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Biochemical and Immunochemical Properties of the Cell Surface of *Renibacterium salmoninarum*

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The biochemical composition of the cell envelope of *Renibacterium salmoninarum* was investigated in a total of 13 strains isolated from different salmonid fish species at various geographical locations of the United States, Canada, and Europe. A marked similarity with the type strain *R. salmoninarum* ATCC 33209 was found both in the peptidoglycan and the cell wall polysaccharide. The primary structure of the peptidoglycan was found to be consistent with lysine in the third position of the peptide subunit, a glycyl-alanine interpeptide bridge between lysine and D-alanine of adjacent peptide subunits, and a D-alanine amide substituent at the α-carboxyl group of D-glutamic acid in position 2 of the peptide subunit. The cell wall polysaccharide contained galactose as the major sugar component which was accompanied by rhamnose, *N*-acetylglucosamine, and *N*-acetylfucosamine. The polysaccharide amounted to more than 60% of the dry weight of the cell walls. It was found to be covalently linked to the peptidoglycan and was released by hot formamide treatment. On gel filtration chromatography the extracted polysaccharide behaved like a homogeneous polymeric compound. The purified cell wall polysaccharide showed antigenic activity with antiserum obtained by immunization of rabbits with heat-inactivated trypsinized cells of *R. salmoninarum*. Immunoblotting experiments with nontrypsinized cell walls and antisera raised against *R. salmoninarum* cells revealed that antigenic proteins were attached to the cell walls.

Among fish diseases, bacterial kidney disease (corynebacterial kidney disease, salmonid kidney disease) is of high importance from both a microbiological and an economic point of view. The causative agent of bacterial kidney disease has been found to be *Renibacterium salmoninarum*, a unique bacterial pathogen infecting specifically members of the family *Salmonidae*: salmon, trout, and char (13). The organism was originally associated with the genus *Corynebacterium* (26). It has subsequently been reclassified on the basis of the G+C content of its DNA (ca. 53 mol%) and criteria corresponding to the cell wall (28). The indicated integrity of the genus *Renibacterium* has been supported by lipid data (9) and most recently by several biochemical characteristics (3). Two cell wall components have also been found to be characteristic of the type strain of *R. salmoninarum*: a unique peptidoglycan and an unusual cell wall polysaccharide having galactose, rhamnose, *N*-acetylglucosamine, and *N*-acetylfucosamine as constituents (19). In the present study several representatives of *R. salmoninarum*, isolated from different salmonid fish species at various geographical locations, were the subject of biochemical investigations of the cell surface. These were designed to examine the coherence of the taxon in this regard and to obtain insight into the biochemistry of somatic antigens among strains of *R. salmoninarum*.

MATERIALS AND METHODS

Organisms and growth conditions. The strains investigated are described in Table 1. The organisms were kindly provided by B. Austin (Fish Diseases Laboratory, Directorate of Fisheries Research, Ministry of Agriculture, Fisheries and Food, Weymonth, Dorset DT4 8UB, United Kingdom), except the type strain *R. salmoninarum* ATCC 33209, which was obtained from the American Type Culture Collection, Rockville, Md. The organisms were cultivated on agar with a semidefined growth medium (8) and in liquid with KDM-2 medium (10) which was modified such that 20% fetal calf serum was replaced with 2.5% calf serum (Serva, Heidelberg, Federal Republic of Germany). Cells were grown aerobically in Erlenmeyer flasks with shaking at 15°C until the early stationary phase of growth (A_{578} = 0.9 to 1.2).

Preparation of cell walls and extraction procedures. Cells were harvested by centrifugation and washed with 0.9% aqueous NaCl after heating at 100°C for 10 min. Cell walls were isolated by the method of Schleifer and Kandler (29).

TABLE 1. Designation and source of *Renibacterium* strains

Strain no.	Source and strain history
ATCC 33209[Ta]	J. L. Fryer, Lea-1-74, from *Oncorhynchus tshawytscha* (United States)
K35	B. Spacey, Burroughs-Wellcome, Kent, England; from *Salmo gairdneri* (England)
K45	D. Groman, College of Forestry, Wildlife and Range Sciences, University of Idaho, Moscow; from *Oncorhynchus nerka* (United States)
K47	NCMB 1112[a]; from *Salvelinus fontinalis* (United States)
K48	NCMB 1114[a]; J. W. Smith in 1962; from *Salmo salar* (River Dee, Scotland)
K63, K70	From *S. gairdneri* (fish farm, Cumbria, England)
K75, K77	B. Souter, Freshwater Institute, Winnipeg, Canada; WS-1 from *S. gairdneri* (Canada), KLAV 75 from *S. gairdneri* (Canada)
K82	F. Bandin-Laurencin, Brest, France; from *Oncorhynchus kisutch* (France)
K83, K84, K86	From *S. gairdneri* (England)

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCMB, National Collection of Marine Bacteria, Aberdeen, Scotland

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TABLE 2. Quantitative amino acid, amino sugar, and ammonia content of trypsinized walls from *R. salmoninarum* strains

Strain	nmol/mg of cells walls								Molar ratio								
	Glu	Gly	Ala	Lys	Mur[a]	$GlcNH_2$[a]	NH_3	$FucNH_2$[a]	Glu	Gly	Ala	Lys	Mur[a]	$GlcNH_2$[a]	NH_3	$FucNH_2$[a]	D-Ala[b]
ATCC 33209	209	233	755	244	236	780	255	908	1.0	1.1	3.6	1.2	1.1	3.7	1.2	4.3	1.9
K35	186	215	703	233	164	656		893	1.0	1.2	3.8	1.3	0.9	3.5		4.8	
K45	242	310	798	228	192	783	304	1,014	1.0	1.3	3.3	0.9	0.9	3.2	1.3	4.2	1.7
K47	199	210	649	214	213	661	230	703	1.0	1.1	3.3	1.1	1.1	3.3	1.2	3.5	
K48	196	211	684	213	220	682	262	777	1.0	1.1	3.5	1.0	1.1	3.5	1.3	4.0	
K63	123	141	432	130	116	469		516	1.0	1.1	3.5	1.0	0.9	3.8		4.2	
K70	176	191	605	190	207	663	232	727	1.0	1.1	3.4	1.1	1.2	3.8	1.3	4.1	1.8
K75	179	186	577	220	136	556	176	563	1.0	1.0	3.2	1.2	0.8	3.1	1.0	3.1	
K77	191	198	632	181	191	653	211	740	1.0	1.0	3.3	0.9	1.0	3.4	1.1	3.9	1.9
K82	171	245	720	160	179	588	192	680	1.0	1.4	4.2	0.9	1.0	3.4	1.1	4.0	1.8
K83	200	217	780	237	174	662	204	706	1.0	1.1	3.9	1.2	0.9	3.1	1.0	3.5	
K84	211	217	809	236	256	786	166	754	1.0	1.0	3.8	1.1	1.2	3.7	0.8	3.6	1.8
K86	176	208	655	188	196	757	185	850	1.0	1.2	3.7	1.1	1.1	4.3	1.0	4.8	

[a] Mur, Muramic acid; $GlcNH_2$, glucosamine; $FucNH_2$, fucosamine.
[b] Determined in separate experiments.

The cell walls were extensively washed with 0.9% aqueous NaCl (nontrypsinized walls) or treated with trypsin (trypsinized walls). Trypsinized walls were further purified with hot 4% (wt/vol) aqueous sodium dodecyl sulfate (SDS) (2).

Polysaccharide covalently bound to the peptidoglycan was removed from cell walls with cold 60% (wt/vol) aqueous hydrofluoric acid (HF; Riedel de Häen, Seelze, Federal Republic of Germany) (11) or hot formamide (14, 27).

Preparation of cell wall polysaccharide. Polysaccharide extracted from cell walls with formamide was freed from the extractant by extensive dialysis against distilled water and subsequently lyophilized. A gel filtration column (145 by 2.5 cm) of Sephacryl S-300 (Pharmacia, Uppsala, Sweden) was used to fractionate the polysaccharide. The column was eluted with 0.1 M ammonium acetate (pH 7.0) at a flow rate of 24 ml/h at room temperature. Fractions of 4 ml were collected.

Analytical methods. For total amino acid and amino sugar analysis, cell walls were hydrolyzed with 4 M HCl for 16 h at 100°C. For sugar analysis, cell walls, HF-extracted material, and formamide-extracted polysaccharide were hydrolyzed with 2 M HCl for 3 h at 100°C.

Quantitative determination of amino acids and amino sugars was performed with an amino acid analyzer (Biotronic LC 6001). The correction for destruction during hydrolysis was based on the analysis of mixtures of known quantities of authentic amino acids and amino sugars which were hydrolyzed under identical conditions as samples. The configuration of alanine was determined with D-alanine oxidase (20). The reaction was monitored with the amino acid analyzer. Phosphorus was measured by the method of Ames (1). Total carbohydrate was determined by the phenol-sulfuric acid method (7). Sugars were identified as their corresponding alditolacetate derivatives by a gas-liquid chromatographic procedure (11). For quantification, external standards consisting of known quantities of reference compounds which were treated in the same manner as samples were used. Galactose was further quantified with galactose dehydrogenase (18). Protein was measured by the method of Lowry et al. (21).

Immunological methods. To prepare a vaccine, heat-inactivated cells were suspended in 0.05 M phosphate buffer (pH 7.8) and treated with trypsin for 3 h at 37°C. Subsequently, the cells were extensively washed with 0.9% aqueous NaCl and stored in this solution at −20°C. The immunization of rabbits and the collection of antiserum were carried out as described by Oeding (24). Serum samples were divided into small aliquots and preserved in a deepfreeze without preservative. Final agglutinin titers of serums, determined by the tube agglutination procedure, were: for strain ATCC 33209, 1:256 (trypsinized cells) and 1:512 (nontrypsinized cells); for strain K82, 1:128 (trypsinized cells) and 1:256 (nontrypsinized cells). A rabbit antiserum raised against living nontrypsinized cells of *R. salmoninarum* ATCC 33209 was provided by C. Pfeil-Putzien (Institute for Zoology and Hydrobiology, University of Munich).

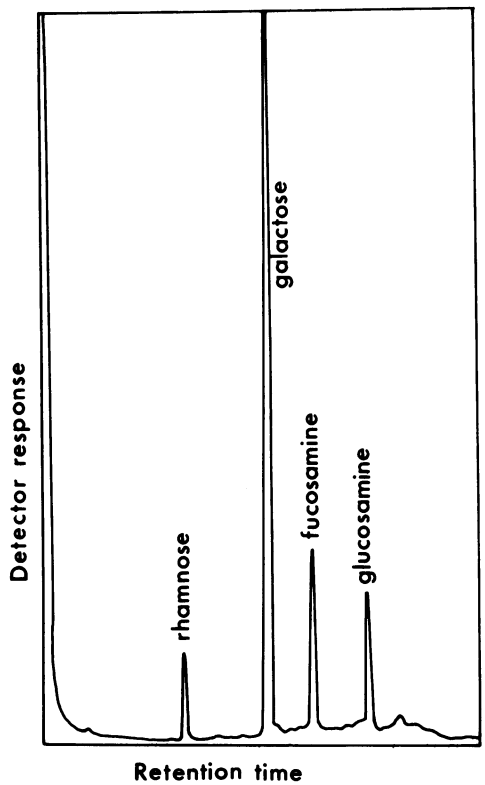

FIG. 1. Sugar components in the hydrolysate (2 M HCl, 100°C, 3 h) of SDS-treated trypsinized cell walls of *R. salmoninarum* ATCC 33209. Derivatization of sugars and detection by gas-liquid chromatography was accomplished as described in Materials and Methods.

The titers were 1:2,048 and 1:512 with nontrypsinized and trypsinized cells, respectively, in the agglutination test.

Agglutination of cells was tested by the tube method (16). Antiserum (200 μl) was subjected to twofold serial dilution in 0.02 M sodium phosphate-buffered saline (pH 7.2) containing 0.04% sodium azide, and 500 μl of cells suspended in 0.9% aqueous NaCl (A_{578} = 1.0) was added to each of the tubes. The mixtures were incubated at 37°C and read after 20 h.

Agglutination of cell walls was checked by the slide method (16), using a phase-contrast microscope (Leitz Dialux 20). Cell walls were suspended in 0.01 M sodium phosphate-buffered saline (pH 7.2) and carefully homogenized with a Branson sonifier (A_{578} = 0.24 to 0.28). Suspended cell walls (5 μl) were added to 5 μl of antiserum diluted 1:10 in 0.01 M sodium phosphate-buffered saline (pH 7.2). In a negative control cell walls were incubated with serum collected before immunization of the rabbit. The slides were incubated in a moist chamber overnight at room temperature. Agglutination of cell walls was demonstrated if aggregates of large square dimensions (more than 20 by 20 μm) were seen.

The quantitative precipitin reaction was done as previously described (16). Various amounts of purified cell wall polysaccharide dissolved in 140 μl of 0.05 M Tris hydrochloride (pH 7.5) containing 0.015% sodium azide were added to 60 μl of rabbit antiserum. The mixtures were preincubated for 60 min at 37°C and then at 4°C for 2 days. The precipitates were washed three times with cold 0.9% aqueous NaCl, and the protein content was determined. Inhibition testing in the quantitative precipitin reaction was done with various sugar compounds applying different concentrations under conditions described above.

For the immunoblotting technique, the method described by Howe and Hershey (15) was followed with some modifications. Proteins adsorbed to cell walls were separated electrophorectically on a 12% SDS-polyacrylamide gel (22). After electrophoresis, the proteins were electrophoretically transferred to a nitrocellulose sheet by using a Bio-Rad blot chamber (60 V, 2 h). The electrode buffer consisted of 25 mM Tris-base, 192 mM glycine, 20% methanol, and 0.1% SDS, pH 8.3. The nitrocellulose sheet was stained with 0.1% amido black in an aqueous solution of 45% methanol–10% acetic acid and destained in 90% methanol–2% acetic acid.

TABLE 3. Composition of polysaccharide eluted from the Sephacryl S-300 column used (sugars were determined by gas-liquid chromatography)

Fraction	Molar ratio			
	Galactose	Rhamnose	Glucosamine	Fucosamine
Ascending part of peak II	1.00	0.18	0.20	0.25
Descending part of peak II	1.00	0.12	0.19	0.16

After washing with distilled water, the nitrocellulose foil was incubated with 3% bovine serum albumin in buffer A (10 mM Tris hydrochloride, 0.5% Triton X-100, 0.2% SDS in saline, pH 7.4) for 1 h at 37°C. Antisera were diluted 1:50 and 1:200 in buffer A containing 1% bovine serum albumin and incubated with the blot overnight with gentle shaking at room temperature. The nitrocellulose blot was washed five times (15 min each) with buffer A and subsequently incubated with a 30-ml solution of ^{125}I-labeled protein A from *Staphylococcus aureus* (2.5 μCi; New England Nuclear Corp., Boston, Mass.) in buffer A containing 1% bovine serum albumin for 3 h at room temperature. Finally, the blots were rinsed five times with buffer A, dried, and monitored by autoradiography with Kodak X-Omat AR 50 film.

RESULTS

Amino acid and amino sugar composition of cell walls. The amino acid and amino sugar composition determined in trypsinized walls prepared from 13 representatives of *R. salmoninarum* is summarized in Table 2. All the organisms exhibited identical amino acids and amino sugars in similar molar ratios which were in line with the earlier study (19). This was also true for the molar ratio of D-alanine/glutamic acid which was found in several samples to be close to 2. This finding and the molar ratios of amino acids and amino sugars were taken as evidence that the unique peptidoglycan structure discovered previously (19) is a general feature of the strains tested. The amino sugar fucosamine and the excess of glucosamine, as compared with glutamic acid and muramic acid, are part of the polysaccharide covalently bound to the peptidoglygan.

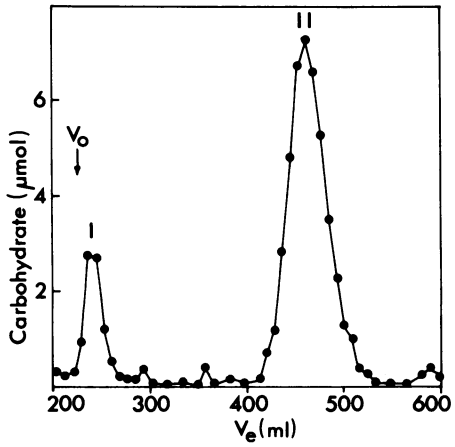

FIG. 2. Chromatography on Sephacryl S-300 of material extracted by formamide from SDS-treated cell walls of *R. salmoninarum* ATCC 33209. The exclusion volume (V_0) of the column was determined in a separate run with blue dextran.

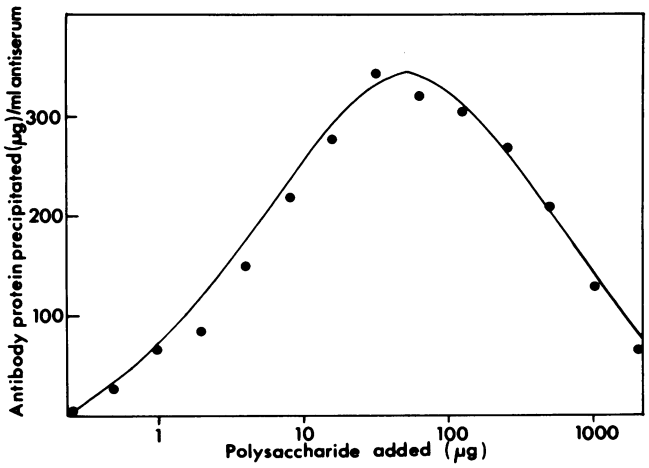

FIG. 3. Quantitative precipitation between antiserum against whole cells of *R. salmoninarum* ATCC 33209 and polysaccharide fractionated as described in the legend to Fig. 2.

TABLE 4. Agglutination of cell walls from various organisms by rabbit antiserum raised against trypsinized cells of *R. salmoninarum* ATCC 33209

Strain	Cell wall preparation	Peptidoglycan[a]	Composition of cell wall polysaccharide[a]	Occurrence of teichoic acid	Agglutination
Renibacterium salmoninarum ATCC 33209	Trypsinized	Lys-Ala-Gly, D-Glu-D-AlaNH_2; A3α (19)	Gal, Rha, GlcNH_2, FucNH_2 (19)	−	+
Renibacterium salmoninarum K84	Nontrypsinized	This paper	This paper	−	+
"*Gaffkya homari*" ATCC 10400	Trypsinized	Lys direct; A1α (23)	Gal, Glc, GlcNH_2, GalNH_2[b], unidentified constituents	−	−
AC480[c]	Trypsinized	m-Dpm direct; A1γ (11)	Gro, Mat, Gal, Glc, Rha, GalNH_2, GlcNH_2, phosphate (11)	+	−
AC577[c]	Trypsinized	m-Dpm direct; A1γ (11)	Gro, Mat, Gal, Glc, GalNH_2, GlcNH_2, phosphate (11)	+	−
Brevibacterium lyticum ATCC 15921	Trypsinized	Lys-D-Ser-D-Asp; A4β (31)	Gal, GalNH_2, GlcNH_2[d] (31)	−	+
Arthrobacter aurescens DSM 20116	Trypsinized, SDS	Lys-Ala-Thr-Ala; A3α (12)	Gal[d]	−	+

[a] Gal, Galactose; GalNH_2, galactosamine; Glc, glucose; GlcNH_2, glucosamine; Gro, glycerol; FucNH_2, fucosamine; Mat, mannitol; Rha, rhamnose; AlaNH_2, alanineamide; A3α, A1α, and A1γ, A4β, peptidoglycan types as described by Schleifer and Kandler (30).
[b] Unpublished data.
[c] Strain of *Brevibacterium linens* (11), isolated by W. H. J. Crombach, Laboratory of Microbiology, Agricultural University, Wageningen, the Netherlands.
[d] Polysaccharide decomposed by 60% aqueous HF (Fiedler and Nordmann, in preparation).

On the basis of a molecular weight of approximately 1,095 for the disaccharide-peptide subunit of this peptidoglycan type and a content of 0.18 to 0.24 μmol of glutamic acid per mg of dry cell walls, it was concluded that only 14 to 27% of the cell wall material corresponds to peptidoglycan. The fact that additional amino acids were not present indicated that polysaccharide covalently linked to pepidoglycan is the predominant component in the cell walls of the *Renibacterium* strains tested.

Cell wall polysaccharide. The determination of phosphorus and sugars in acid hydrolysates (2 M HCl, 100°C, 3 h) and hydrolyzed HF extracts (2 M HCl, 100°C, 3 h) of SDS-treated trypsinized walls made from all the test organisms confirmed the earlier results (19). The phosphate content of dry cell walls consistently did not exceed 0.15 μmol/mg, and polyols were not detected. This observation indicated that the *Renibacterium* strains tested do not possess teichoic acids in the cell walls. There is also evidence that lipoteichoic acids do not occur in the cytoplasmic membranes of the organisms (G. Ruhland and F. Fiedler, unpublished data). The composition of the polysaccharide is shown in Fig. 1. Galactose was uniformly the principal sugar component and was accompanied by rhamnose, glucosamine, and fucosamine. The polysaccharide was partially hydrolyzed by 60% HF yielding mainly monomeric galactose and significant amounts of fucosamine. This decomposition by HF was not caused by the presence of phosphodiester bonds but by the occurrence of highly acid-labile glycosidic linkages of galactosyl residues in furanoid configuration (25; F. Fiedler and P. Nordmann, manuscript in preparation). The polysaccharide was cleaved from cell walls of the type strain with hot formamide. When subjected to column chromatography on Sephacryl S-300, the water-soluble polysaccharide was separated as shown in Fig. 2. The small peak I, appearing close to the void volume of the column, contained peptidoglycan components, whereas fractions corresponding to peak II exclusively contained the constituents of the polysaccharide. Phosphorus was not measured in significant amounts. The fact that neither the isolated polysaccharide nor the fucosamine cleaved by HF from the polysaccharide reacted with ninhydrin indicated that the amino groups of glucosamine and fucosamine were blocked. Therefore, it was assumed that the amino sugars occur as *N*-acetylglucosamine and *N*-acetylfucosamine residues in the polysaccharide. The chromatographic behavior of the material corresponding to peak II was similar to that found with a dextran of a molecular weight of 10,000 (dextran T 10; Pharmacia). This property and its rather condensed elution from the column are consistent with those expected from a polymeric homogeneous material. This was essentially supported by analytical data obtained by measuring fractions in the ascending and descending parts of peak II (Table 3), although the molar ratios are not perfectly equal.

Immunological experiments. To define the antigenicity of components at the cell surface, we raised antibodies against heated and trypsinized cells from the type strain of *R. salmoninarum* and strain K82 in rabbits. The serums obtained agglutinated both trypsinized and nontrypsinized cells of the organisms used for immunization of the rabbits as well as cells from other strains used. The titers read were in the range of 1:128 to 1:512.

To check the antigenicity of the polysaccharide preparation (Fig. 2), we carried out a quantitative precipitin reaction between polysaccharide and rabbit antiserum (Fig. 3). When about 40 μg of antigen was reacted with 60 μl of antiserum, the largest amounts of precipitate were measured. Inhibition testing in the quantitative precipitin reaction was followed with various monosaccharides constituting the polysaccharide, oligosaccharides containing these sugars in different linkages, and methyl-D-galactoside compounds. However, none of the compounds assayed showed significant hapten activity in the inhibition test.

The antigenicity of the cell wall polysaccharide was further examined in agglutination experiments reacting trypsinized cell walls and rabbit antiserum. Both trypsinized and nontrypsinized walls from different *Renibacterium* strains were agglutinated by the antiserum (Table 4). Some cell walls prepared from other gram-positive organisms, exhibiting both various peptidoglycan types and accessory cell wall components, did not react with this antiserum. However, the cell walls from *Arthrobacter aurescens* DSM 20116 and *Brevibacterium lyticum* ATCC 15921 showed a high reactiv-

ity with the antiserum against *Renibacterium* cells. While the peptidoglycan types of these cell walls were different from that existing in *Renibacterium*, the compositions of the polysaccharide portions of the cell walls were similar in two ways with that found in *Renibacterium* strains. On the one hand, galactose was the predominant component, and on the other, the polysaccharides present were decomposed by 60% aqueous HF. These findings indicated that the polysaccharide linked to peptidoglycan in cell walls of *R. salmoninarum* is a somatic antigen in which galactosyl residues obviously function as antigenic determinants.

To further define antigenic properties of the cell surface of *R. salmoninarum*, we performed an immunoblotting experiment, subjecting nontrypsinized cell walls from strain K84 to polyacrylamide gel electrophoresis in the presence of SDS. Proteins noncovalently attached to cell walls migrated into the gel, and antigenic proteins were detected by an immunoblotting procedure with antisera which were elicited in rabbits against trypsinized and nontrypsinized cells of the type strain of *R. salmoninarum*. Several proteins were detected in the gel by staining with Coomassie blue (Fig. 4). A predominant protein corresponding to an approximate molecular weight of 70,000, based on a molecular weight standard, was present which strongly reacted with the antisera used. The significantly stronger reactivity observed with the antiserum raised against nontrypsinized cells possibly points to the location of this protein on the cell surface where it is functioning as an antigen. Using nontrypsinized cells for the immunization of rabbits, some other proteins obviously attached with cell walls to a minor extent raised antibodies which were also visualized in this experiment. The proteins detected in the cell wall preparation of *R. salmoninarum* K84 may not originate from the growth medium since trypsinized cell walls of the same organism did not adsorb proteins from the KDM-2 medium.

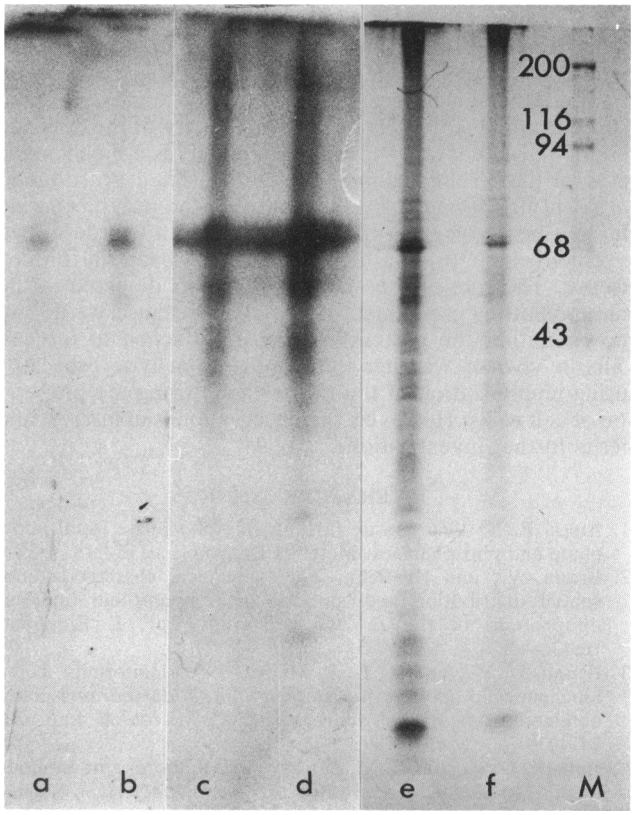

FIG. 4. Gel electrophoresis and immunoblotting of proteins attached to cell walls of *R. salmoninarum* K84. Lanes a and b: 50 and 100 μg of nontrypsinized walls, respectively, reacted with antiserum against inactivated trypsinized cells of *R. salmoninarum* ATCC 33209 and ^{125}I-labeled protein A of *S. aureus*. Lanes c and d: Identical with lanes a and b, except that antiserum raised against nontrypsinized cells of *R. salmoninarum* ATCC 33209 was used. Lanes e and f: 100 and 50 μg of nontrypsinized walls, respectively, stained with Coomassie blue. Lane M, Molecular weight standards: myosin (200,000); α-galactosidase (116,500); phosphorylase *b* (94,000); bovine serum albumin (68,000); ovalbumin (43,000).

DISCUSSION

The peptidoglycan and the accessory cell wall polysaccharide from 13 representatives, including the type strain of *R. salmoninarum*, isolated from six fish species of the family *Salmonidae* at different locations in the United States, Canada, and Europe turned out to be uniform. These findings are in perfect accordance with other studies (3, 9) demonstrating *Renibacterium* as a coherent genus. On the basis of the numerous fish pathogenic isolates studied serologically (5) and most recently biochemically (3), the genus obviously consists only of the species *R. salmoninarum* which may cause bacterial kidney disease among different salmonid fishes. It is interesting to note that this was additionally indicated by the cell wall polysaccharide. In contrast to other genera of gram-positive bacteria, the polysaccharide behaved invariably similarly among the representatives tested in respect to both its quantity in the cell walls and the chemical composition. To the best of our knowledge this is a unique polysaccharide found in *Renibacterium* strains. However, structural features have yet to be elucidated. Because the component *N*-acetylfucosamine seems to be rare in cell walls of gram-positive bacteria, its presence in the *Renibacterium* polysaccharide may be used to identify *Renibacterium* strains.

The data presented allow us to describe some features of the organization of the cell surface of *R. salmoninarum*. The walls consist of the peptidoglycan described previously (19) and the specific polysaccharide. Remarkably, this polysaccharide amounts to about 60 to 70% of the dry weight of trypsinized wall preparations and obviously includes similar molecular types each having an approximate molecular weight of 10,000. The polysaccharide does not merely coexist with the peptidoglycan but is covalently bound to it. The low amount of phosphorus occurring in cell walls and a compound in the acid hydrolysate of cell walls showing a chromatographic behavior similar to muramylphosphate (data not shown) indicate that the polysaccharide molecules are linked to the hydroxyl at C-6 of *N*-acetylmuramyl residues via phosphodiester bridges. On the basis of a molecular weight of approximately 10,000 for the polysaccharide and the content measured for its constituents per milligram of dry cell walls, it can be assumed that about 120 nmol of polysaccharide is present in 1 mg of these cell walls. Since muramic acid amounts to about 230 nmol/mg of cell walls in the type strain *R. salmoninarum* ATCC 33209, it can be concluded that roughly every second *N*-acetylmuramyl residue of the glycan strands of the peptidoglycan is substituted. This assumption seems to be supported both by the amount of phosphate found in the cell walls and by the failure to lyse cells and cell walls with lysozyme or mutanolysin (data not shown). As a consequence of the low amount of peptidoglycan in the cell wall and the high degree

of substitution of *N*-acetylmuramyl residues, it seems that the surface of a *Renibacterium* cell is formed mainly by its unique polysaccharide. Hence, the polysaccharide might be of importance in determining pathogenic and serological properties of *R. salmoninarum*. Antigenicity of the polysaccharide in rabbits was shown and probably is a biochemical basis for the serological methods used to detect *R. salmoninarum* in fish tissues (4, 17). However, the polysaccharide may not be involved in the immunodiffusion test described by Chen et al. (6) for diagnosing overt bacterial kidney disease. The role of the antigenic proteins detected in the immunoblotting experiment is not clear because we do not know whether the proteins are really attached to the cell walls in vivo or whether this results from lytic processes during immunization of the rabbits and during the preparation of cell walls. However, the observation is of interest and merits further investigation.

LITERATURE CITED

1. **Ames, B. N.** 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. **3**:115–118.
2. **Braun, V., and K. Rehn.** 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *E. coli* cell wall. Eur. J. Biochem. **10**:426–438.
3. **Bruno, D. W., and A. L. S. Munro.** 1986. Uniformity in the biochemical properties of *Renibacterium salmoninarum* isolates obtained from several sources. FEMS Microbiol. Lett. **33**: 247–250.
4. **Bullock, G. L., and H. M. Stuckey.** 1975. Fluorescent antibody identification and detection of the *Corynebacterium* causing kidney disease of salmonids. J. Fish. Res. Board Can. **32**:2224–2227.
5. **Bullock, G. L., H. M. Stuckey, and P. K. Chen.** 1974. Corynebacterial kidney disease of salmonids: growth and serological studies on the causative bacterium. Appl. Microbiol. **28**:811–814.
6. **Chen, P. K., G. L. Bullock, H. M. Stuckey, and A. C. Bullock.** 1974. Serological diagnosis of corynebacterial kidney disease of salmonids. J. Fish. Res. Board Can. **31**:1939–1940.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350–356.
8. **Embley, T. M., M. Goodfellow, and B. Austin.** 1982. A semidefined medium for *Renibacterium salmoninarum*. FEMS Microbiol. Lett. **14**:299–301.
9. **Embley, T. M., M. Goodfellow, D. E. Minnikin, and B. Austin.** 1983. Fatty acid, isoprenoid quinone and polar lipid composition in the classification of *Renibacterium salmoninarum*. J. Appl. Bacteriol. **55**:31–37.
10. **Evelyn, T. P. T.** 1977. An improved growth medium for the kidney disease bacterium and some notes on using the medium. Bull. Off. Int. Epizooties **87**:511–513.
11. **Fiedler, F., M. J. Schäffler, and E. Stackebrandt.** 1981. Biochemical and nucleic acid hybridization studies on *Brevibacterium linens* and related strains. Arch. Microbiol. **129**:85–93.
12. **Fiedler, F., K. H. Schleifer, and O. Kandler.** 1973. Amino acid sequence of the threonine-containing mureins of coryneform bacteria. J. Bacteriol. **113**:8–17.
13. **Fryer, J. L., and J. E. Sanders.** 1981. Bacterial kidney disease of salmonid fish. Annu. Rev. Microbiol. **35**:273–298.
14. **Fuller, A. T.** 1938. The formamide method for the extraction of polysaccharides from haemolytic streptococci. Br. J. Exp. Pathol. **19**:130–139.
15. **Howe, J. G., and J. W. B. Hershey.** 1981. A sensitive immunoblotting method for measuring protein initiation factor levels in lysates of *Escherichia coli*. J. Biol. Chem. **256**:12836–12839.
16. **Kabat, E. A., and M. M. Mayer.** 1971. Experimental immunochemistry. Charles C Thomas, Publisher, Springfield, Ill.
17. **Kimura, T.** 1978. Bacterial kidney disease of salmonids. Fish Pathol. **13**:43–52.
18. **Kurz, G., and K. Wallenfels.** 1970. D-Galactose. UV-Test mit Galactose-Dehydrogenase, p. 1241–1244. *In* H. U. Bergmeyer (ed.), Methoden der enzymatischen Analyse, vol. 2. Verlag Chemie, Weinheim.
19. **Kusser, W., and F. Fiedler.** 1983. Murein type and polysaccharide composition of cell walls from *Renibacterium salmoninarum*. FEMS Microbiol. Lett. **20**:391–394.
20. **Larson, D. M., D. C. Snetsinger, and P. E. Waibel.** 1971. Procedure for the determination of D-amino acids. Anal. Biochem. **39**:395–401.
21. **Lowry, D. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Lugtenberg, B., J. Meijers, R. Peters, P. van der Hoek, and L. van Alpen.** 1975. Electrophoretic resolution of the "major outer membrane protein" of *Escherichia coli* K-12 into four bands. FEBS Lett. **58**:254–258.
23. **Nakel, M., J. M. Ghuysen, and O. Kandler.** 1971. Wall peptidoglycan in *Aerococcus viridans* strains 201 Evans and ATCC 11563 and in *Gaffkya homari* strain ATCC 10400. Biochemistry **10**:2170–2175.
24. **Oeding, P.** 1978. Genus *Staphylococcus*. Methods Microbiol. **12**:127–176.
25. **Op den Camp, H. J. M., J. H. Veerkamp, A. Oosterhof, and H. van Halbeek.** 1984. Structure of the lipoteichoic acids from *Bifidobacterium bifidum* ssp. *pennsylvanicum*. Biochim. Biophys. Acta **795**:301–313.
26. **Ordal, E. J., and B. J. Earp.** 1956. Cultivation and transmission of the etiological agent of kidney disease in salmonid fishes. Proc. Soc. Exp. Biol. Med. **92**:85–88.
27. **Perkins, H. R.** 1965. The action of hot formamide on bacterial cell walls. Biochem. J. **95**:876–882.
28. **Sanders, J. E., and J. L. Fryer.** 1980. *Renibacterium salmoninarum* gen. nov., sp. nov., the causative agent of bacterial kidney disease in salmonid fishes. Int. J. Syst. Bacteriol. **30**:496–502.
29. **Schleifer, K. H., and O. Kandler.** 1967. Zur chemischen Zusammensetzung der Zellwand der Streptokokken. I. Zur Aminosäuresequenz des Mureins von *Str. thermophilus* and *Str. faecalis*. Arch. Mikrobiol. **57**:335–365.
30. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36**:407–477.
31. **Stackebrandt, E., F. Fiedler, and O. Kandler.** 1978. Peptidoglykantyp und Zusammensetzung der Zellwandpolysaccharide von *Cellulomonas cartalyticum* und einigen coryneformen Organismen. Arch. Microbiol. **117**:115–118.