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Classification of the "Rhodochrous" Complex and Allied Taxa Based upon Deoxyribonucleic Acid Reassociation

M. MORDARSKI, M. GOODFELLOW, K. SZYBA, G. PULVERER, AND A. TKACZ

Department of Biosynthesis, Institute of Immunology and Experimental Therapy, Wrocław, Poland; Department of Microbiology, The Medical School, The University, Newcastle upon Tyne NE1 7RU, United Kingdom; and Institute of Hygiene, University of Cologne, Cologne, Federal Republic of Germany

The degree of binding was determined between deoxyribonucleic acid (DNA) preparations from nocardiae and "rhodochrous" strains and uracil-labeled DNA from three reference strains, *Nocardia asteroides* N668 and rhodochrous strains N11 and N54. In all cases, good congruence was found between the DNA reassociation data and that from numerical phenetic studies. Only a small degree of nucleotide sequence homology was found between the *N. asteroides* reference system and the other taxa studied, and there was evidence that *N. asteroides* is genetically heterogeneous. The moles percent guanine plus cytosine for the rhodochrous strains was within the range 58 to 67; the corresponding range for nocardiae was 64 to 68 mol%.

The confused and tortuous taxonomic history of bacteria variously known as "*Mycobacterium*" *rhodochrous*, the "rhodochrous" complex, and the "rhodochrous" taxon was reviewed by Bousfield and Goodfellow (5), who concluded that these organisms form a recognisable taxon equivalent in rank to the genera *Corynebacterium*, *Nocardia*, and *Mycobacterium*. The internal structure of the rhodochrous complex is still obscure, but the results of chemical (2, 3, 17), numerical phenetic (17, 39), deoxyribonucleic acid (DNA) reassociation (10), genetic recombination (1), and serological (24) studies show that subgroups exist.

The heterogeneity of the rhodochrous complex is most clearly seen in numerical phenetic studies in which two or more homogeneous phena have been recognised (6, 16, 17, 22, 35). However, since few strains are common to all of these investigations, it is difficult to determine whether or not, and to what extent, the phena overlap. In an extensive numerical taxonomic study, Goodfellow and Alderson (J. Gen. Microbiol., in press) divided 150 representative rhodochrous strains into 10 homogeneous subclusters (1A through 1J). Subcluster 1A was equated with *N. rubra* (6), *Gordona rhodochroa* (38), and phenon 1a (17); subclusters 1B and 1E were equated with phena 14B and 14A (16), respectively; subcluster 1D was equated with *N. pellegrino* (28); subclusters 1F and 1G were equated with phena 1c and 1b (17), respectively; subcluster 1H was equated with *N. erythropolis* (6) and phena 14D (16) and F3 (22); and subcluster 1J was equated with bacteria given the trivial name "Lspi" (large, spored, pink, irregular) (15). Subcluster 1C can be subdivided further and contains strains of *G. bronchialis*, *G. rubra*, and *G. terrae* (37).

Good congruence has been observed between nucleotide sequence homology and numerical phenetic data in a number of genera including actinomycete taxa (13, 19, 34). In most cases, taxospecies share at least 70% DNA homology, and lower binding values are considered to reflect significant genetic divergence (12). In an earlier DNA reassociation study, Mordarski et al. (29) found that representatives of *G. bronchialis*, *G. rubra*, *G. terrae*, *N. pellegrino* (28), and phenon 1a (17) formed DNA-homology groups. In view of these encouraging results, we have extended our DNA reassociation assays on nocardioform bacteria and in this study show the degree of binding between DNA preparations from nocardiae and rhodochrous strains and uracil-labeled DNA from three reference systems including the type strain of "*Mycobacterium*" *rhodochrous*.

MATERIALS AND METHODS

Test strains and growth conditions. Details of strains and their sources are given in Table 1. All cultures were maintained routinely on yeast extract agar at room temperature.

For DNA extraction, organisms were grown in shake cultures on a reciprocal shaker (160 strokes per min) for 30 to 40 h at 37°C in modified Sautons medium (27) or in peptone-yeast extract broth (7). Cultures were checked for purity, harvested by centrifugation, washed in 0.15 M saline-0.1 M ethylenediaminetetraacetate (pH 8.0), and stored at −20°C.

Extraction of DNA. DNA was extracted from harvested bacteria by using the materials and methods described previously (29). All DNA preparations

TABLE 1. *Designation, sources, and DNA base composition of test strains*[a]

Cluster	Strain/source	G+C (mol%)
1[b] (rhodochrous complex)	Subcluster 1A[b]	
	N5: Rhodochrous strain (*Nocardia salmonicolor*), NCIB 97019	65
	N54: Rhodochrous strain ("*Mycobacterium*" *rhodochrous*), ATCC 13808; R.E. Gordon, Rutgers University, New Brunswick, N.J. 372 (*Rhodococcus rhodochrous*); type strain	62
	N61: Rhodochrous strain ("*Mycobacterium*" *rhodochrous*); R.E. Gordon, W3408 (*Nocardia corallina*); ATCC 4273	66
	N84: Rhodochrous strain (*N. corallina*); S.T. Williams, Botany Department, University of Liverpool, Liverpool, England	69
	Subcluster 1B[b]	
	N31: Rhodochrous strain ("*Mycobacterium*" *rhodochrous*); R.E. Gordon, W21	65
	Subcluster 1C[b]	
	N451: Rhodochrous strain (*N. corallina*); R. Bönicke, Institut für experimentelle Biologie und Medizin, Borstel, West Germany, SN5302	64
	Subcluster 1D[b]	
	N365: Rhodochrous strain (*Nocardia rubra*)	66
	N420: Rhodochrous strain (*Nocardia pellegrino*); A. Tacquet, Pasteur Institute, Lille, France, 906	58
	N447: Rhodochrous strain (*N. pellegrino*); R. Bönicke, SN5108	68
	N454: Rhodochrous strain (*N. rubra*); R. Bönicke, SN5201	66
	Subcluster 1E[b]	
	N324: Rhodochrous strain (*N. pellegrino*), ATCC 15998	68
	N325: Rhodochrous strain (*N. pellegrino*); G. Castelnuovo, Instituto Superiore Di Sanita, Rome, Italy, 106B	68
	Subcluster 1G[b]	
	R35: Rhodochrous strain, ATCC 25689	65
	R43: Rhodochrous strain, ATCC 25699	63
	R46: Rhodochrous strain (*Mycobacterium rubrum*), ATCC 25702; R.E. Gordon, 384	67
	N146: Rhodochrous strain (*Corynebacterium fascians*), M. Turner, Nottingham University, Nottingham, England, 39	62
	Subcluster 1H[b]	
	N11: Rhodochrous strain (*Nocardia erythropolis*), NCIB 9158	67
	Subcluster 1I[b]	
	N443: Rhodochrous strain (*Nocardia rhodnii*); P. Hill, Edinburgh University, Edinburgh, Scotland, A1	66
	Subcluster 1J[b]	
	N650: Rhodochrous strain (*Rhodococcus coprophilus*); T. Rowbotham, Bradford University, Bradford, England, CUB 628	64
	N651: Rhodochrous strain (*R. coprophilus*); T. Rowbotham, CUB 118	64
	N652: Rhodochrous strain (*R. coprophilus*); T. Rowbotham, N38	60
	N653: Rhodochrous strain (*R. coprophilus*); T. Rowbotham, N19	63
	Subgroup 14E[c]	
	N124: Rhodochrous strain (*Nocardia opaca*), CBS 330.61	65
1[c] (*Nocardia asteroides* complex)	N13: *N. asteroides*, NCTC 8595, ATCC 14759	64
	N668: *N. asteroides*; S.G. Bradley, Department of Microbiology, Virginia Commonwealth University, Richmond, Va., VAC 462	67
	N670: *N. asteroides;* S.G. Bradley, VAC 324	66
	N671: *N. asteroides;* S.G. Bradley, VAC 330	68
	N672: *N. asteroides;* S.G. Bradley, VAC 333	67

TABLE 1—*Continued*

Cluster	Strain/source	G+C (mol%)
	N675: *Nocardia carnea;* R.E. Gordon 3419; ATCC 6847	68
	N676: *N. carnea;* R.E. Gordon, 3419; ATCC 6847	64
	N537: *Nocardia salmonicida,* NRRL B-2778; type strain	66
	N680: *Nocardia transvalensis;* R.E. Gordon, IMRU 3246; NCTC 2392; ATCC 6865	67
2[c] (*Nocardia caviae*)	N36: *N. caviae,* NCTC 1934; ATCC 14629; type strain	67
	N231: *N. caviae;* R. Olds, Pathology Department, Cambridge University, Cambridge, England, CN749	67
	N313: *N. caviae;* F. Mariat, Institut Pasteur, Paris, 751	66
	N563: *N. caviae;* D. Frey, The Royal North Shore Hospital of Sydney, St. Leonards, N.S.W., Australia, 1912	66
5[c] (*Nocardia brasiliensis*)	N318: *N. brasiliensis,* ATCC 19296; type strain	67
7[c] (*Actinomadura madurae*)	N306: *A. madurae;* F. Mariat, 767	65
	Strain not included in the numerical analyses	
	N667: *Nocardia amarae;* M. Lechevalier, IMRU W3960; ATCC 27808; type strain	65

[a] ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centralbureau voor Schimmelcultures, Baarn, The Netherlands; NCIB, National Collection of Industrial Bacteria, Aberdeen, Scotland; NCTC, National Collection of Type Cultures, London, England; NRRL, Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill.

[b] Phena recovered by Goodfellow and Alderson (in press).

[c] Phena recovered by Goodfellow (16).

used for reassociation showed $E_{260}/E_{280} > 1.9$ and $E_{225}/E_{230} > 2.1$, and gave a negative Lowry test for proteins and a negative concanavaline A diffusion test for carbohydrate contamination. Reaction with orcinol for contaminating RNA indicated less than 5% ribonucleic acid (RNA). The purified DNA was stored at 5°C in 0.1× SSC (SSC = 0.15 M NaCl-0.015 M trisodium citrate) containing a few drops of chloroform.

Preparation of radioactive DNA for homology studies. [2-^{14}C]uracil (Nuclear Research Institute, Swierk, Poland; specific activity, 9.6 to 12.3 mCi/mol) was added to inoculated media to give a final concentration of 1 μgCi/ml. Labelled organisms were centrifuged and the DNA was extracted and purified as described previously (29).

Labelled reference DNA was prepared from three strains: *N. asteroides* N668 and rhodochrous strains N11 (NCIB 9158) and N54 (ATCC 13808). The specific activities of the reference DNA preparations are shown in Table 2.

Analysis of base compositions. The moles percent guanine plus cytosine content was determined by the method of Huang and Rosenberg (21).

Fixation of denatured, high-molecular-weight DNA on membrane filters and DNA:DNA pairing. The relationships between the test strains were established by determining the extent to which immobilised single-stranded DNA bound labelled reference DNA in solution. The reassociation was performed on nitrocellulose membranes (Sartorius SM-1140) in 2× SSC solution plus 30% (vol/vol) dimethyl sulphoxide for 18 h at 65°C. The methods used were described in detail elsewhere (29).

Thermal stability of DNA:DNA duplexes. The thermal stability of duplexes formed between filter-bound DNA and the reference DNA preparations was determined from the $T_{m(e)}$ values. [$T_{m(e)}$ is the temperature at which half of the reassociated reference DNA becomes dissociated and eluted from the test DNA bound to the filter.] The midpoint of the thermal elution, $\Delta T_{m(e)}$, was found by subtracting the $T_{m(e)}$ of hybrids of the heterologous system from the $T_{m(e)}$ of homologous hybrids (30).

After pairing was completed, the filters were dried and then eluted with 0.1x SSC solution at temperature increments of 5°C over the range 70 to 100°C. The radioactive samples were assayed in the scintillation fluid of Bray (11).

RESULTS

DNA base compositions. The moles percent guanine plus cytosine (G+C) for the rhodochrous strains was 58 to 67; the corresponding range for the *Nocardia* strains was 64 to 68 mol% (Table 1).

DNA:DNA pairing. The DNA preparations from all of the subcluster 1A strains bound significant amounts of reference DNA from the rhodochrous type strain (N54) but showed little affinity with that from rhodochrous strain N11 and *N. asteroides* N668. Rhodochrous strains N31 and N454, from subclusters 1B and 1D, respectively, exhibited a high degree of homology with the reference DNA from rhodochrous strain N54; a much smaller degree of nucleotide sequence homology was found between this ref-

TABLE 2. *Reassociation of DNA samples from Nocardia, Actinomadura, and rhodochrous strains with the DNA from reference strains Nocardia asteroides N668 and rhodochrous strains N11 and N54*[a]

DNA source	Labelled DNA source								
	Rhodochrous strain N11			Rhodochrous strain N54			*Nocardia asteroides* N668		
	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)
Subcluster 1A									
N5: Rhodochrous strain	21	1		83	2	5	19	2	
N54: Rhodochrous strain	16	1		100			18	2	
N61: Rhodochrous strain	18	1		85	4	1	19	1	
N84: Rhodochrous strain	13	<1		96	6	1	15	1	
Subcluster 1B									
N31	40	6	>15	130	15	0	28	4	11
Subcluster 1C									
N451: Rhodochrous strain	31	1	>15	18	4		19	0	
Subcluster 1D									
N420: Rhodochrous strain	15	1		20	1		11	1	
N447: Rhodochrous strain	17	2		25	2		19	1	
N454: Rhodochrous strain	18	4		65	2	6.5	17	1	
Subcluster 1E									
N324: Rhodochrous strain	11	0		13	1		13	1	
N325: Rhodochrous strain	11	1		18	4		14	1	
Subcluster 1G									
R35: Rhodochrous strain	10	2		12	2		14	0	
R43: Rhodochrous strain	26	<1		14	0		27	1	
R46: Rhodochrous strain	32	0	>15	14	1		116	12	0
N146: Rhodochrous strain	21	1		10	0		11	<1	
Subcluster 1H									
N11: Rhodochrous strain	100			13	3		20	2	
Subcluster 1I									
N443: Rhodochrous strain	11	1		23	1		19	1	
Subcluster 1J									
N650: Rhodochous strain	30	14		27	3		25	1	11
N651: Rhodochrous strain	30	4		26	2		23	4	
N652: Rhodochrous strain	25	4		11	3		43	2	9
N653: Rhodochrous strain	24	7		35	5	12	23	1	
Subgroup 14E									
N124: Rhodochrous strain	21	2		20	2		29	4	
Cluster 1									
N13: *Nocardia asteroides*	21	0		16	0		37	3	6
N668: *N. asteroides*	17	2		9	1		100		
N670: *N. asteroides*	47	2		14	2		80	0	0
N671: *N. asteroides*	34	7	13	11	1		79	1	2
N672: *N. asteroides*	27	5		18	6		92	1	1
N676: *N. carnea*	29	1		14	1		35	0	4
N537: *N. salmonicida*	44	4		23	3		45	1	9

TABLE 2—*Continued*

DNA source	Labelled DNA source								
	Rhodochrous strain N11			Rhodochrous strain N54			*Nocardia asteroides* N668		
	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)	Relative DNA bound (%)	± Standard error	$\Delta T_{m(e)}$ (°C)
Cluster 2									
N36: *Nocardia caviae*	>100	15	5.5	10	0		32	1	5
N231: *N. caviae*	61	3	6	6	0		20	2	
N313: *N. caviae*	>100	15	0	10	1		35	1	11
N563: *N. caviae*	>100	10	1	14	0		34	<1	9
Cluster 5									
N318: *Nocardia brasiliensis*	25	1		10	1		23	6	
Cluster 7									
N306: *Actinomadura madurae*	35	5	>15	9	1		28	1	

[a] The extent of binding between DNA from test strains and labelled DNA from reference strains is expressed as a percentage relative to the extent of binding between labelled and unlabelled DNA from the reference strain itself. $\Delta T_{m(e)}$, a measure of the thermal stability of the DNA:DNA duplexes, was obtained by subtracting $T_{m(e)}$, 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 labelled DNA was 1:15. The background, imp/minute per membrane, did not exceed 1 to 2% of the value in the homologous system. Specific activities of the DNA samples from the reference strains were (counts per minute per microgram): rhodochrous N11, 7,117; rhodochrous N54, 2,066; *N. asteroides* N668, 5,310.

erence system and the nocardiae (9 to 23%) and the remaining rhodochrous strains (20 to 35%) (Table 2).

With few exceptions the DNA preparations from the test strains showed a low degree of homology with the reference DNA from rhodochrous strain N11. The exceptions were the very high nucleotide sequence homologies found between this reference system and the DNA from the *N. caviae* strains. Apart from rhodochrous strain R46, a low degree of homology was found between DNA from the rhodochrous strains and reference DNA from *N. asteroides* N668. However, four of the five *N. asteroides* strains bound between 79 and 100% of the reference *N. asteroides* DNA (Table 2). The remaining strain, *N. asteroides* N13, bound 37% of the reference *N. asteroides* DNA; similar results were obtained between the latter and DNA from *N. brasiliensis, N. carnea, N. caviae, N. salmonicida,* and *Actinomadura madurae.*

The DNA reassociation rates of the reference strains are shown in Table 3.

TABLE 3. *DNA:DNA reassociation rates of the reference strains*[a]

Code no.	Reassociation rate					
	N11		N54		N668	
	N	H	N	H	N	H
N11						
H	100		16		17	
N				13		20
N54						
H			100		17	
N						18
N668						
H					100	
N						

[a] H, Labelled DNA; N, unlabelled DNA. The reassociation conditions are given in Table 2, footnote *a*.

DISCUSSION

The small degree of genetic relatedness between the *N. asteroides* reference system and the rhodochrous strains is in good agreement with earlier findings (8, 29). The DNA reassociation data, together with that from chemical, numerical and serological studies (2, 17,32, 39), support the view that the rhodochrous complex be considered a taxon equivalent to the genera *Corynebacterium, Nocardia,* and *Mycobacterium* (5, 14). The genus *Gordona* (37) was also equated with the genera *Nocardia* and *Mycobacterium*; however, the status of this taxon is not settled. We consider that the suggestion of classifying some rhodochrous strains in the ge-

nus *Gordona* (37, 38) was premature. Recently, *Gordona* strains were classified in a rhodochrous phenon for which the earlier name *Rhodococcus* was proposed (Goodfellow and Alderson, in press).

There is much evidence indicating that *N. asteroides* forms a heterogeneous taxon whereas *N. brasiliensis* and *N. caviae* appear to be good taxospecies (34). Most of the data are derived from numerical taxonomic studies (16, 23, 36), but additional evidence is provided by serological (25, 26), DNA reassociation (7, 10), phage typing (31), and phenetic (4, 20, 33) studies.

Little is known about the properties of *N. carnea* and *N. salmonicida*, but the latter seems to be a distinct species (18). The small amount of genetic homology between the *N. asteroides* reference system and the representatives of *N. brasiliensis*, *N. carnea*, *N. caviae*, *N. salmonicida*, and *A. madurae*, and the recovery of *N. asteroides* as a genetically heterogeneous taxon, are in line with the earlier findings. Clearly more extensive numerical and nucleotide sequence homology analyses are required to determine the structure of the *N. asteroides* taxon.

It was very encouraging to find that the representatives of subcluster 1A (Goodfellow and Alderson, in press) formed a DNA-homology group in which the strains shared 83% or more DNA with reference DNA from the type strain N54. These findings confirm our earlier study (29) and show that subcluster 1A forms a taxon on the basis of genetic, numerical phenetic, chemical, and serological data (2, 17). However, the DNA base composition range (59 to 69 mol% G+C) of the constituent strains is wider than that reported for *N. rubra* (6).

Subcluster 1A and 1B strains share a high phenetic similarity (16; Goodfellow and Alderson, in press) and failure to classify them in a single taxon could be the result of test and sampling error. Since representatives of the two subclusters form a single homology group and contain similar mycolic acids (2), subcluster 1B should be merged into subcluster 1A.

Subcluster 1H appears to be the same taxon as *N. erythropolis* (6). On the basis of DNA association, the latter was considered to be a homogeneous species (8–10). The only representative of subcluster 1H (Goodfellow and Alderson, in press), rhodochrous strain 11, showed little genetic homology with strains representing the other rhodochrous subclusters. In light of the numerical phenetic and earlier homology work , this is an encouraging result. However, additional representatives of subcluster 1H need to be examined to determine whether they share a high DNA homology with the rhodochrous N11 reference system.

It appears that at least five (1A, 1C, 1D, 1G, and 1H) of the rhodochrous subclusters (Goodfellow and Alderson, in press) are, or contain, genetically homogeneous taxa, and our studies are being extended to consider the remaining subclusters.

REPRINT REQUESTS

Address reprint requests to: Dr. M. Goodfellow, Department of Microbiology, The Medical School, The University, Newcastle upon Tyne NE1 7RU, U.K.

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