Deoxyribonucleic Acid Reassociation in the Classification of the Genus *Rhodococcus* Zopf 1891 (Approved Lists, 1980)

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We determined the degree of binding between deoxyribonucleic acid (DNA) preparations from representative rhodococci and thymine-labeled DNAs (derived from labeled uracil) from the following six reference strains: *Rhodococcus coprophilus* (Approved Lists, 1980) strain N744, *R. corallinus* (Bergey et al. 1923) Goodfellow and Alderson 1979 (Approved Lists, 1980) strain N657, *R. equi* (Approved Lists, 1980) strain C7, *R. rhodochrous* (Approved Lists, 1980) strain N54, *R. ruber* (Approved Lists, 1980) strain N361, and *R. rubropertinctus* (Hefferan 1904) Goodfellow and Alderson 1979 (Approved Lists, 1980) strain N4. Although the congruence between the DNA reassociation and earlier numerical phenetic data was good, there was evidence that some strains had been misclassified in the numerical phenetic study. In particular, strains labeled *R. corallinus* (including the type strain of *R. corallinus*) and *R. rubropertinctus* belonged to a single DNA homology group. The former name is thus a later, subjective synonym of the latter. The guanine plus cytosine contents of the DNAs of the rhodococci fell within the range of 65 to 73 mol%.

The genus *Rhodococcus* Zopf 1891 (34) (Approved Lists, 1980 [29]) was reintroduced (9), was introduced the basis of numerical phenetic data, for some organisms previously classified as *Corynebacterium* (2), *Gordona* (32), *Jensenia* (1), *Proactinomyces* (4, 18), *Nocardia* (2, 20), and "Mycobacterium" *rhodochrous* (2, 15, 16, 28) or assigned to the "rhodochrous" complex (2, 10, 11). In addition to the type species, *Rhodococcus rhodochrous*, nine species were recognized, including *R. coprophilus* (9, 26), which contains strains found in herbivore dung (27).

Because numerical relationships between strains can be distorted by factors such as sampling and test errors, differences in growth rate, etc., it is important to evaluate numerical classifications by using other powerful taxonomic methods (30). Thus, after numerical analysis, representative strains can be selected from defined clusters and examined by using more analytical methods which cannot readily be applied to many strains. The agreement found among data derived from the application of several independent taxonomic methods is a measure of the reliance that can be placed in a classification (14, 30).

Good agreement has been found between deoxyribonucleic acid (DNA)-DNA pairing and numerical phenetic data in a number of genera, including some of those in the order *Actinomycetales* (Approved Lists, 1980 [29]) (30, 31). In

most instances, strains classified in well-defined taxospecies have approximately 70% DNA in common, with lower homology values reflecting significant genetic divergence (7). However, DNA homology values should not be regarded as absolute values, for they are not free from experimental error and are especially sensitive to the temperature at which reassociation is conducted (3, 13).

In earlier DNA-DNA pairing assays, representatives of several species of *Rhodococcus* formed genetically homogeneous taxa (23, 24). In this investigation these preliminary experiments were extended to include additional representative rhodococci and reference systems in order to help establish the infrageneric classification of the genus.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The test organisms and their sources are given in Table 1. Detailed descriptions exist for most of the strains (8, 9). The procedures for cultivating strains and preparing biomass and the method used for obtaining purified DNA have all been described in detail elsewhere (23).

Preparations of radioactive DNA for homology studies. Uracil-labeled cells were grown and centrifuged, and the thymine-labeled DNA derived from the uracil was extracted and purified as described previously (23). Labeled reference DNA was prepared from the following six strains: *R. coprophilus* N744

TABLE 1. *Designations, sources, and DNA base compositions of test strains*

Strain and source ^a	Laboratory no.	G+C content of DNA (mol%)
<i>R. coprophilus</i> Rowbotham and Cross 1979 (Approved Lists 1980 [29])		
T.J. Rowbotham, Bradford University, England, CUB 628; L.G. Wil- loughby, lake mud, Blelham Tarn, Cumbria	N650	71.1
T.J. Rowbotham, CUB 118; T. Cross, lake mud, Blelham Tarn	N651	67.4
T.J. Rowbotham, N38	N652	72.0
T.J. Rowbotham, N19	N653	69.8
NCIB 11211; T.J. Rowbotham, CUB 687	N744 ^b	69.3
<i>R. corallinus</i> (Bergey et al. 1923) Goodfellow and Alderson 1979 (Ap- proved Lists, 1980 [29])		
R. Bönicke, Institut für Experimentelle Biologie und Medizin, Borstel, Federal Republic of Germany, SN5302	N451	67.1
NCTC 10668; M. Tsukamura, 3605	N657 ^b	69.0
<i>R. egui</i> (Magnusson 1923) Goodfellow and Alderson 1979 (Approved Lists, 1980 [29])		
NCTC 1621	C7 ^b	70.4
ATCC 25694	R39	70.8
ATCC 25728; R.E. Gordon, 1256	R70	69.2
ATCC 25729; R.E. Gordon, 1621	R71	70.3
<i>R. rhodochrous</i> (Zopf 1891) Tsukamura 1974 (Approved Lists, 1980 [29])		
NCIB 9701	N5	69.8
R.E. Gordon, Rutgers University, New Brunswick, N.J., W21	N31	69.1
ATCC 13808; R.E. Gordon, 372	N54 ^b	69.5
R.E. Gordon, W3408	N61	70.1
S.T. Williams, Botany Department, Liverpool University, England, E41	N84	72.7
R.E. Gordon, W3639	N110	68.6
ATCC 999	R4	69.8
ATCC 4001	R5	68.6
ATCC 14341	R10	67.5
ATCC 14349	R14	69.1
ATCC 25722; R.E. Gordon, 1022	R64	68.2
ATCC 25725; R.E. Gordon, 1082S	R67	67.0
ATCC 25731; R.E. Gordon, W3407	R73	67.7
<i>R. ruber</i> (Kruse 1896) Goodfellow and Alderson 1979 (Approved Lists, 1980 [29])		
ATCC 15998	N324	71.7
M. Tsukamura, Chuba Chest Hospital, Obu, Aichi-ken, 474, Japan, M-1	N361	72.3
M. Tsukamura, M-192; R.E. Gordon, R-680	N363	72.6
A. Tacquet, Pasteur Institute, Lille, France, 906	N420	71.4
R. Bönicke, SN5108	N447	71.5
R. Bönicke, SN5201	N454	70.4
R. Bönicke, SN5102	R179	72.2
R. Bönicke, SN5103	R180	72.0
R. Bönicke, SN5104	R181	72.5
R. Bönicke, SN5105	R182	71.5
R. Bönicke, SN5107	R183	70.5
R. Bönicke, SN5108	R184	72.4
R. Bönicke, SN5109	R185	70.5
R. Bönicke, SN5110	R186	71.5
R. Bönicke, SN5112	R187	70.8
<i>R. rubropertinctus</i> (Hefferan 1904) Goodfellow and Alderson 1979 (Ap- proved Lists, 1980 [29])		
NCIB 9664	N4 ^b	67.7
V.B.D. Skerman, Queensland University, Australia, 134	N113	69.1
M. Turner, Nottingham University, England, 39	N146	65.1
ATCC 25689	R35	68.2

^a ATCC, American Type Culture Collection, Rockville, Md.; CUB, Collection of the University of Bradford, Bradford, England; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland.

^b Type strain (29).

(=NCIB 11211), *R. corallinus* N657 (=NCTC 10668), *R. equi* C7 (=NCTC 1621), *R. rhodochrous* N54 (=ATCC 13808), *R. ruber* N361 (=M. Tsukamura M-1), and *R. rubropertinctus* N4 (=NCIB 9664). The specific activities of the reference DNA preparations are shown in Tables 2 and 3.

Analysis of DNA base compositions. The guanine plus cytosine (G+C) contents of the DNAs were determined by the thermal denaturation method of Marmur and Doty (19); the denaturations were performed in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M trisodium citrate).

Fixation of denatured, high-molecular-weight DNA on membrane filters and DNA-DNA pairing. Homology values were determined by establishing the extent to which immobilized, single-stranded DNA bound labeled reference DNA in solution. The pairing was performed for 18 h at 64°C on nitrocellulose membranes (Sartorius SM-1140) in 2× SSC containing 30% (vol/vol) dimethyl sulfoxide. The thermal denaturation (T_m) values of the DNAs used in these experiments varied depending on the G+C content; the mean optimal temperature was 86°C. Further details of the methods used have been described elsewhere (24).

Thermal stability of DNA-DNA duplexes. The thermal stability of duplexes formed between filter-bound and reference DNAs was determined from $T_{m(e)}$ values. ($T_{m(e)}$ was the temperature at which one-half of the reassociated reference DNA became dissociated and eluted from the test DNA bound to the filter.) The $T_{m(e)}$ was found by subtracting the $T_{m(e)}$ of the hybrid of a heterologous system from the $T_{m(e)}$ of a homologous duplex (25). Thus, once pairing was completed, the filters were dried and then eluted with 0.1× SSC at temperature increments of 5°C over the range 70 to 100°C. The radioactive samples were assayed in the scintillation fluid of Bray (6). The thermal stability of duplexes was determined only where reassociation values of more than 30% were obtained.

RESULTS AND DISCUSSION

The case for the reintroduction of the genus *Rhodococcus* (9) has been supported by the

results of further numerical phenetic, chemical, and genetic studies (10, 11, 21, 27, 33). In preliminary studies on representatives of some of the recognized *Rhodococcus* species, good congruence was found between DNA-DNA homology and numerical phenetic data (23, 24). Thus, although there are difficulties in relating homology and similarity values, *R. bronchialis* (Approved Lists, 1980 [29]), *R. erythropolis* (Gray and Thornton 1928) Goodfellow and Alderson 1979 (Approved Lists, 1980 [29]), *R. rhodochrous*, *R. ruber*, and *R. terrae* (Approved Lists, 1980 [29]) appeared to be good species on both numerical phenetic and genetic grounds (12). The results of the present study confirm and extend these earlier observations (Tables 1, 3, and 4). The DNA reassociation rates of the reference strains are shown in Table 2. It is evident that *R. corallinus* N657 and *R. rubropertinctus* N4 are closely related and that the remaining reference systems are quite distinct.

The DNA-DNA pairing data confirmed the homogeneity of the type species, *R. rhodochrous*, and showed that three strains were misplaced in this taxon (Table 3). *R. rhodochrous* R67 shares little genetic similarity with any of the reference systems and should be included in future comparative studies, whereas *R. rhodochrous* N84 and N110 should be reclassified as *R. ruber*. In contrast, *R. ruber* N454, together with *R. equi* R70, should be transferred to *R. rhodochrous*. Since *R. rhodochrous* and *R. ruber* share a high intercluster similarity (9), factors such as test or sampling error might account for the observed misclassification of strains between these species. All of the *R. rhodochrous* strains have a narrow DNA base composition range of 67.5 to 70.4 mol% G+C (Table 1). It is well known that base ratio estimations are influenced by the methods and formulas used (5); the lower

TABLE 2. DNA-DNA reassociation rates of the reference strains^a

Laboratory no. of strain	DNA	% Reassociation with strain:											
		C7		N4		N54		N361		N657		N744	
		Unlabeled DNA	Labeled DNA	Unlabeled DNA	Labeled DNA	Unlabeled DNA	Labeled DNA	Unlabeled DNA	Labeled DNA	Unlabeled DNA	Labeled DNA	Unlabeled DNA	Labeled DNA
C7	Labeled	100		8		16		13		8		13	
	Unlabeled				8		11		13		6		13
N4	Labeled			100		10		9		58		8	
	Unlabeled						9		10		74		7
N54	Labeled					100		17		9		15	
	Unlabeled							23		8		20	
N361	Labeled							100		10		13	
	Unlabeled									6		14	
N657	Labeled									100		7	
	Unlabeled											7	
N744	Labeled											100	

^a The reassociation conditions are described in Table 3, footnote a.

TABLE 3. Reassociation of DNA samples from *Rhodococcus* strains with DNAs from reference strains *R. rhodochrous* N54, *R. ruber* N361, and *R. coprophilus* N744^a

DNA source		Labeled DNA source					
Species	Strain	<i>R. coprophilus</i> N744 ^b		<i>R. rhodochrous</i> N54 ^b		<i>R. ruber</i> N361	
		Relative amt of DNA bound (%)	$T_{m(e)}$ (°C)	Relative amt of DNA bound (%)	$T_{m(e)}$ (°C)	Relative amt of DNA bound (%)	$T_{m(e)}$ (°C)
<i>R. coprophilus</i>	N744 ^b	100		15 ± 0 ^c		13 ± 1	
	N651	68 ± 3	1	20 ± 0.5		14 ± 0.5	
	N650	51 ± 1	1	20 ± 0.5		99 ± 2	3
	N653	43 ± 2	0.5	20 ± 1		17 ± 0.5	
	N652	8 ± 1		11 ± 0.5		13 ± 1	
<i>R. rhodochrous</i>	N54 ^b	20 ± 1		100		23 ± 0.5	
	N61	18 ± 0.5		93 ± 2	1	26 ± 1	
	N31	21 ± 1		92 ± 3	0	22 ± 1	
	R10	15 ± 1		84 ± 4	0	19 ± 1	
	R14	17 ± 0		79 ± 0	1	22 ± 1	
	R73	19 ± 0		72 ± 0	1.5	23 ± 1	
	R4	16 ± 1		62 ± 2	1.5	16 ± 1	
	R64	20 ± 1		58 ± 0	4.5	25 ± 1	
	N5	11 ± 0.5		50 ± 0	0	15 ± 0.5	
	R5	7 ± 0		40 ± 0	3.5	10 ± 0.5	
	R67	7 ± 0.5		26 ± 0		7 ± 0	
	N84	14 ± 0		23 ± 0.5		96 ± 4	1
	N110	12 ± 1		15 ± 1		89 ± 3	1
	N361	14 ± 1		17 ± 0		100	
	N447	16 ± 2		26 ± 1		114 ± 1	3
	R181	15 ± 1		23 ± 1		104 ± 3	1
	R184	13 ± 0		20 ± 0.5		104 ± 5	0
<i>R. ruber</i>	R186	15 ± 1		24 ± 0		104 ± 3	0
	N420	11 ± 0		17 ± 0		102 ± 3	0
	R179	14 ± 0.5		22 ± 0.5		100 ± 1	3
	N363	14 ± 0		20 ± 0		97 ± 3	1
	R180	13 ± 0.5		18 ± 0.5		97 ± 0.5	2
	R182	15 ± 0.5		18 ± 0.5		91 ± 2	1
	R187	13 ± 0		19 ± 0		89 ± 1	0.5
	R183	13 ± 0		23 ± 0.5		87 ± 4	0
	N324	9 ± 0		16 ± 1		79 ± 5	1.5
	R185	8 ± 1		13 ± 0		71 ± 3	0
	N454	21 ± 0.5		65 ± 1	4.0	27 ± 1	
	N657 ^b	7 ± 0.5		9 ± 0.5		10 ± 0	
	N451	6 ± 0		8 ± 0		9 ± 0.5	
	C7 ^b	13 ± 1		11 ± 0		13 ± 0.5	
	R39	11 ± 0.5		15 ± 0		18 ± 0.5	
	R71	10 ± 0		13 ± 0.5		33 ± 1	0
	R70	19 ± 0.5		74 ± 0	0.5	25 ± 0.5	
<i>R. rubroperitinctus</i>	N4 ^b	7 ± 0.5		9 ± 0		10 ± 0	
	R35	7 ± 0		8 ± 1		9 ± 0	
	N113	6 ± 0.5		8 ± 0.5		7 ± 0	
	N146	7 ± 0.5		8 ± 0		9 ± 0.5	

^a The extent of binding between the DNA from a test strain and labeled DNA from a reference strain is expressed as a percentage relative to the extent of binding between labeled and unlabeled DNAs from the reference strain itself. $\Delta T_{m(e)}$, a measure of the thermal stability of the DNA-DNA duplexes, was obtained by subtracting the $T_{m(e)}$ (the midpoint of thermal elution) of the molecular hybrids of the heterologous system from the $T_{m(e)}$ of the homologous hybrids. The ratio of labeled DNA to unlabeled DNA was 1:15. The background imp per minute per membrane did not exceed 1 to 2% of the value in the homologous system.

^b Type strain (29). The specific activities of DNA samples from the reference strains were as follows: *R. coprophilus* N744, 6,288 cpm/μg; *R. rhodochrous* N54, 4,571 cpm/μg; and *R. ruber* N361, 2,473 cpm/μg.

^c Mean ± standard error.

TABLE 4. Reassociation of DNA samples from *Rhodococcus* strains with DNAs from reference strains *R. corallinus* N657, *R. equi* C7, and *R. rubropertinctus* N4^a

DNA source		Labeled DNA source					
Species	Strain	<i>R. corallinus</i> N657 ^b		<i>R. equi</i> C7 ^b		<i>R. rubropertinctus</i> N4 ^b	
		Relative amt of DNA bound (%)	<i>T</i> _{m(e)} (°C)	Relative amt of DNA bound (%)	<i>T</i> _{m(e)} (°C)	Relative amt of DNA bound (%)	<i>T</i> _{m(e)} (°C)
<i>R. coprophilus</i>	N744 ^b	7 ± 0 ^c		13 ± 0		8	
	N651	6 ± 1		10 ± 0.5		12	
	N650	6 ± 0		16 ± 1		12	
	N653	6 ± 0		11 ± 0.5		11	
	N652	6 ± 0		78 ± 2	1	11	
<i>R. corallinus</i>	N657 ^b	100		8 ± 0		58	1
	N451	71 ± 3	4	10 ± 0		68	0
<i>R. equi</i>	C7 ^b	6 ± 0		100		8	
	R39	7 ± 0		99 ± 3	0.5	10	
	R71	7 ± 0.5		98 ± 2	0.5	10	
	R70	7 ± 0		15 ± 1		10	
<i>R. rubropertinctus</i>	N4 ^b	74 ± 4	3	8 ± 0		100	
	R35	53 ± 1	1	10 ± 0.5		43	2.5
	N113	52 ± 4	1	7 ± 0		38	2
	N146	5 ± 0		10 ± 0.5		8	
<i>R. rhodochrous</i>	N54 ^b	8 ± 0		16 ± 0		10	
	N61	7 ± 0		15 ± 1		11	
	N31	8 ± 0		16 ± 0.5		9	
	R10	6 ± 0.5		14 ± 0.5		9	
	R14	8 ± 0		17 ± 0		11	
	R73	7 ± 0		15 ± 0.5		13	
	R4	6 ± 0		14 ± 0.5		8	
	R64	9 ± 0.5		18 ± 1		12	
	N5	4 ± 0		10 ± 0		7	
	R5	3 ± 0		7 ± 0		5	
	R67	4 ± 0		7 ± 0.5		6	
	R84	8 ± 0.5		20 ± 0.5		12	
	N110	6 ± 0		15 ± 0.5		8	
	N361	6 ± 1		15 ± 1		9	
	N447	9 ± 0.5		19 ± 1		16	
	R181	7 ± 0.5		20 ± 0		13	
	R184	6 ± 0		15 ± 0.5		11	
<i>R. ruber</i>	R186	9 ± 0.5		17 ± 0		13	
	N420	5 ± 0		12 ± 0.5		11	
	R179	8 ± 0		20 ± 0.5		13	
	N363	9 ± 0		16 ± 0		12	
	R180	6 ± 0		15 ± 0.5		10	
	R182	7 ± 0		15 ± 1		12	
	R187	6 ± 0.5		12 ± 1		8	
	R183	7 ± 0.5		15 ± 1		12	
	N324	4 ± 0		12 ± 1		7	
	R185	4 ± 0		9 ± 0		6	
	N454	8 ± 0		16 ± 0		14	

^a See Table 3, footnote a.^b Type strain (29). The specific activities of DNA samples from the reference strains were as follows: *R. corallinus* N657, 7,000 cpm/μg; *R. equi* C7, 1,551 cpm/μg; and *R. rubropertinctus* N4, 7,596 cpm/μg.^c Mean ± standard error.

values recorded in earlier studies on rhodococci were based upon a chemical method (17), whereas those in the present study were determined by using the thermal denaturation method (19).

The recovery of *R. ruber* as a genetically homogeneous species is in good agreement with earlier nucleotide homology data (24) and with the results from chemical and fermentation studies (22). *R. coprophilus* N650 is almost identical genetically to *R. ruber* N361, but it also shares a lot of DNA in common with the *R. coprophilus* reference system. The *R. ruber* strains have a narrow base composition range (68.6 to 72.7 mol% G+C).

The DNA homology data support the status of *R. coprophilus* and *R. equi* as distinct species (9, 26) inasmuch as the type strains of each of these taxa have little DNA in common with one another or with the other reference systems studied (Tables 3 and 4). It is evident that *R. coprophilus* N652, which was not included in the numerical phenetic study of Rowbotham and Cross (26), should be transferred to *R. equi*. The *R. coprophilus* and *R. equi* strains form homogeneous groups on the basis of base composition, falling within the ranges of 67.4 to 69.8 and 70.3 to 72.0 mol% G+C, respectively.

In the numerical classification of Goodfellow and Alderson (9), subcluster 1G was labeled *R. rubropertinctus* because the name *Nocardia rubropertincta* was considered to have priority over other validly published names carried by type strains recovered in the subcluster. Most of the strains in subcluster 1G shared very high similarity values, but a few less closely related organisms found at or toward the periphery of the subcluster also showed a high affinity with the *R. corallinus* strains of subcluster 1C (9). Organisms found toward the periphery of the *R. rubropertinctus* subcluster included the type strain of *R. rubropertinctus*, strain N4, and strain R35. Subsequently, the analysis of menaquinone and mycolic acid composition provided further evidence of the heterogeneity of *R. rubropertinctus* (21).

It is evident from the DNA-DNA pairing data (Table 4) that *R. corallinus* N451 and N657 (the type strain of *R. corallinus*) should be classified in the same species as *R. rubropertinctus* N4, R35, and N113. Since *R. rubropertinctus* (Hefner 1904) Goodfellow and Alderson has priority over *R. corallinus* (9), the latter becomes a later subjective synonym of *R. rubropertinctus*. Strains classified in this revised taxon have a DNA base composition within the range of 67.1 to 69.1 mol% G+C.

The good agreement found between the DNA homology and numerical phenetic data is en-

couraging, but the results of the present study emphasize the importance of evaluating numerical classifications by using other powerful taxonomic methods. Although taxometric techniques provide a relatively quick and accurate way of determining zones of variation among poorly classified strains, it is not always easy to lay down sharp boundaries between defined clusters. It is clear from the present study, however, that the numerical phenetic and DNA reassociation methods are complementary and together provide a powerful way of clarifying the infrageneric relationships of ill-defined taxa.

The numerical phenetic and genetic data indicate that *R. bronchialis*, *R. coprophilus*, *R. equi*, *R. erythropolis*, *R. rhodochrous*, *R. ruber*, and *R. terrae* are good species. The DNA homology results show that the name *R. corallinus* should be reduced to the status of a junior synonym of *R. rubropertinctus*, with strain N4 (=NCIB 9664) as the type strain of the redefined species. Our studies are being extended to determine whether *R. rhodnii* (Approved Lists, 1980 [29]) and the rhodococci remaining in subcluster 1G form distinct homology groups.

REPRINT REQUESTS

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