Distribution and Application of Mycobactins for the Characterization of Species within the Genus *Rhodococcus*

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Representatives of 11 species of *Rhodococcus* were examined for their ability to synthesize mycobactin, a lipid-soluble siderophore, following iron-limited growth on solidified glycerol/asparagine medium. *Rhodococcus bronchialis*, *R. terrae* and *R. rubropertinctus* formed mycobactins, whereas the remaining species (*R. coprophilus*, *R. equi*, *R. erythropolis*, *R. rhodnii*, *R. rhodochrous*, *R. ruber*, *R. maris* and *R. luteus*) failed to synthesize these compounds even under conditions of strictly iron-limited growth. The mycobactins from *R. terrae* and *R. rubropertinctus* showed close similarity by thin-layer chromatography and high-performance liquid chromatography and could be easily distinguished from that of *R. bronchialis*.

INTRODUCTION

The genus *Rhodococcus* (Zopf, 1891; Tsukamura, 1974) is now well-established and forms a phylogenetically recognizable group together with mycolic acid-containing actinomycetes belonging to the genera *Caseobacter*, *Mycobacterium*, *Nocardia* and *Rhodococcus* (Goodfellow & Cross, 1984). The organisms in these taxa can be distinguished using a combination of morphological and chemical properties (Goodfellow & Minnikin, 1981) but there is evidence that the genus *Rhodococcus* is heterogeneous. Thus, all of the species originally assigned to the genus '*Gordona*' (Tsukamura, 1971), namely *Rhodococcus bronchialis*, *R. terrae* and *R. rubropertinctus*, have mycolic acids with between 48 and 66 carbons and major amounts of dehydrogenated menaquinones with nine isoprene units, whereas the remaining species, *R. coprophilus*, *R. equi*, *R. erythropolis*, *R. rhodnii*, *R. rhodochrous*, *R. ruber*, *R. maris* and *R. luteus*, have shorter mycolic acids and MK-8(H₂) as the major isoprenologue. The two aggregate groups can also be recognized on the basis of antibiotic sensitivity patterns (Goodfellow & Orchard, 1974), delayed skin reactions on sensitized guinea pigs and by polyacrylamide gel electrophoresis (PAGE) of cell extracts (Hyman & Chaparas, 1977).

Methods for the identification of bacteria assigned to the genus *Rhodococcus* are as yet incomplete (Goodfellow & Schaal, 1979). In this study we have cultured rhodococcal species under conditions of iron-limitation in order to encourage the possible production of the lipid-soluble siderophore, mycobactin. Mycobactins have a conserved structure and are species-specific for members of *Mycobacterium* and *Nocardia* (Ratledge & Patel, 1976*a, b*; Hall & Ratledge, 1984, 1985). The aim of this study was, therefore, to evaluate whether mycobactins are of value in rhodococcal systematics.

METHODS

Cultures and cultivation. Test strains (Table 1) were maintained on glucose/yeast extract/agar slopes (Gordon & Mihm, 1962) or as dense suspensions of cells frozen in 20% (v/v) glycerol at -20 °C (Wellington & Williams, 1978). Rhodococci were initially cultured in liquid glycerol/asparagine medium (Ratledge & Hall, 1971) or

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Table 1. *Rhodococci examined for mycobactin production*

Laboratory no.*	Designation and source of strains†
N361	<i>R. ruber</i> ; M. Tsukamura, M-1. Type strain
N54	<i>R. rhodochrous</i> ; ATCC 13808. Type strain
N445	<i>R. rhodnii</i> ; P. Hill, A1. Type strain
N11	<i>R. erythropolis</i> ; NCIB 9158. Type strain
C7	<i>R. equi</i> ; D. Jones, C48; NCTC 1621. Type strain
N744	<i>R. coprophilus</i> ; NCIB 11211. Type strain
N659	‡ <i>R. terrae</i> ; NCTC 10669; M. Tsukamura, 3612. Type strain
N4	‡ <i>R. rubropertinctus</i> ; NCIB 9664. Type strain
N654	‡ <i>R. bronchialis</i> ; NCTC 10667. Type strain
N1021	<i>R. maris</i> ; IMV 53; soil
N1025	<i>R. maris</i> ; IMV 678
N1026	<i>R. luteus</i> ; IMV 29; soil
N1027	<i>R. luteus</i> ; IMV 27; soil
N1028	<i>R. luteus</i> ; IMV 187; soil
N1029	<i>R. luteus</i> ; IMV 202; soil
N1032	<i>R. luteus</i> ; IMV 419; soil
N1034	<i>R. luteus</i> ; IMV 587; soil
N1035	<i>R. luteus</i> ; IMV B 868

* Laboratory numbers are those of the collection of Dr M. Goodfellow, Department of Microbiology, University of Newcastle upon Tyne, UK.

† *R. maris* and *R. luteus* strains were received via Dr Goodfellow from Dr O. A. Nesterenko (D. K. Zabolotny Institute of Microbiology and Virology of the Academy of Sciences of the Ukrainian SSR, 252143 Kiev, USSR). Addresses: M. Tsukamura, National Chubu Hospital, Obu, Aichi, Japan; P. Hill, Edinburgh University, UK; D. Jones, Leicester University, UK.

‡ Strains synthesizing mycobactins.

Table 2. *TLC of mycobactins isolated from the genus Rhodococcus*

Species	Percentage mycobactin yield*	R_F values for TLC systems†			
		I	III	V	VI
<i>R. terrae</i> N659	3.1	0.26	0.38	0.78	0.48
<i>R. rubropertinctus</i> N4	3.7	0.26	0.40	0.77	0.46
<i>R. bronchialis</i> N654	2.6	0.30	0.44	0.77	0.41

* Yield expressed as a percentage of cell dry weight.

† Solvent systems employed: (I) silica gel G, 20 cm $\times$ 20 cm (Analtech, Newark, USA) developed with petroleum spirit (b.p. 60–80 °C)/ethyl acetate/n-butanol (2:3:3, by vol.); (III) high performance thin-layer plates, 10 cm $\times$ 10 cm, including a 2.5 $\times$ 10 cm concentrating zone (Merck, 13748) developed with same solvents as system I; (V) plates as for system I but using methanol/ethyl acetate (4:1, v/v) for development; (VI) plates as for system I but using propan-1-ol as solvent (Hall & Ratledge, 1984, 1985).

glucose/yeast extract broth with thiamin (1 μ g ml⁻¹). Growth was at 30 °C with orbital shaking (200 r.p.m.) for 4 to 5 d, after which time a 0.2 ml sample was removed and inoculated on glycerol/asparagine medium solidified with 2% (w/v) Lab. Magar (London Analytical and Bacteriological Media, London, UK) as previously described (Hall & Ratledge, 1982). Under such conditions the bacteria were stressed for iron and so were encouraged to synthesize the iron-chelating compound mycobactin. Initially five plates (9 cm diam.) were inoculated for each strain. However, where mycobactin synthesis was low or not evident, 20 plates were used. *R. rhodnii* and *R. coprophilus* failed to grow readily on glycerol/asparagine medium unless glucose (10 g l⁻¹) was added.

Mycobactin isolation and characterization. Bacteria were harvested when growth was confluent. Mycobactins were isolated and yields determined following partial purification (Hall & Ratledge, 1982, 1984). Those strains which consistently failed to produce any mycobactin on solid media were grown in 2 litre iron-deficient glycerol/asparagine medium (Ratledge & Hall, 1971) and shaken at 30 °C at 200 r.p.m.

Mycobactins were initially analysed by thin-layer chromatography (TLC), in a single dimension, employing a range of systems previously described in detail (Table 2; Hall & Ratledge, 1984, 1985). High-performance liquid chromatography (HPLC) analysis of the mycobactins was done using a Spectra-Physics SP8000 instrument (Hall & Ratledge, 1984, 1985).

Fig. 1. HPLC of mycobactins isolated from (a) *R. terrae* N659, (b) *R. rubropertinctus* N4 and (c) *R. bronchialis* N654. Peaks are labelled with retention times in seconds and percentage peak areas are given, in parentheses, where $>3\%$ of final chromatogram. Separation was achieved using a Lichrosorb RP18 reverse-phase column with a solvent gradient changing from methanol/water (80:20, v/v) over 30 min to methanol alone (Hall & Ratledge, 1985).

RESULTS

Mycobactins were synthesized only by *R. terrae*, *R. rubropertinctus* and *R. bronchialis* and were characterized by TLC and HPLC (Table 2 and Fig. 1). Examination of the mycobactins isolated by TLC showed that they migrated as a single component in each of four different solvent systems (Table 2). In each case, the R_F values of the mycobactins from *R. terrae* and *R. rubropertinctus* were close, if not identical. Mycobactins from *R. bronchialis* could be readily differentiated in three of the four systems employed. On examination by HPLC (Fig. 1) the mycobactins could be separated into a number of components, thus providing characteristic 'fingerprints' which appear to be highly species-specific. Again the mycobactins from *R. terrae* and *R. rubropertinctus* showed close similarity, the majority of peaks having identical retention times. However, the proportions of each peak present clearly differed. *R. bronchialis* mycobactin again could be rapidly differentiated from that of the other producing species.

None of the remaining *Rhodococcus* spp. examined produced a detectable mycobactin (limit of detection about $0.26\text{ }\mu\text{g}$ on TLC and 1 ng on HPLC) even following bulk growth on solidified medium or in liquid culture under conditions of strict iron-limitation (see Methods).

DISCUSSION

The results of the present study provide further evidence of the heterogeneity of the genus *Rhodococcus*. Preliminary work (Ratledge & Patel, 1976a) showed that a few strains, later classified as rhodococci (Goodfellow & Alderson, 1977), produced no lipid-soluble iron-binding compounds, whereas a single strain classified as '*Gordona*' *rubra* did synthesize such a molecule. In the present study only those species previously assigned to the genus '*Gordona*' synthesized a detectable mycobactin, i.e. *R. terrae* ('*Gordona*' *terrae*), *R. bronchialis* ('*G.*' *bronchialis*) and *R. rubropertinctus* (syn. *R. corallinus* $\equiv$ '*G.*' *rubra*). The mycobactins produced by *R. terrae* and *R. rubropertinctus* appeared to be identical, confirming earlier serological data (Ivshina *et al.*, 1982), but that of *R. bronchialis* was clearly different. The remaining species in the genus did not synthesize any detectable amount of mycobactin.

This apparent division in the genus *Rhodococcus* is also supported by chemical and serological data. Mycolic acids, long-chain 2-branched 3-hydroxy fatty acids, isolated from *R. terrae*, *R. bronchialis* and *R. rubropertinctus* have between 48 and 66 carbon atoms. These strains also contain dihydrogenated menaquinones with nine isoprene units. On the other hand, *R.*

coprophilus, *R. erythropolis*, *R. equi*, *R. rhodnii*, *R. rhodochrous* and *R. ruber* produce shorter-chain mycolic acids (C_{30} to C_{52}) and have, predominantly, menaquinones with eight isoprene units. The mycolic acids of *R. luteus* and *R. maris* have not yet been examined but their major menaquinone is the MK-8(H_2) type (Minnikin & Goodfellow, 1980; Collins *et al.*, 1982; Goodfellow & Cross, 1984). The two groups can also be differentiated on the basis of delayed skin test reactions on sensitized guinea pigs and also by PAGE of bacterial extracts (Hyman & Chaparas, 1977).

It is possible that further studies might reinforce the separation between the two aggregate taxa, as indicated by the presence and absence of mycobactin, and hence raise the question of whether the two groups can be contained within one genus.

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