Morphological, Biochemical, and Serological Characterization of *Rothia dentocariosa*

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Fifty strains of *Rothia dentocariosa* Georg and Brown were characterized morphologically, biochemically, and serologically. All strains had characteristics agreeing with previous morphological descriptions of this organism, although there was greater biochemical and serological strain variation than previously reported. Four biotypes were established on the basis of variability in reduction of nitrite, production of urease, hydrolysis of esculin, and formation of acid from lactose, mannitol, mannose, raffinose, rhamnose, salicin, and trehalose. Three serotypes and a group of fluorescent antibody-negative strains were identified on the basis of the fluorescent antibody technique. A relationship between the four biotypes and the three serotypes was established.

The monospecific genus *Rothia*, with type species *Rothia dentocariosa*, was proposed by Georg and Brown (2) to accommodate microorganisms previously designated as *Actinomyces dentocariosus*, *Nocardia dentocariosus*, and *Nocardia salivae*. Brown, Georg, and Waters (1) subsequently studied 50 isolates having the morphological and biochemical characteristics of *Rothia*. Hammond (3) showed that all of his strains of *R. dentocariosa* contained a soluble polysaccharide antigen (RPS), and the detection of this antigen by the fluorescent-antibody (FA) technique is useful in identifying this organism.

Rothia is included in the family *Actinomycetaceae* because of its branching, filamentous morphology and its ability to septate into bacillary, diphtheroidal, or coccoid cells, or a mixture of these; its colonial morphology, including spider microcolonies and mature colonies which may be heaped and rough or entirely smooth; and its cell wall composition. However, *Rothia* differs from the genera *Actinomyces*, *Arachnia*, *Bifidobacterium*, and *Bacterionema* in its aerobic tendencies, its production of catalase, its lack of growth stimulation by CO₂, and its production of lactose as the major end product from glucose fermentation.

Among the organisms isolated from dental calculus in this laboratory were a number of filamentous, aerobic, gram-positive, catalase-positive strains resembling *Rothia* morphologically but possessing variant biochemical reactions and not reacting with antisera

prepared with the neotype strain of *R. dentocariosa*, ATCC 17931. Similar aberrant strains have been submitted by others to the Center for Disease Control for identification (L. Georg, personal communication).

By comparing the morphological, biochemical, and serological characteristics of *Rothia* with *Rothia*-like organisms, we hope to clarify the uncertain status of the latter.

MATERIALS AND METHODS

Bacterial strains. The sources and designations of the fifty strains used in this study are given in Table 1.

All cultures were maintained by monthly transfer on tryptic soy agar (TSA) (Difco) slants. Prior to use, the cultures were transferred twice in tryptic soy broth (TSB) (Difco).

Colonial morphology. TSB cultures were streaked onto two TSA plates and incubated aerobically and anaerobically at 37 C. The anaerobic plate was incubated in a Torbal jar containing N₂:H₂:CO₂ (80:20:10). Microcolonies were observed after 18 and 24 h of incubation at a magnification of ×100 to ×400. Mature colonies were observed at ×25 to ×40 after 7 days of incubation.

Cellular morphology. Gram-stained smears and wet mounts for dark-field microscopy were prepared and examined from 2- and 7-day-old cultures in TSB and on TSA.

Biochemical Tests. Tests for catalase, indole, nitrate reduction, nitrite reduction, esculin hydrolysis, gelatin liquefaction, hydrogen sulfide production using BHI agar, urease production, and the production of acid from carbohydrates were done by the methods of Brown et al. (1).

Additional biochemical tests used were growth on

Sabouraud dextrose agar (Difco), liquefaction of Loeffler coagulated serum (BBL) slants, digestion of casein, hydrolysis of hippurate, deoxyribonuclease production, production of ammonia from arginine, and the oxidation-fermentation (O-F) test (5).

DNase test agar (BBL) was inoculated and incubated for 7 days. After incubation, the plates were flooded with 1 N HCl and observed for the formation of clear zones around the colonies.

TSB broth containing 1.0% sodium hippurate was inoculated and incubated for 7 days. The cultures were centrifuged, and 1.0 ml of supernatant was added to 1.5 ml of 50% H₂SO₄. After 4 h at room temperature, the tubes were observed for the

formation of flat crystals of benzoic acid.

The production of ammonia from arginine was tested after 7 and 14 days. The basal medium contained the following: yeast extract, 0.1%; Trypticase, 0.1%; K₂PO₄, 0.03%; and NaCl, 0.5%. The test medium also contained 0.3% arginine. Duplicate tubes of basal and test media were inoculated and tested for ammonia production by mixing 1.0 ml of each medium with 1.0 ml of Nessler reagent and observing for the formation of a deep orange to brick-red color in the presence of ammonia.

The oxidation-fermentation test was performed as described by Hugh and Leifson (5). The medium used to test for fermentation of glucose contained 24 g of

TABLE 1. *Sources of strains studied*

WVU ^a number	Previous designation and source
<i>Rothia dentocariosa</i>	
477	CDC 808 ^b , leg-stump drainage
478	CDC W876, blood
479	CDC W853, throat
1317	WVU isolate, dental calculus
1489	ATCC ^c 17931, type strain human dentine
1524	ATCC 14189
1525	ATCC 14190
1526	ATCC 14191
1560	I. R. Rothenberg 25684, sputum
1562	I. R. Rothenberg 28342, sputum
<i>Rothia</i> -like	
804, 841, 842	WVU isolates, dental calculus
858, 874, 918	
936, 945, 972	
992, 997, 999	
1013, 1027, 1072	
1073, 1088, 1192, 1200	CDC W874a, throat
1532	
1533	
1534	
1535	
1536	
1537	
1538	
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1549	
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1557	
1558	
1559	

^a WVU, West Virginia University, Morgantown, W. Va.

^b CDC, Center for Disease Control, Atlanta, Ga.; all cultures provided by L. Georg.

^c American Type Culture Collection, Rockville, Md.

fluid thioglycolate medium without glucose or indicator (BBL) per liter in addition to the components suggested by Hugh and Liefson (5).

Serological tests. Strains (WVU 477, 936, 999, 1088, and 1489) were used for antiserum production. Cells grown in TSB at 37 C for 24 h were harvested by centrifugation, washed twice in sterile saline, resuspended in saline to a turbidity of a no. 4 MacFarland tube, and stored in 4-ml quantities at -30 C.

For immunization, one vial of antigen was thawed prior to injection. The immunization schedule is given in Table 2.

The procedures used for determining the working titer of the conjugate and for staining the smears were essentially the same as those described by Slack, Landfried, and Gerencsek (6).

Sorption of the antisera was done by incubating 1 ml of a 1:2 dilution of the conjugate with 0.1 ml of washed, packed cells of the sorbing strain at 56 C for 1 h followed by overnight refrigeration. The conjugate was removed from the cells by centrifugation, and the sorption was repeated. A sorbed antiserum was considered satisfactory if it stained the homologous strain with an intensity of 4+ at a final dilution of 1:40 and did not stain the sorbing strain at 1:2.

RESULTS

Cellular morphology. Cells in TSA and TSB were gram positive and pleomorphic; coccoid, cocco-bacillary, and filamentous forms were present. Cultures would occasionally be completely coccoid or diphtheroidal. Aging cultures tended to become gram negative.

Colonial morphology. Young (18- to 24-h-old) colonies on TSA plates averaged 1 mm in diameter. When examined with a microscope, young colonies grown aerobically were granular with entire borders, whereas those grown anaerobically were granular with filamentous borders.

TABLE 2. Immunization schedule for production of antiserum^a

Injection	Time interval (days)
1	1
2	3
3	7
4	10
5	14
6	17
7	25
8	28
9	31
10 ^b	35

^a Amount of antigen injected was 1 ml. This same schedule used for all antigens prepared.

^b Animals were bled by cardiac puncture 1 week after last injection.

TABLE 3. Biochemical reactions of 50 *Rothia* strains

Test	No. positive	% Positive
Catalase production	50	100
Indole production	0	0
Nitrate reduction	48	96
Nitrite reduction (0.001%)	44	88
Nitrite reduction (0.01%)	33	66
Esculin hydrolysis	47	94
Urease production	10	20
Deoxyribonuclease production	45	90
Gelatin liquefaction	0	0
Casein hydrolysis	0	0
Serum liquefaction	0	0
Acid from: ^a		
Glucose	50	100
Glycerol	50	100
Lactose	11	22
Maltose	50	100
Mannose	46	92
Mannitol	10	20
Raffinose	10	20
Rhamnose	14	28
Ribose	35	70
Salicin	42	84
Sucrose	50	100
Trehalose	44	88

^a None of the strains fermented adonitol, arabinose, cellobiose, glycogen, inositol, sorbitol, starch, or xylose.

Mature colonies (7 to 14 days) were creamy white in color and varied from 1 to 4 mm in diameter and from smooth to rough. Smooth colonies were convex with a finely granular surface and entire edges; rough colonies were convex with convoluted, highly cerebriform surfaces, and undulate or scalloped edges. The texture of both colony types varied from mucoid to friable. Both forms could be seen in the same culture.

Biochemical tests. The results of the biochemical tests are shown in Table 3. All strains were catalase positive, indole negative, and hippurate-hydrolysis negative; none of the strains produced ammonia from arginine or liquefied gelatin, digested casein, or liquefied Loeffler serum slants.

Nitrite reduction was variable and was dependent on the concentration of the KNO₂ used in the test medium. In the original test for nitrite reduction, which required 0.01% KNO₂, only 66% of the strains were positive. Further testings using the same medium, with the nitrite concentration reduced to 0.001% KNO₂, resulted in better growth and increased positive reactions (88%).

Serology. The results of the cross-staining and the reciprocal-sorption studies with the five strains used for antiserum production are shown in Table 4. These results suggested that strains 936 and 999 were serologically distinct from the other strains and were closely related, if not identical. Relationships between the other three strains were more complex and have not been completely worked out; however, the sorption studies suggest that the type strain, 1489, is different from strains 477 and 1088. The latter strains are not identical but are more closely related to each other than to 1489. With this as a basis, all 50 strains were tested with each of the antisera, and positive strains were retested with the appropriate sorbed sera. Six strains were FA negative with all five sera. Nine strains reacted to titer with either 936 or 999 sera, or with both. Eight of these strains showed no reactions or minimal reactions with the other sera. One strain reacted strongly with 1489 as well as with 936 and 999 sera. The remaining 35 strains reacted with one or more of the 1489, 477, and 1088 sera. When tested with sorbed sera, some strains stained only with 1489 or with 477 or 1088 sera, again suggesting that these strains represent two serotypes. However, some cultures which had been positive with unsorbed sera did not react with any of the sorbed sera, making their placement in one of the two serotypes uncertain. At present these 35 strains can be divided into two serotypes: type 1 with 10 strains and type 2 with 15 strains. A group of 10 strains showed relationship to both serotypes.

Classification. Using both biochemical and serological results, the 50 strains could be divided into four biotypes and three serotypes (Table 5).

The establishment of the four biotypes involved variability in the reduction of NO_2 when the KNO_3 concentration in the basal medium was 0.01%, the production of urease, the hydrolysis of esculin, and the production of acid from mannose, salicin, trehalose, lactose, mannitol, raffinose, and rhamnose. Biotype 1 strains were characterized by biochemical reactions which agreed with those previously reported for the species in that they reduced NO_2 (100%), were urease negative (100%) and esculin positive (100%), produced acid from mannose (100%), salicin (100%), trehalose (100%), and rhamnose (4%), and did not produce acid from lactose, mannitol, or raffinose. Biotype 2 strains did not reduce NO_2 , were urease negative and esculin positive (100%), produced acid from mannose (57%), salicin (28%), trehalose (57%), lactose (28%), and rhamnose (14%), and did not produce acid

TABLE 4. Serological reactions of strains of *Rothia*^a

Antiserum	Sorbed with	Strain				
		477	1088	1489	936	999
1489		—	—	+	—	—
	477	—	—	+	—	—
	1088	—	—	+	—	—
477		+	+	+	—	—
	1088	+	—	—	—	—
	1489	+	—	—	—	—
1088		+	+	—	—	—
	477	—	—	—	—	—
	1489	+	+	—	—	—
936		—	—	—	+	+
999		—	—	—	+	+
	936	—	—	—	—	—

^a Symbols: + = or greater fluorescence at working titer (unsorbed sera) or at 1:2 (sorbed sera); — = less than 2+ fluorescence undiluted (unsorbed) or 1:2 (sorbed).

from mannitol or raffinose. Biotype 3 strains reduced NO_2 (63%), were urease positive (100%) and esculin positive (86%), and produced acid from mannose (86%), salicin (71%), trehalose (71%), lactose (50%), mannitol (14%), raffinose (14%), and rhamnose (29%). Biotype 4 strains reduced NO_2 (80%), were urease positive (27%) and esculin positive (82%), and produced acid from mannose (100%), salicin (91%), trehalose (91%), lactose (50%), mannitol (82%), raffinose (82%), and rhamnose (91%). In all biotypes, any strain which was anomalous in a sugar fermentation usually differed with respect to several sugars so that a strain which failed to ferment mannose usually also failed to ferment ribose, salicin, and trehalose; strains which fermented a sugar such as lactose often also fermented rhamnose.

Serologically, 25 of the 39 strains included in biotypes 1 to 3 belonged to serotypes 1 or 2, and 10 strains showed some relationship to both serotypes. Since strains in biotypes 2 and 3 were serologically related to biotype 1, all 39 strains were identified as *Rothia dentocarios*.

Biotype 4 strains showed some intertype variability in the tests used in separating biotypes 1 to 3, especially the urease test. However, these strains were consistently more active in fermentation reactions than were strains of biotypes 1 to 3. Ten of the 11 strains fermented either mannitol or raffinose, and seven fermented both. These seven strains also

TABLE 5. *Characteristics of biotypes of Rothia*

Test	Biotype							
	1 (25 strains)		2 (7 strains)		3 (7 strains)		4 (11 strains)	
	No. positive	% positive	No. positive	% positive	No. positive	% positive	No. positive	% positive
Catalase	0	0	0	0	0	0	0	0
Indole	0	0	0	0	0	0	0	0
Nitrate	25	100	6	85	6	6	11	100
Nitrite (0.001%)	25	100			5	63	8	80
Nitrite (0.01%)	21	84	4	57	4	50	4	40
Esculin	25	100	7	100	6	86	9	82
Urease	0	0	0	0	7	100	3	27
Deoxyribonuclease	24	96	4	57	6	86	11	100
Gelatin liquefaction	0	0	0	0	0	0	0	0
Casein hydrolysis	0	0	0	0	0	0	0	0
Serum liquefaction	0	0	0	0	0	0	0	0
Acid from: ^a								
Glucose	25	100	7	100	7	100	11	100
Glycerol	25	100	7	100	7	100	11	100
Lactose	0	0	2	28	4	50	5	50
Maltose	25	100	7	100	7	100	11	100
Mannose	25	100	4	57	6	86	11	100
Mannitol	0	0	0	0	1	14	9	82
Raffinose	0	0	0	0	1	14	9	82
Rhamnose	1	4	1	14	2	29	10	91
Ribose	22	88	1	14	2	29	10	91
Salicin	25	100	2	28	5	71	10	91
Sucrose	25	100	7	100	7	100	11	100
Trehalose	25	100	4	57	5	71	10	91
Serotype 1 1489	6		2		2		0	
Serotype 2 477	9		3		3		0	
Serotype 3 936	0		0		0		9	
Serotype FA negative	3		1		0		2	
1 & 2 related	7		1		2		0	

^a All strains were negative in adonitol, arabinose, cellobiose, glycogen, inositol, sorbitol, starch, and xylose.

fermented lactose. Nine of the strains were apparently identical serologically and were unrelated to the serotypes found in biotypes 1 to 3.

DISCUSSION

The 50 strains of gram-positive bacteria studied conform to the morphological description of the genus *Rothia* (1,2). All strains were pleomorphic with coccoid, cocco-bacillary, and filamentous forms. There was a distinct tendency for some cultures to be completely coccoid or diphtheroidal. Among the *Actinomycetaceae*, only *Arachnia propionica* shares with *Rothia* this characteristic of converting to completely coccoid morphology.

The colonies produced by the strains studied presented a variety of types, but both microcolony and mature colony types were within the range of types described for the species by Brown et al. (1).

The results of the physiological tests were more strain variable than those reported by Brown et al. (1).

All strains were aerobic and catalase positive and fermented carbohydrates (O-F test) with the production of acid with no gas. On the basis of these characteristics and their morphology, all 50 strains were classified as belonging to *Rothia*.

In view of the remarkably uniform reactions of strains of *R. dentocariosa*, the single species in this genus, the major consideration was

whether biochemically variant strains should be placed in this species and, if so, how much variation should be accepted.

All strains of biotypes 1 to 3 (Table 5) were identified as *Rothia dentocariosa*. Biotype 1 presented the uniform biochemical picture expected of *R. dentocariosa* and generally agreed with previous descriptions of the species. Biotypes 2 and 3 were related to biotype 1 serologically, so it was decided to include them in *R. dentocariosa*.

An increased strain variation can be expected in biochemical tests, and inclusion of such variable strains in the species should make it easier for laboratories to identify fresh isolates. It should be remembered that these strains tend to have several aberrant characters, especially the failure to ferment three or all of the sugars mannose, ribose, salicin, and trehalose.

The serological relationships within biotypes 1 to 3 need to be clarified. The four FA-negative strains and the 10 strains showing some relationship to both serotypes 1 and 2 suggest that further serotypes may exist. Identification of the distinctive polysaccharide antigen (RPS) described by Hammond (3) in these strains might help to elucidate the antigenic relationships.

The 11 strains included in biotype 4 were serologically distinct. Only one strain in biotypes 1 to 3 showed any serological cross-reaction with biotype 4 antiserum. Another serotype 1 strain resembled biotype 4 strains in fermenting mannitol and raffinose. For these reasons, we feel that it is definitely premature to exclude these 11 strains from *R. dentocariosa*, but it also seems undesirable to classify them as such at present. Therefore, we would prefer to retain them as *Rothia* sp. biotype 4.

Before a final decision is made as to the suggestion of a new species with the genus

Rothia for biotype 4 strains, further studies, including deoxyribonucleic acid base ratios and homologies, are required.

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REPRINT REQUESTS

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