DNA Base Composition and Homology Values in the Classification of Some *Rhodococcus* Species

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Similar DNA homology values were recorded when a modified S1 nuclease technique and a standard nitrocellulose membrane filter method were applied to representative strains of *Rhodococcus*. The DNA homology data showed that *R. globerulus*, *R. luteus* and *R. sputi* form distinct genomic species. The congruence between the DNA homology and earlier numerical phenetic data was good, but there was evidence that some strains had been misclassified in the previous studies. In particular, the type strains of *R. obuensis* and *R. sputi* belong to a single genomic species. The former name is thus a later, subjective synonym of the latter. The guanine plus cytosine content of the DNA of the rhodococci fell within the range 61 to 72 mol%.

INTRODUCTION

The genus *Rhodococcus* (Zopf, 1891) continues to provide a niche for actinomycetes previously assigned to genera such as *Arthrobacter*, *Brevibacterium*, *Corynebacterium*, *Mycobacterium* and *Nocardia* (Goodfellow & Cross, 1984). In the current edition of *Bergey's Manual of Systematic Bacteriology* (Goodfellow, 1986) fourteen species of *Rhodococcus* are recognized and five taxa listed as *species incertae sedis*. Most of these species were circumscribed in numerical phenetic surveys (Tsukamura, 1971, 1982; Goodfellow & Alderson, 1977; Goodfellow *et al.*, 1982*a, b*) but it can be important to evaluate such numerically defined taxospecies in light of results from DNA homology studies (Mordarski *et al.*, 1980). DNA relatedness studies have shown that *R. bronchialis*, *R. coprophilus*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. ruber*, *R. rubropertinctus* and *R. terrae* form distinct genomic species (Mordarski *et al.*, 1976, 1977, 1980).

The present nucleic acid pairing experiments were primarily designed to establish the taxonomic status of representatives of some recently described rhodococcal species and numerically defined clusters and to find suitable conditions for determining DNA homologies amongst rhodococci in the presence of formamide using the S1 nuclease method.

METHODS

Bacterial strains and growth conditions. The test strains and their sources are given in Table 1. The procedures for cultivating the strains and preparing biomass, and the method used for obtaining purified DNA, have been described previously (Mordarski *et al.*, 1976).

Preparation of reference radioactive DNA. At the onset of exponential growth, [2-¹⁴C]- or [5,6-³H]uracil was added to a sucrose/yeast extract/peptone/mineral salts medium (Stackebrandt *et al.*, 1981) and labelled DNA was extracted and purified as described previously (Mordarski *et al.*, 1977). Labelled reference DNA was prepared from *Rhodococcus globerulus* R58, *Rhodococcus luteus* N1008, *Rhodococcus sputi* N930 and *Rhodococcus* strains N865 and N914. The latter were representatives of two groups, clusters 1L and 1K, defined in a numerical phenetic survey (Goodfellow *et al.*, 1982*a*). The specific activities of the reference DNAs were approximately 10⁵ c.p.m. per µg DNA.

Table 1. *Designation, source and DNA base composition of test strains*

Strain and source	Laboratory no.	G + C content of DNA (mol%)†
<i>Rhodococcus aichiensis</i> Tsukamura 1982		
M. Tsukamura, Chubu Chest Hospital, Obu, Aichi, Japan, E 7776; sputum	N933	68.6
M. Tsukamura, E 9028; sputum	N934*	65.4
<i>Rhodococcus equi</i> (Magnusson) Goodfellow and Alderson, 1977		
R. E. Gordon, Rutgers University, USA, S. A. Waksman; M. P. Lechevalier, 569p	N812	72.4
R. E. Gordon, 1241; T. D. Stout, R842; soil	N814	69.8
M. Mutimer, Department of Veterinary Pathology and Public Health, University of Queensland, Brisbane, Australia, 1389	R123	69.0
M. Mutimer, UQV 1302; bovine rumen	R128	69.0
R. E. Gordon, 1068	R155	66.0
R. E. Gordon, 1070W	R156	67.2
M. Barton, School of Veterinary Science, University of Melbourne, Australia, 24; porcine isolate	R164	70.5
M. Barton, 31; soil	R167	69.2
M. Barton, 156; bovine strain	R172	70.4
<i>Rhodococcus globerulus</i> Goodfellow <i>et al.</i> 1982b		
ATCC 25699	R43	67.1
ATCC 25710; R. E. Gordon, 494	R55	61.6
ATCC 25711; R. E. Gordon, 495	R56	62.2
ATCC 25714; R. E. Gordon, 544	R58*	61.4
<i>Rhodococcus luteus</i> (Sohngen) Nesterenko <i>et al.</i> 1982		
O. A. Nesterenko, D. K. Zabolotny, Institute of Microbiology and Virology, Kiev, USSR, IMV 385; soil	N1008*	65.2
<i>Rhodococcus maris</i> (Harrison) Nesterenko <i>et al.</i> 1982		
O. A. Nesterenko, IMV 195; soil	N1009*	71.9
<i>Rhodococcus obuensis</i> Tsukamura, 1982		
M. Tsukamura, E 8183; sputum	N935*	65.8
<i>Rhodococcus rhodnii</i> Goodfellow and Alderson, 1977		
P. Hill, Edinburgh University, UK, B/O; intestine of <i>Rhodnius prolixus</i>	N445*	72.3
<i>Rhodococcus rubropertinctus</i> (Hefferan) Tsukamura, 1974		
R. E. Gordon, 1240; A. J. Crowle	N59	70.6
R. E. Gordon, 8139	N66	68.7
ATCC 25689	R35	66.5
ATCC 25702; R. E. Gordon 384	R46	69.5
ATCC 25705; R. E. Gordon 462	R49	70.1
ATCC 25723; R. E. Gordon 1054	R65	68.2
<i>Rhodococcus sputi</i> (ex Tsukamura, 1978) Tsukamura and Yano, 1985		
M. Tsukamura, E 3884; sputum	N930*	66.7
M. Tsukamura, E 6130; sputum	N931	66.2
M. Tsukamura, E 6520; sputum	N932	67.6
<i>Rhodococcus</i> strains		
R. E. Gordon, 1067; Tarshis, SM-CV2/3/(cluster 1L; Goodfellow <i>et al.</i> , 1982a)	N865	67.4
R. E. Gordon, 1068; Tarshis, CM-CV3 (Cluster 1L)	N866	66.0
R. E. Gordon, 1070; Tarshis, INH-CV3 (Cluster 1L)	N868	66.3
R. E. Gordon, variant of 1070 (cluster 1L)	N869	66.2
E. G. Jeffreys, ICI Pharmaceuticals Division, Macclesfield, UK, strain GN smooth (cluster 1K, Goodfellow <i>et al.</i> , 1982a)	N913	66.7
E. G. Jeffreys, strain GN, rough (cluster 1K)	N914	65.0
M. Tsukamura, 8773; sputum (cluster Y)	N937	67.4
M. Tsukamura, 9138 (cluster Y)	N938	67.2

ATCC, American Type Culture Collection, Rockville, Md, USA.

* Type strains.

† The mean standard deviation for these results was 0.8 mol%.

DNA base composition studies. The thermal denaturation method of Marmur & Doty (1962) was used to determine the guanine (G) plus cytosine (C) content of the DNA extracted from the test organisms. The mol% G + C was calculated after Mandel & Marmur (1968), using the following equation: mol% G + C = $(T_m - 53.9)2.44$, where T_m is the melting temperature. Determinations were performed in $0.1 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M-NaCl plus $0.015 \text{ M-trisodium citrate}$, pH 7.0). Experiments were done three or four times; reference DNA was taken from '*Micrococcus lysodeikticus*' (*M. luteus*) ATCC 4696 and *Pseudomonas aeruginosa* ATCC 27853.

DNA homology experiments. Levels of DNA relatedness between the test strains were obtained using a nitrocellulose membrane filter technique (Mordarski *et al.*, 1976) and the S1 nuclease method of Crosa *et al.* (1973) as modified by Popoff & Coynault (1980) and Gładek *et al.* (1985). Optimal conditions, about 25°C below the melting temperature (T_m), were achieved using a formamide concentration of 20% (v/v) and a salt concentration above 0.4 M-NaCl (Wetmur & Davidson, 1968). Hybridization experiments were done at 62°C for 24 h in a hybridization mixture (0.5 ml) containing formamide (20%, v/v), NaCl (0.42 M), unlabelled, denatured DNA (150 µg) and sheared denatured labelled DNA (0.1 µg). After incubation, hybridization mixtures were cooled in an ice bath then supplemented with 1 ml ice-cold S1 nuclease buffer (0.05 M-NaCl, 0.045 M-sodium acetate, pH 4.6; 0.05 M- $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) containing 30 µg carrier (sheared denatured calf thymus DNA) ml^{-1} . Two samples of each mixture (0.5 ml) were treated with S1 nuclease (20 units) and a third sample (0.5 ml) left untreated; samples were incubated for 20 min at 58°C . S1-nuclease-treated duplexes were adsorbed onto DE-81 cellulose filters (Whatman, size 2.3 cm) and free nucleotides eluted with $0.14 \text{ M-Na}_2\text{HPO}_4$ buffer, pH 8.2. Additional steps in the procedure have been described in detail elsewhere (Popoff & Coynault, 1980; Gładek *et al.*, 1985).

Thermal stability of DNA : DNA duplexes. The thermal stability of duplexes formed between donor and reference DNA preparations were determined from $T_{m(e)}$ values as described previously (Mordarski *et al.*, 1976); $T_{m(e)}$ is the temperature at which half of the reassociated reference DNA becomes dissociated and eluted from test DNA bound to filters. $\Delta T_{m(e)}$ values were obtained by subtracting the $T_{m(e)}$ of duplexes of the heterologous systems from those of the homologous systems (Okanishi & Gregory, 1970). The thermal stability of duplexes was determined only when reassociation values of more than 30% were obtained.

RESULTS

DNA base compositions

The DNA base composition of the *Rhodococcus* strains ranged from 61 to 72 mol% G + C (Table 1), which is in agreement with previously published data (Mordarski *et al.*, 1980). Similarly, the DNA base composition of the control strains, '*M. lysodeikticus*' (*M. luteus*) ATCC 4696 (72 mol%) and *Pseudomonas aeruginosa* ATCC 27853 (66.4 mol%), is in line with earlier studies (Fasman, 1976).

DNA : DNA pairing : S1 nuclease/DE-81 method

The T_m of rhodococcal DNA was reduced by 14°C , from 101°C to 87°C , in the presence of 20% formamide. Under the optimal reassociation conditions described (20% formamide, 0.42 M-NaCl for 24 h at 62°C i.e. 25°C below T_m), over 90% of the single-stranded DNA hybridized in the homologous system. The corresponding value in the absence of formamide, and at the higher hybridization temperature of 76°C , was around 60%. Over 95% of single-stranded DNA and less than 5% of double-stranded DNA was digested by S1 nuclease in the presence of formamide. The concentration of formamide after the addition of the S1 nuclease buffer was 6.7% (v/v). Under these conditions good congruence was found between the results obtained using the S1 nuclease and the nitrocellulose membrane filter methods (Table 2). The hybridization values obtained using the two methods are the means of assays done in triplicate. Equally reproducible results were found using the two techniques.

DNA : DNA pairing : nitrocellulose membrane filter method

It is evident from Table 2 that *Rhodococcus* strains N913 and N914 belong to the *R. luteus* DNA homology group. It is also interesting that DNA from the remaining test strains had little affinity with reference DNA preparations from *Rhodococcus* strain N914 and *R. luteus* N1008. Similarly, all of the test organisms showed little DNA in common with reference DNA from the type strain of *R. globerulus* (Table 3). In contrast, several organisms showed high homology values with reference DNA from *R. sputi* N930. The duplexes formed between the *R. sputi*

Table 2. Levels of DNA relatedness amongst rhodococci determined using the nitrocellulose membrane filter and S1 nuclease/DE-81 methods with reference strains *R. luteus* N1008* and *Rhodococcus* N914

Source of unlabelled DNA		DNA binding (%)†			
		Source of labelled DNA:			
		<i>R. luteus</i> N1008*		<i>Rhodococcus</i> strain N914 (cluster 1K)	
Species/cluster	Strain	Nitrocellulose filter method	S1/DE-81 method	Nitrocellulose filter method	S1/DE-81 method
<i>R. globerulus</i>	R58*	9	10	11	15
<i>R. sputi</i>	N930*	8	9	9	5
<i>R. rubropertinctus</i>	R65	9	11	9	10
<i>Rhodococcus</i> cluster Y	N937	13	9	9	13
<i>Rhodococcus</i> cluster IL	N869	8	12	7	6
<i>Rhodococcus</i> cluster IL	N865	7	10	7	11
<i>R. obuensis</i>	N935*	8	8	9	13
<i>Rhodococcus</i> cluster Y	N938	13	4	10	15
<i>R. sputi</i>	N931	6	9	6	7
<i>Rhodococcus</i> cluster IL	N886	11	12	5	13
<i>R. equi</i>	R155	8	7	6	13
<i>R. rubropertinctus</i>	R49	6	14	5	16
<i>R. equi</i>	R156	9	14	8	11
<i>R. sputi</i>	N932	9	10	9	12
<i>Rhodococcus</i> cluster IL	N868	4	5	4	6
<i>R. aichiensis</i>	N933	14	8	9	7
<i>R. aichiensis</i>	N934*	9	6	8	8
<i>R. globerulus</i>	R43	8	8	9	10
<i>Rhodococcus</i> cluster IK	N913	69 (0.5)	74	100	100
<i>Rhodococcus</i> cluster IK	N914	84	81(1.0)	81 (0)	83
<i>R. luteus</i>	N1008*	100	100	81 (0)	77
<i>R. equi</i>	N812	12	12	7	13
<i>R. equi</i>	R814	13	10	10	5
<i>R. equi</i>	R128	19	9	4	7
<i>R. equi</i>	R123	16	8	15	10
<i>R. equi</i>	R164	11	8	15	10
<i>R. equi</i>	R167	14	13	13	15
<i>R. equi</i>	R172	12	ND	9	ND
<i>R. globerulus</i>	R55	15	8	13	7
<i>R. globerulus</i>	R56	9	12	9	12
<i>R. maris</i>	N1009*	7	8	6	7
<i>R. rhodnii</i>	N445*	12	10	11	15
<i>R. rubropertinctus</i>	R35	12	12	10	10
<i>R. rubropertinctus</i>	R46	13	6	7	7
<i>R. rubropertinctus</i>	R59	14	12	12	21
<i>R. rubropertinctus</i>	R66	9	9	9	11

ND, Not determined.

* Type strains.

† The figures in parentheses show $\Delta T_{m(e)}$, i.e. the difference between the thermal stability of the homologous and heterologous hybrids.

reference system and *R. equi* R155 and R156, *R. obuensis* N935, *R. rubropertinctus* R49 and R65, and all but one of the representatives of clusters 1L and Y showed high $T_{m(e)}$ values. On the basis of these DNA pairing data all of these strains can be assigned to the *R. sputi* homology group. Similarly, all of these strains, including *R. sputi* N930, exhibited high DNA homology values with the reference system based on *Rhodococcus* strain N865. The type strains of *R. luteus* and *R. maris* had little DNA in common with one another or with that of any of the other reference systems. This was also the case for most of the *R. equi* and *R. rubropertinctus* strains.

Table 3. Levels of DNA relatedness amongst rhodococci determined using the nitrocellulose filter method with reference strains *R. globerulus* R58*, *R. sputi* N930* and *Rhodococcus* N865

Source of unlabelled DNA		DNA binding (%)†		
		Source of labelled DNA:		
Species/cluster	Strain	<i>R. globerulus</i> R58*	<i>R. sputi</i> N930*	<i>Rhodococcus</i> strain N865 (cluster 1L)
<i>R. globerulus</i>	R58*	100	12	5
<i>R. sputi</i>	N930*	12	100	85 (0)
<i>R. rubropertinctus</i>	N65	14	101 (2.0)	69 (4.5)
<i>Rhodococcus</i> cluster Y	N937	12	98 (1.0)	39 (4.5)
<i>Rhodococcus</i> cluster 1L	N869	12	97 (2.5)	79 (1.5)
<i>Rhodococcus</i> cluster 1L	N865	13	94 (3.0)	100
<i>R. obuensis</i>	N935*	10	92 (0)	42 (5.5)
<i>Rhodococcus</i> cluster Y	N938	15	91 (0.5)	45 (5.5)
<i>R. sputi</i>	N931	13	89 (2.5)	49 (6.5)
<i>Rhodococcus</i> cluster 1L	N886	10	80 (3.5)	85 (0)
<i>R. equi</i>	R155	13	75 (2.5)	78 (1.0)
<i>R. rubropertinctus</i>	R49	10	71 (3.5)	50 (3.0)
<i>R. equi</i>	R156	15	68 (4.0)	39 (5.5)
<i>R. sputi</i>	N932	15	58 (10.5)	33 (9.5)
<i>Rhodococcus</i> cluster 1L	N868	11	54 (6.5)	44 (3.0)
<i>R. aichiensis</i>	N933	12	39 (8.0)	18
<i>R. aichiensis</i>	N934*	11	38 (10.0)	27
<i>R. globerulus</i>	R43	18	35 (6.0)	71 (1.0)
<i>Rhodococcus</i> cluster 1K	N913	15	12	7
<i>Rhodococcus</i> cluster 1K	N914	12	12	6
<i>R. luteus</i>	N1008*	13	8	5
<i>R. equi</i>	N812	11	9	4
<i>R. equi</i>	N814	21	13	7
<i>R. equi</i>	N128	14	19	5
<i>R. equi</i>	N123	17	15	6
<i>R. equi</i>	R164	15	15	7
<i>R. equi</i>	R167	15	14	6
<i>R. equi</i>	R172	15	15	8
<i>R. globerulus</i>	R55	16	10	4
<i>R. globerulus</i>	R56	8	10	3
<i>R. maris</i>	N1009*	12	11	4
<i>R. rhodnii</i>	N445*	18	11	7
<i>R. rubropertinctus</i>	R35	15	27	17
<i>R. rubropertinctus</i>	R46	13	23	11
<i>R. rubropertinctus</i>	R59	11	15	6
<i>R. rubropertinctus</i>	R66	11	28	19

* Type strain.

† The figures in parentheses show $\Delta T_{m(e)}$ (see Table 2).

DISCUSSION

The action of the S1 nuclease was only slightly altered in the presence of formamide, a result in line with previous reports (Furamai *et al.*, 1984; Shadhu & Datta, 1984). The modified S1 nuclease technique should be of value not only in DNA homology studies of rhodococci and related actinomycetes but also in similar investigations on micro-organisms with DNA rich in G + C.

In previous studies good congruence was found between DNA homology and numerical phenetic data though instances of misclassification were found, especially where strains lay towards the periphery of numerically circumscribed clusters (Mordarski *et al.*, 1976, 1977, 1980).

In most cases, members of numerically defined taxospecies show at least 60% DNA homology with reference DNA from cluster representatives, although DNA pairing values tend to fall to quite low levels with relatively small decreases in overall similarity values (Mordarski *et al.*, 1986). The results of the current investigation confirm and extend these earlier studies. Thus, the DNA relatedness data support the recognition of the species *R. globerulus*, *R. luteus* and *R. sputi*, although strains labelled *R. globerulus* had little DNA in common with the type strain of that species despite sharing relatively high similarity values with the latter (Goodfellow *et al.*, 1982*b*). It is also clear that the strains comprising clusters 1K and 1L (Goodfellow *et al.*, 1982*a*) should be classified as *R. luteus* and *R. sputi*, respectively.

Rhodococcus sputi was proposed by Tsukamura (1978) for strains assigned to a cluster defined in a numerical phenetic survey. The taxon, subsequently redescribed by Tsukamura & Yano (1985), is listed as a *species incertae sedis* in the current edition of *Bergey's Manual of Systematic Bacteriology* (Goodfellow, 1986). It is, however, evident from the present study that *R. sputi* merits species status. In contrast, the DNA homology data indicate that *R. obuensis* Tsukamura 1982 should be reduced to a subjective synonym of *R. sputi*.

The results of the present experiments show the importance of interpreting numerical taxonomies in light of DNA relatedness data obtained on representatives of numerically defined clusters. Indeed, the DNA pairing data underline the dangers inherent in proposing new species of *Rhodococcus* solely on the basis of small differences in overall similarity values. Nevertheless, it is clear that the numerical taxonomic and DNA homology methods are complementary, as *R. globerulus*, *R. luteus* and *R. sputi* are good species on the basis of both molecular genetic and numerical phenetic data. DNA relatedness studies need to be extended to confirm the status of *R. fascians*, *R. marinonascens*, *R. maris*, *R. rhodnii* and the recently described species *R. chlorophenolicus* (Apajalahti *et al.*, 1986).

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