	8	1		3	6		24	3
DSM 43338 ^T	<i>R. ruber</i>	2	4	2	20	25	2	4	3	3	16	1	18	1

^a Examples for abbreviations: 14:0, straight-chain tetradecanoic acid; 18:1 *cis*9, monounsaturated octadecenoic acid (oleic acid); 18:0 10Me, 10-methyloctadecanoic acid (tuberculoheptanoic acid).

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