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Isolation and Serological Characterization of a Cell Wall Antigen of *Rothia dentocariosa*

BENJAMIN F. HAMMOND

Department of Microbiology, University of Pennsylvania School of Dental Medicine and Forsyth Dental Center, Philadelphia, Pennsylvania 19104

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A soluble polysaccharide antigen from the cell wall of *Rothia dentocariosa* ATCC 17931 has been isolated, purified and characterized by serological and chemical procedures. The polysaccharide (RPS) was found to be located at the surface of cells grown under diverse environmental conditions, and could be easily detected on cells in pure culture or in clinical samples from humans and experimentally infected hamsters by fluorescent-antibody techniques. Fructose, glucose, galactose, and ribose were the major constituents of RPS. Although purified RPS was not immunogenic in rabbits, it was presumed to be a major antigen of the cell because it could specifically absorb approximately one-third of the antibody nitrogen in antisera prepared against whole cells of *R. dentocariosa*. Haptene inhibition studies indicated that fructose was the principal determinant of serological specificity in RPS. This polysaccharide was found to be serologically unique and did not cross-react with the polysaccharides and surface polymers of other oral actinomycetes and filamentous organisms.

The chemical and immunological characterization of a major antigen in bacterial cell walls provides a reasonable basis for recognizing the natural relationships of bacteria and for helping to establish a given bacterial species as an ecological biotype. Among the actinomycetes, the correlation between cell wall structure and taxonomic status is particularly well known and of great interest (3, 4, 11). One of the principal reasons for this interest is the assumption that cell wall structure is related in some way (i) to the extreme pleomorphism characteristic of members of this group and (ii) to differences in biological properties (ecological habitat, pathogenic potential, etc.).

An interesting actinomycete exhibiting marked variation in the shape of its cell wall is *Rothia dentocariosa*, a gram-positive, filamentous organism frequently isolated from the human oral cavity (6) and now presumed to be pathogenic for man (13). It is the only known species of the newly created genus *Rothia* (6), and it is closely related morphologically and physiologically to other oral filamentous organisms, including *Actinomyces* species and members of the genus *Nocardia*. The biochemical differentiation of these three groups is possible (2); however, the procedures involved are tedious, time-consuming, and subject to considerable variation among strains. It therefore seemed worthwhile to investigate the possibility that a stable antigenic property of *Rothia* could be utilized in a rapid yet specific serological procedure. In addition to the need for such an identification procedure, it is also clear that basic information about cell wall structure (as reflected in its antigenic wall polymers) is necessary to put this newly recognized group into a proper context of the taxonomy, ecology, and physiology of the actinomycetes.

This report describes the isolation and characterization of a stable, major antigen in the cell wall of *R. dentocariosa* and presents evidence that the antigen is suitable as a specific marker in fluorescent-antibody procedures for identifying strains of *R. dentocariosa* obtained from diverse ecological sources and in the presence of related microorganisms.

MATERIALS AND METHODS

Organism and culture procedures. The reference organism used in this study for biochemical tests, vaccine production, and cell wall preparation was *R. dentocariosa* ATCC 17931. Other cultures of *Rothia* were kindly supplied by Lucille K. Georg and H. V. Jordan, and from the stock collection of oral isolates Department of Microbiology, School of Dental Medicine, University of Pennsylvania. All procedures used in the maintenance of stock cultures,

biochemical tests, carbohydrate fermentation, and in determinations of colonial morphology and oxygen requirements were the same as those described by Brown, Georg, and Waters (2).

Cell wall preparation. Cells from 30 liters of a 48-hr culture in Trypticase Soy Broth (BBL) were harvested by centrifugation, washed three times in saline, mixed with an equal volume of glass beads, and subjected to sonic treatment for 30 min in a sonic disintegrator (MSE, Westlake, Ohio). After the glass beads settled out, the cell sediment was separated from the opalescent extract by centrifugation at 2,500 × *g* in 40% sucrose according to the procedure of Yoshida et al. (16). The cell walls were found in the middle layer of this cell-sucrose mixture. The intact cells were at the bottom of the tube, and a whitish membrane-associated fraction was at the top. The middle layer was carefully removed by pipetting, and was again centrifuged in the 40% sucrose. The wall layer obtained was washed 10 times with saline and 10 times in distilled water before it was treated with trypsin and ribonuclease (4). The enzyme-treated preparation was again washed 10 times each in saline and distilled water, and finally was lyophilized.

Preparation of crude antigens. The lyophilized cell wall preparation (5 g) was extracted with cold 10% perchloric acid for 2 hr, the extract was precipitated with two volumes of acetone at 4 C for 24 hr, and the resulting precipitate, after being solubilized in a small amount of distilled water, was dialyzed for 48 hr against eight changes of distilled water. This cell wall extract was found to contain small amounts of protein, most of which were removed by further precipitation with 10% trichloroacetic acid; the extract was then redialyzed as before. The final preparation was then lyophilized prior to chemical analysis and column chromatography.

Production of antiserum. A high-titered antiserum was prepared by use of a live vaccine of *R. dentocariosa* ATCC 17931 as described earlier by Hammond (7) for *Lactobacillus casei*, except that the immunization was prolonged to 5 weeks instead of 4 weeks. Six different rabbits (2.7 kg, New Zealand white) were used.

Other serological procedures. Fluorescent-antibody determinations on pure cultures and carious dentin samples were carried out by means of a slightly modified Coons technique (7). A Zeiss fluorescence microscope equipped with an Osram HBO 220 mercury vapor lamp and apochromatic and fluorite objectives was used to examine all specimens. An OG-12 exciter filter transmitting light at about 490 nm was attached to the lamp, and an OG 4 yellow filter (529 nm) was used as a barrier filter. Negative fluorescent-antibody controls included: (i) normal serum control, (ii) heterologous cell control (*L. casei*, 32-11), (iii) autofluorescence control, and (iv) test serum absorbed with homologous cells. The positive control used to adjust the light on the fluorescence microscope was a fluorescent antibody-labeled slide of *Escherichia coli* (Difco). Haptene inhibition studies of *Rothia* polysaccharide (RPS) precipitation were performed according to the procedure of Ullmann and Cameron (15), except that immunodiffusion slides rather than Ouchterlony plates were used. Immunodiffusion methods followed the general procedures outlined by Hammond (8) and Campbell et al. (2).

Antibody nitrogen in the precipitate resulting from addition of the RPS to *Rothia* antiserum was determined by nesslerization according to the procedure of Campbell et al. (3). Correction was made for the nitrogen present in the purified antigen preparation prior to the addition of the antiserum. Experiments were carried out in triplicate; results were considered satisfactory if the experimental error did not exceed ± 5%.

Chemical methods. Chemical determinations on the polysaccharide (RPS) hydrolysates and paper chromatography of carbohydrates were performed as described previously by Hammond (8). Additional standards for paper chromatography included the ketohexoses tagatose and sorbose. Amino acid chromatography of samples after hydrolysis in 12 N HCl was done by the procedures of Montague and Knox (12). Muramic acid was determined by a modification of the Barker-Summerson lactic acid technique (10). Gas-liquid chromatography of the hydrolyzed RPS samples was performed according to the procedure of Sawardeker, Sloneker, and Jeanes (14), with deoxyglucose as the internal standard. The quantitation of fructose by gas-liquid chromatography was done by analyzing duplicate samples (1.0 ml) of the RPS, one of which was pretreated with glucose oxidase (Worthington Biochemical Corp.); the other was untreated. Since sorbitol was formed from the reduction of both glucose and fructose, it was necessary to subtract the sorbitol value due to glucose from the total sorbitol to obtain the value due to fructose alone.

RESULTS

Preparation and characterization of the partially purified antigen. Antisera prepared against whole cells of *R. dentocariosa* (ATCC 17931) were found to contain at least four bands on immunoelectrophoresis with the water-soluble perchloric acid cell wall extract (Fig. 1). A procedure for partial purification and separation of these antigens was devised by use of ascending chromatography on a Sephadex G-200 column (2.5 by 100 cm; bed volume, 497 ml) with a Büchler polystaltic pump. The column was eluted with phosphate-buffered saline (*p*H 6.8) at a flow rate of 15.6 ml/hr, and 2.5-ml effluent fractions were collected with a Warner-Chilcott drop count fractionator. Alternate lyophilized fractions were monitored by immunoelectrophoresis with the use of antiserum against whole cells of *R. dentocariosa* (ATCC 17931). The recovery profile of the antigens is shown in Fig. 2. Antigen no. 2 was observed to come off the column beginning with fraction 44, and fractions showing this single band were pooled

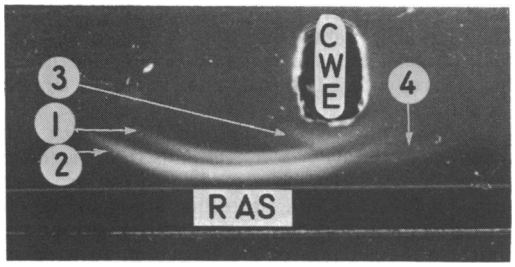

FIG. 1. *Immunoelectrophoretic pattern of Rothia dentocariosa ATCC 17931 cell wall extract (CWE) before separation of antigens by Sephadex G-200 chromatography. The various antigen bands are designated 1, 2, 3, and 4; RAS represents antiserum prepared against whole cells of R. dentocariosa.*

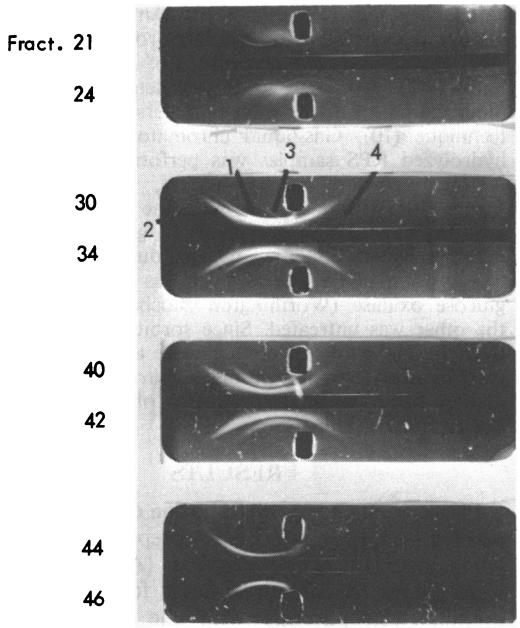

FIG. 2. *Separation of R. dentocariosa (ATCC 17931) cell wall antigen (no. 2) by chromatography of cell wall extract on Sephadex G-200. Antiserum in the center trough was prepared against whole cells of R. dentocariosa.*

for further study. Fraction 44 gave a reaction of identity on double-diffusion plates with a band in the original unfractionated extract. This particular antigen was chosen for three reasons. (i) It is a common antigen found in the cell wall extracts of all 26 strains of *R. dentocariosa* examined. (ii) It appeared to be a major antigen accounting for 31% (range, 28 to 33%) of the antibody nitrogen produced in response to the injection of whole cell vaccines in each of six different rabbits. (However, the purified antigen was not immunogenic in rabbits.) (iii) The antigen was stable (see below).

Preliminary chemical analysis of the dialyzed untreated material indicated that the antigen (designated RPS) was carbohydrate in nature, with a small amount of protein (Table 1). After hydrolysis in 0.2 N HCl glucose, galactose and a ketohexose were detected by chemical methods, with the ketohexose accounting for approximately 60% of the material on a dry weight basis. Paper chromatography with the use of two solvent systems confirmed the presence of fructose and revealed the presence of a sugar having an R_F and a color reaction identical to those of ribose (Table 2). A large spot with an R_F value similar to those of both glucose and galactose was also observed; since both sugars had been detected earlier by the glucose and galactose oxidase procedures, it was assumed that this large spot represented the fusion of the two sugars. The sugars in the hydrolysate were converted to their corresponding alditol acetates and then were analyzed by gas-liquid chromatography. The following sugars were observed: glucose (sorbitol), fructose (sorbitol and mannitol), ribose

TABLE 1. *Chemical analysis of Rothia dentocariosa cell wall polysaccharide (RPS)*

Sample	Component	Per cent (dry wt)
Untreated (10 mg)	Carbohydrate	94.0
	Nitrogen	0.1
	Protein	4.2
After hydrolysis[a]	Glucose (glucose oxidase)	4
	Galactose (galactose oxidase)	7
	Ketohexose (Roe method)	60

[a] A 10-mg sample was hydrolyzed by autoclaving in a sealed ampoule with 2 ml of 0.5 N HCl.

TABLE 2. *Chromatographic analysis of Rothia dentocariosa cell wall polysaccharide (RPS)*

Component	Paper chromatography	Gas-liquid chromatography[a]
Fructose	+	64
Ribose	+	11
Glucose	+	9
Galactose	+	0
Anhydroribitol	0	4

[a] Dry weight values from the hydrolysate in Table 1. Total per cent recovery of carbohydrate in gas-liquid chromatography = 89%.

(ribitol), and a small amount of anhydroribitol. Galactose was not detected as a separate entity, and it is possible that the glucose peak (with a similar R_F) also contains the galactose moiety of RPS (14). Muramic acid and hexosamine were not detected in the polysaccharide, but qualitative amino acid chromatography indicated the presence of alanine, lysine, and glutamic acid, suggesting that fragments of the wall mucopeptide may be attached to this polysaccharide.

Because it is unusual to find fructose as a major sugar in bacterial cell wall antigens, a new batch of *Rothia* cell walls was prepared without the use of centrifugation in 40% sucrose, and the presence of fructose in the cell wall hydrolysates was rechecked. A ketohexose having an absorption maximum identical to that of the control sample of fructose (515 nm) in the Roe resorcinol procedure (5) was observed. Since tagatose and sorbose could not be differentiated from fructose on this basis, an area on the paper chromatogram of the hydrolysate corresponding to the position of the "fructose" spot on a duplicate chromatogram was cut out, and the sugar was eluted from the paper with distilled water. The eluate was then concentrated, and a spectral analysis was performed on the color complex resulting from the secondary cysteine reaction of hexoses (5). The spectrum was identical to that of the fructose standard and was clearly different from the spectra of either tagatose or sorbose. Fructose and the test sample produced a greenish blue color in this reaction, whereas tagatose gave a yellowish color and sorbose gave a dark blue color.

Agar-gel precipitation analysis of the cell wall RPS is indicated in Table 3. In this qualitative haptene inhibition test, only fructose was observed to be effective in blocking precipitation, and it was presumed to be the determinant sugar. Control Ouchterlony gels containing fructose (1%), but with the use of a different antigen-antibody system (*Lactobacillus casei* glycogen with homologous antibody), did not show inhibition of precipitation, indicating that fructose was specific in its inhibition of RPS precipitation. Similarly, the addition of fructose to the RPS antiserum for 10 min prior to its use in the indirect fluorescent-antibody technique resulted in marked diminution or complete loss of fluorescence of organisms which would otherwise give a positive fluorescence.

The stability of this antigen under various growth conditions was also checked. The cell wall extracts of cells grown under various conditions invariably contained the RPS band on immunoelectrophoresis. Some of the conditions included: (i) growth at *p*H 5.5; (ii) aerobic and anaerobic incubation in Trypticase Soy Broth; (iii) growth at 25, 37, and 40 C; (iv) growth in fermentation medium base (2) containing glucose, maltose, salicin, sucrose, or lactose, and growth in nitrate medium (Difco); (v) using log-phase (12-hr) and stationary-phase cells (48-hr); and (vi) growth in the presence of 4% NaCl. Indeed, this antigen was even detected in cells grown in the presence of small amounts of sterile homologous antiserum.

TABLE 3. *Haptene inhibition in Rothia dentocariosa antiserum*[a]

Sugar (1%)	Inhibition of precipitation
Fructose	+
Glucose	−
Galactose	−
Ribose	−
Anhydroribitol	−
Ribitol	−
Sucrose	−
Control (no sugar added)	−

[a] A 0.1-ml amount of a 200 ng/ml solution of the extracted RPS in normal saline was added to the outer well of an Ouchterlony plate, and 0.1 ml of undilute antiserum was added to the center well. Final sugar concentration in the agar gel was 1%.

Occurrence of RPS in strains of R. dentocariosa and other actinomycetes. Different strains of *Rothia* were obtained from various investigators, and these strains were screened by the indirect fluorescent-antibody technique with the use of our test antiserum. The results in Table 4 indicate that all of the known strains of *R. dentocariosa* gave positive fluorescence reactions before sorption with RPS, whereas after sorption with RPS these strains became negative. However, if the antisera were sorbed with the other antigens in the cell wall (antigens 1, 3, and 4, as shown in Fig. 1), only minor changes in the fluorescence of the known *Rothia* strains were observed. There was a slight diminution in the intensity of staining (from 4+ to 2+ or 3+) after sorption, but these changes were clearly different from control cells or cells stained with RPS-sorbed sera. Similarly, no significant cross-reactions were observed between this reference antiserum and other filamentous organisms, including *Actinomyces naeslundii* strain I, *A. viscosus* strain T6, *A. odontolyticus* SS-1, and eight *Nocardia* strains. Also, as indicated previously, the same RPS antigen was detected in the cell wall extracts of the known *Rothia* strains by use of immunodiffusion techniques.

TABLE 4. *Fluorescent-antibody reactions of known strains with Rothia dentocariosa (ATCC 17931) antiserum*

Strain	Clinical source	17931 antiserum	
		Before sorption with RPS	After sorption with RPS
R. dentocariosa ATCC 17931	Carious teeth	4+	0
R. dentocariosa X303	Saliva	4+	0
R. dentocariosa X346	Carious teeth	4+	0
R. dentocariosa X356	Saliva	4+	0
R. dentocariosa X368	Cerebral spinal fluid	4+	0
R. dentocariosa X614a	Throat	2+	±
R. dentocariosa W841	Gums	4+	0
R. dentocariosa W1008	Leg ulcers	3+	+
Actinomyces naeslundii (1)	Gingiva (human)	0	0
A. viscosus (T6)	Hamster	±	0
A. odontolyticus	Carious dentin	0	0
Nocardia strains (eight)[a]	Unknown	0	0
Anaerobic diphtheroid (4st)	Saliva	0	0

[a] The eight *Nocardia* strains included: *N. asteroides* (three), *N serratia*, *N. brasidiensis* (two), *N. pellegrino*, and *N. corallina*.

The detection of RPS by the fluorescent-antibody method was also satisfactory for screening unknown isolates in clinical samples. From a group of 30 unidentified oral filamentous organisms isolated from human subjects (saliva and carious dentin), 7 were shown to give positive fluorescence. Six of these seven gave biochemical reactions which were consistent with *Rothia* identification (Table 5), whereas the remaining 23 gave no fluorescence and turned out to have biochemical properties or combinations of biochemical properties clearly different from those of *Rothia*. Thus, the validity of a serological identification was supported by confirmatory biochemical and cultural criteria.

In addition, samples of carious dentin from Syrian golden hamsters infected with *Rothia* were obtained from S. S. Socransky and were checked with the fluorescent-antibody technique. These samples gave clear positive reactions (3+ or 4+), whereas the control animals not infected with *Rothia* gave questionable (±) or negative (0) fluorescence. *Rothia*-type organisms were also subcultured from the clinical sample to tubes of Trypticase Soy Broth, and isolates from Trypticase Soy Agar plates were subjected to the same biochemical tests listed in Table 5. The isolates having colonial and cellular morphologies characteristic of *Rothia* strains were shown to have biochemical properties identical to those of the type strain used originally to infect the animals.

DISCUSSION

In the light of increasing evidence (9, 12) pointing to the involvement of gram-positive filamentous organisms in human oral disease (periodontal pathology and root-surface caries), there is a clear need for some means of identifying these slow growing organisms in vitro and in vivo. The need is equally obvious for some stable property which can be used as a group marker in ecological and epidemiological studies. The present investigation has yielded data indicating that a fructose-containing cell wall antigen satisfies such a need for the genus *Rothia*.

The presence of such an antigenic marker also offers another means of studying the relationships of this newly created taxonomic group to other members of the family *Actinomycetaceae*. Since this antigen appears to be unique to all strains of *Rothia* examined, it could be claimed that the taxonomic separation of this group from other filamentous organisms is justified on serological as well as physiological grounds. By extension, it should be possible to determine quantitatively the degrees of serological relatedness to the type strain or the serological uniqueness, of freshly isolated *Rothia*-type filaments in clinical samples. The results of the fluorescent-antibody determinations indicated clearly that degrees of positive fluorescence do occur (from 2+ to 4+) even among known strains of *Rothia*. Thus, the relative ability of purified RPS to inhibit the precipitation of an antigenic extract from an unidentified filament could be taken as a measure of serological relatedness between the unidentified strain and the type strain. In addition, some knowledge of the chemical nature of the cross-reacting antigen could be deduced, because the chemical nature (and determinant sugar) of the reference antigen is known.

The chemical composition of this new antigen presents several features not previously associated with the genus *Rothia*. In contrast to a previous report indicating that galactose was the sole monosaccharide in *Rothia* cell walls (6), the cell wall antigen described here contains fructose,

TABLE 5. *Biochemical reactions of isolates showing positive fluorescent-antibody reactions with Rothia dentocariosa antiserum*

Strain designation[a]	Catalase	Nitrate reduction	Nitrite reduction	Indole	Gelatinase	H_2S	Lactose fermentation	CO_2 requirement	Esculin hydrolysis
B8	+[b]	+	+	0	0	+	0	0	+
B14	+	+	0	±	+	±	0	0	0
RC27	+	+	+	0	0	+	0	0	+
RC29	+	+	+	0	0	+	0	0	+
RC30	+	+	+	0	0	+	0	0[c]	+
RC37	+	+	+	0	0	+	0	0	±
RC44	+	+	+	0	0	+	±	0	+
R. dentocariosa ATCC 17931	+	+	+	0	0	+	0	0	+

[a] All of the above strains fermented glucose, maltose, sucrose, and salicine; none fermented mannitol, starch, arabinose, xylose, or inositol; all required a source of organic nitrogen for growth.

[b] Reactions: +, positive; 0, negative; ±, weak.

[c] Strain RC30 did not show an absolute requirement for added CO_2, but growth was clearly stimulated by it under anaerobic conditions.

glucose, and ribitol components as well. In fact, galactose would appear to be a minor component, as it accounts for only a small percentage of the total dry weight (7%) and does not inhibit specific precipitation of the antigen in the presence of immune serum. Fructose, by contrast, accounts for a major portion of the total dry weight and appears to be a determinant of serological specificity. Since fructose has not been reported in the cell walls of any other actinomycete, it is not surprising that antibodies against this antigen fail to cross-react with the antigens of other actinomycetes. It therefore seems reasonable to conclude that fructose is the characteristic sugar for the cell wall of *Rothia*, in much the same way that rhamnose is characteristic of the cell walls of streptococci and lactobacilli.

Another extremely interesting possibility is the relationship of these cell wall antigens to the morphology of the cell wall. *Rothia* is known to have both a coccal form and a filamentous form, although the chemical and antigenic changes in the cell wall accompanying this morphological alteration have not been reported. There is an equally wide gap in our knowledge of the environmental factors responsible for or associated with such changes. The loss or gain of some chemically defined antigen might provide an important clue regarding the mechanism of this structural rearrangement of the cell wall. In a manner analogous to the loss of cell wall antigens by bacterial L-forms, it is conceivable that the coccal phase of *Rothia* could result from a deficiency in the manufacture of serologically active cell wall polymers. It has not yet been possible to determine such differences experimentally, because it is not technically feasible to get pure cultures of the coccal phase for purposes of comparison.

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