Analysis of Antigens of *Rhodococcus* Species by Rocket Immunelectrophoresis

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Seventeen representative strains of *Rhodococcus* and related taxa were analyzed for antigenic relationships by rocket immunelectrophoresis. A good correlation was found between data from this form of analysis and previously reported numerical phenetic and deoxyribonucleic acid reassociation data. *Rhodococcus ruber* and *Rhodococcus erythropolis* strains formed distinct clusters in a dendrogram produced by average linkage analyses for dissimilarity matrices. *Rhodococcus equi*, *Nocardia asteroides*, and an unidentified *Rhodococcus* strain formed single-member clusters, whereas *Rhodococcus coprophilus* was found to be heterogeneous. In contrast, *R. erythropolis* and *R. ruber* were found to be distinct species. The discrepancies between deoxyribonucleic acid pairing and rocket immunelectrophoresis results can be explained on the basis of suboptimal antigen or antiserum preparations.

The genus *Rhodococcus* was redefined (6, 13) to include some organisms of the "rhodochrous" complex which formed a heterogeneous cluster that separated distinctly from aggregate phenotypes corresponding to the genera *Mycobacterium* and *Nocardia*. Goodfellow and Alderson (6) recognized 10 species in the genus, including the type species, *Rhodococcus rhodochrous*. In studies of antigenic relationships among species of *Mycobacterium* (2, 3), the fused rocket immunelectrophoretic procedure was found to be a valuable adjunct in confirming suggested taxonomic relationships. In this study we applied this procedure to an analysis of antigens in selected *Rhodococcus* strains.

MATERIALS AND METHODS

Bacterial strains. The strains studied (Table 1) were received from Michael Goodfellow and have been described previously (5, 7).

Antigen preparation. Primary cultivation was on oleic acid albumin and Lowenstein-Jensen egg yolk slants for 2 to 4 days, after which cells were inoculated into Blake bottles containing Long synthetic medium, harvested, and sonicated as described previously (7). Immunological studies were performed with centrifuged supernatants.

Production of antibody. Rabbits were immunized with a Freund-type water-in-oil emulsion consisting of an aqueous phase of sonic extract and an equal volume of Arlcel A-Drakeol 6VR (35:65) containing 10 mg of dried organisms per 1.0 ml. The emulsion was prepared by repeated passage of the mixture through a double-hub needle connected to two 6.0-ml syringes.

Each rabbit received a total of 1.0 ml of an emulsion distributed equally into the four footpads and subcutaneously into six lateral positions. Subsequent injections consisted of 0.5 ml of sonic extract given subcutaneously every 2 weeks in the nuchal area until precipitin production appeared to be maximal.

Fused rocket immunelectrophoresis. We used a modification of the fused rocket immunelectrophoresis procedure of Svendsen (12), as described previously (2, 3); antigen was placed in overlapping oval antigen wells and was forced electrophoretically into an antibody-containing region of Veronal-buffered agar (pH 8.6; ionic strength, 0.075 M). Electrophoresis was performed for 5 h at 2.7 V/cm and 10 mA.

Antigenic and computer analyses. Precipitin bands developed with homologous and heterologous antigen preparations by means of the fused rocket immunelectrophoresis procedures were analyzed by the percent qualitative sharing (PQS) method (2), as follows: $PQS = (\text{number of bands with heterologous preparation}) / (\text{number of bands with homologous preparation}) \times 100$. This method takes into account whether antigenic determinants are present and weighs weak bands as much as intense bands.

The data in Table 2 were examined by cluster analysis via C-lab (maintained by the Division of Computer Research and Technology, National Institutes of Health, Bethesda, Md.). The values were entered into a 17-by-17 matrix. The values within the lower triangle formed by the homologous diagonal of values were read into another file which contained the similarity triangle matrix. C-lab was written to accept dissimilarity matrices; thus, the similarity matrix was converted into a dissimilarity matrix. The dendrogram prepared for dissimilarity matrices (see Fig. 1) was based on average linkage. The distance of each point from every other point was calculated, and clusters

TABLE 1. Strains used in this study

Strain	Species	No. and cluster of Goodfellow and Alderson ^a
1	<i>R. ruber</i>	N324/1E
2	<i>R. ruber</i>	N325/1E
3	<i>R. ruber</i>	N361/1D
4	<i>R. ruber</i>	N420/1D
5	<i>R. coprophilus</i>	N650/ <i>R. ruber</i> - <i>R. coprophilus</i>
6	<i>R. coprophilus</i>	N651/1J
7	<i>R. equi</i>	N652 <i>R. equi</i>
8	<i>R. erythropolis</i>	N38/1H
9	<i>R. erythropolis</i>	N53/1H
10	<i>R. erythropolis</i>	N41
11	<i>R. rhodochrous</i>	N5/1A
12	<i>R. rhodochrous</i>	N54/1A
13	<i>Rhodococcus</i> species	N124 ^b /14E
14	<i>R. coprophilus</i>	N653 ^c
15	<i>R. corallinus</i>	N655/1C
16	<i>N. asteroides</i>	N99
17	<i>R. terrae</i>	N656/1C

^a See reference 6.^b Not assigned to any species.^c Species identification not clear.

were formed with the closest points. Average distances of proximal clusters were determined.

Skin tests. Guinea pigs were sensitized and skin tested as previously described (1); each subcutaneous injection of a water-in-oil emulsion contained 2 mg of dried organisms. At 6 weeks after sensitization, guinea pigs were skin tested with 0.1 ml of a saline solution containing homologous and heterologous antigen preparations. The dose of each antigen used was predetermined to render a reaction size of about 13 mm in the homologically sensitized animals.

Specificity differences (SPD) for each reaction were determined by the method of Magnusson (8), as follows: SPD = (Aa + Bb) - (Ab + Ba), where Aa and Bb are the diameters of the homologous reactions of two species and Ab and Ba are the diameters of the heterologous reactions for the same two species.

RESULTS

With the strains studied, the number of precipitin bands produced with cell extracts and homologous antisera in fused rocket immunoelectrophoresis varied from 13 to 50, except for *Rhodococcus terrae* N656 (formerly *Gordona terrae*), which produced only 7 bands (Table 2). Table 2 also shows the PQS values. A method has not yet been devised to quantitate accurately the antigens in multiple-component systems in which shared epitopes that react in precipitation reactions might be on different types of carrier molecules. Occasionally, the PQS value of a heterologous antigen can exceed or equal 100% due either to a close similarity of species or differences in the potencies of antigen preparations or both. For computer analyses, one value of 107% was reduced to 100%.

FIG. 1. Average linkage for 16 strains of *Rhodococcus* and 1 strain of *N. asteroides*.

The dendrogram in Fig. 1 illustrates the average linkage values for the 17 strains studied. It also demonstrates the general relationship of most of the organisms. All of the organisms defined by Goodfellow and Alderson (6) as *Rhodococcus ruber* formed a closely related cluster as determined by precipitin analysis and were clearly separated from the three clustered *Rhodococcus erythropolis* strains. Two of the three *Rhodococcus coprophilus* strains appeared to be very closely related, whereas the other strain (strain N653) was less related. The *R. terrae* strain appeared to be closely related to *Rhodococcus corallinus* on the dendrogram, but the *R. rhodochrous* strains were separated from one another. The single *Nocardia asteroides* strain examined appeared to have affinity to *R. coprophilus* N650 and N651; the reason for this apparent discrepancy is discussed below.

Because of biological variation both in the preparation of sonic extracts of organisms and in the antibody responses of rabbits, the PQS values of any two related species may be disparate (Table 2). Some of the discrepancies between two species were lessened when values were averaged to obtain the reciprocal average PQS (RAP), as follows: RAP = (PQS of A with serum B + PQS of B with serum A)/2. The RAP values are shown in Table 3, together with the corresponding skin test SPDs (see above).

In general, there was good agreement between the results obtained by skin testing and those obtained by antigen analysis; discrepancies were observed in only 27 of 136 heterologous values (Table 3), either when a low SPD (<3 mm) was accompanied by an RAP of 55% or less or when an SPD of >3 mm corresponded to an RAP value greater than 55%. Possible reasons for these discrepancies are discussed below.

DISCUSSION

Analyses of precipitinogens detectable with homologous and heterologous antisera are sub-

TABLE 2. PQS values of precipitin bands of *Rhodococcus* species

Strain	No. of homologous bands ^a	PQS (%) ^b							
		<i>R. ruber</i> N324/1E	<i>R. ruber</i> N325/1E	<i>R. ruber</i> N361/1D	<i>R. ruber</i> N420/1D	<i>R. coprophilus</i> N650	<i>R. coprophilus</i> N651/1J	<i>R. coprophilus</i> N653	
<i>R. ruber</i> N324/1E	50	100	80	87	93	80	107	80	
<i>R. ruber</i> N325/1E	13	92	100	92	77	85	92	54	
<i>R. ruber</i> N361/1D	16	63	69	100	94	75	63	75	
<i>R. ruber</i> N420/1D	40	68	93	83	100	80	20	58	
<i>R. coprophilus</i> N650	33	91	97	88	100	100	97	39	
<i>R. coprophilus</i> N651/1J	17	53	41	41	53	88	100	65	
<i>R. coprophilus</i> N653	29	48	48	48	48	55	28	100	
<i>R. equi</i> N652	20	30	35	35	40	55	15	45	
<i>R. erythropolis</i> N38/1H	25	56	60	40	40	80	64	60	
<i>R. erythropolis</i> N53/1H	39	36	31	36	31	28	31	23	
<i>R. erythropolis</i> N41	19	32	47	21	58	53	21	16	
<i>Rhodococcus</i> species N124/14E	37	65	46	27	38	32	35	30	
<i>R. rhodochrous</i> N5/1A	23	43	35	26	35	52	48	43	
<i>R. rhodochrous</i> N54/1A	36	17	11	8	14	14	11	11	
<i>R. corallinus</i> N655/1C	23	30	35	30	22	35	26	22	
<i>R. terrae</i> N656/1C	7	29	29	29	29	29	29	29	
<i>N. asteroides</i> N99	19	11	11	11	26	53	79	26	

67	60	53	67	80	53	47	20	27
77	38	38	46	77	46	46	38	31
56	38	38	38	63	63	44	19	38
45	65	50	20	60	45	28	10	18
91	73	64	55	70	79	39	39	33
65	59	35	35	47	76	24	59	82
72	38	28	34	55	55	28	28	24
100	70	25	25	65	30	20	10	25
108	100	96	72	80	88	44	8	12
33	67	100	31	33	26	21	13	15
16	79	100	26	11	11	11	5	5
27	57	54	100	43	27	27	11	11
43	30	39	30	100	83	17	4	9
17	33	8	14	33	100	8	3	14
22	30	22	22	17	17	100	26	13
29	29	14	29	14	29	100	100	57
21	16	11	16	26	63	16	37	100

^a Numbers of bands developed with homologous antisera (actual numbers of bands with heterologous antigens).
^b See text for method of calculation.

48	43	66	62	35	39	25	19	50	11.5
1.8	2.8	-1.1	1.6	0.3	5.4	5.6	8.9		
35	43	46	56	28	41	34	21	13	12.5
7.4	5.3	2.4	1.6	3.8	6.6	5.8	8.8		
37	30	33	50	30	37	24	25	16	12.0
5.1	4.4	3.6	2.8	1.9	6.5	5.8	8.0		
42	56	34	53	30	25	20	22	40	12.9
7.0	6.1	4.6	5.5	3.3	7.9	8.5	9.6		
43	59	44	61	47	37	34	43	33	13.5
6.6	4.5	2.8	2.9	1.4	7.1	1.7	7.9		
39	28	35	48	44	25	44	81	17	12.1
5.2	5.6	2.8	1.5	-1.0	6.5	6.1	0.88		
26	22	32	49	33	25	28	25	29	14.4
7.6	5.8	2.5	4.3	2.9	6.7	7.3	6.1		
29	21	26	54	24	21	20	23	20	13.9
6.9	6.9	2.3	4.8	2.4	5.8	8.0	7.6		
82	88	70	55	60	37	19	14	25	12.1
0.54	2.4	3.5	2.1	1.3	4.7	6.7	7.7		
100	100	40	36	19	22	14	13	39	13.1
0	0.38	1.8	6.0	4.4	8.6	7.1	9.8		
	100	40	25	10	17	17	13	19	13.9
	0	4.5	3.9	5.0	8.0	6.7	12.6		
		100	37	21	25	20	14	37	13.3
		0	3.1	4.0	8.0	5.5	7.6		
			100	58	17	9	18	23	12.9
			0	0.13	8.6	9.3	6.8		
				100	13	16	39	36	12.5
				0	5.6	6.5	4.7		
					100	63	15	23	
					0	3.5	12.5		
						100	47	7	11.8
						0	10.0		
							100	19	13.5
							0		

^a RAP and SPD are defined in the text. The upper value is the RAP (percent), and the lower value is the SPD.

ject to variations in the antigenic preparations and in the immune responses. Despite such variations, the procedures described above offer a powerful means for evaluating taxonomic relationships revealed in numerical phenetic analyses. Theoretically, if the maximum number of antigens could be prepared consistently for all preparations and the maximum humoral and cell-mediated immune responses were induced for each antigen, it should be possible to derive optimal relationships between species by this procedure. Realistically however, we must settle for less. In fact, potent heterologous antigen preparations may produce larger numbers of precipitin bands than less potent homologous antigen preparations. A skin reaction is the summation of a number of reactions induced by specifically sensitized lymphocytes reacting with possibly 50 or more antigens. Despite this, analyses of homologous and heterologous skin test reactions produce results that are very discriminating for species (4, 7, 8). However, occasionally results may be inconsistent with other taxonomic criteria. This is the case with the relationship between *N. asteroides* N99 and *R. coprophilus* N651. A close relationship is indicated by the SPD (0.88) (Table 3) and by the high RAP value (81%). Thus, these two strains appear to be closely related antigenically; however, on the basis of numerical phenetic data they are clearly different (6). The antisera used in the analysis contained from 17 to 19 bands, far from the potentially maximal number of 60 or so. Antibodies may have been raised principally to shared dominant antigens. The potential errors due to antibody preparations are more vividly seen with *R. corallinus* N655 and *R. terrae* N656. Mordarski et al. (11) found little deoxyribonucleic acid homology between strains of these two species, but in our analyses a high degree of relatedness was noted (Fig. 1). However, this apparent relationship can be explained by the poor antiserum for *R. terrae* (Table 2); only seven precipitin bands were produced, and all were shared with *R. corallinus*. A truer relationship is seen with *R. corallinus* antiserum, which produced 23 bands, only 26% of which were shared. From Table 2 it is clear that *R. corallinus* is a distinct entity. In a recent study (9), *R. corallinus* and *Rhodococcus rubropertinctus* formed a single deoxyribonucleic acid homology group, and the former name was reduced in status to a synonym of the latter. An advantage of analyses by precipitation in gels is that individual precipitating antigens can be detected and related to a homologous or heterologous system. Sharing of a high percentage of bands generally reflects a close relationship. However, a poor antiserum or antigen preparation can produce misleading results. In skin

testing it is possible that a potent sensitizing skin-reactive component present in a relatively high concentration may dominate an observed skin reaction.

R. ruber and *R. erythropolis* each formed distinct clusters (Fig. 1), a result consistent with both numerical phenetic and deoxyribonucleic acid pairing data (9). *Rhodococcus* strain N124 did not fit clearly into any of the clusters recovered by Goodfellow and Alderson (6) and did not cluster clearly in this study; this strain has yet to be identified to species. *Rhodococcus equi* also clustered by itself in the dendrogram (Fig. 1).

Mordarski et al. (11) found that much of the deoxyribonucleic acid of *R. rhodochrous* N54 is homologous with that of strain N5. Although these two strains did not appear to be closely related in the dendrogram based on skin test analyses (Table 3), they appeared to be closely related, as suggested by a low SPD. Antiserum for strain N54 was one of the best antisera in that in rocket immunoelectrophoresis it produced 36 bands with the homologous system. Most of these bands appeared to be strain specific and reduced the PQS value for strain N5. Thus, excellent antisera and antigen preparations can be as misleading as poor ones.

Overall, the skin testing and precipitating procedures which we used were effective in determining the relationships of the species in the genus *Rhodococcus*, and the results obtained were generally consistent with and confirmed the results of Goodfellow and Alderson (6). Of importance is the production of good quality antisera and antigen preparations in reference quantities for each of the type species. Such reagents should be useful in the classification and identification of bacteria.

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REPRINT REQUESTS

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