Purification, and biochemical and structural characterization of a fimbrial haemagglutinin of *Renibacterium salmoninarum*

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Renibacterium salmoninarum was shown to possess peritrichous fimbriae. Electron microscopy of strains FMV 84-01 and ATCC 33209^T revealed short, flexible fimbriae less than 2 nm in diameter. These surface appendages were isolated from the bacteria by a procedure involving water extraction and urea solubilization. The fimbrin was purified to homogeneity by Fast Pressure Liquid Chromatography, and shown by SDS-PAGE to be a protein of 57 kDa. Isoelectric focusing under non-denaturing conditions indicated a pI of 4.8. The protein had an amino acid composition rich in glycine, Asx (aspartic acid and asparagine), valine and alanine; methionine was absent. Approximately 33% of the amino acid residues were hydrophobic. Immunoblotting using a polyclonal antiserum raised against whole cells showed that the 57 kDa protein was the immunodominant antigen on the cell surface. Immunogold labelling using polyclonal antibodies raised against the fimbrin revealed an alignment of gold particles along the fimbriae. Purified fimbriae caused agglutination of rabbit erythrocytes and antifimbrial serum inhibited this haemagglutination. Altogether the results indicate that the fimbriae on the surface of *R. salmoninarum* are responsible for the haemagglutinating activity.

Introduction

Renibacterium salmoninarum is the aetiological agent of bacterial kidney disease and is responsible for significant mortality in salmonid fish throughout the world (Fryer & Sanders, 1981). *R. salmoninarum* represents a unique bacterial pathogen in that the isolates obtained from several sources around the world show uniformity in biochemical properties (Bruno & Munro, 1986) and antigenicity (Bullock *et al.*, 1974). Getchell *et al.* (1985) claimed that the isolates shared seven antigens. Two cell wall components have been found to be characteristic of the type strain (ATCC 33209^T): a unique peptidoglycan and an unusual cell wall polysaccharide having galactose, rhamnose, *N*-acetylglucosamine and *N*-acetyl-fucosamine as constituents (Kusser & Fiedler, 1983). More recently, a study of the biochemical composition of the cell envelope indicated a marked similarity in the peptidoglycan and polysaccharides of various isolates and the type strain (Fiedler & Draxl, 1986). Interestingly, the polysaccharide amounted to more than 60% of the dry weight of the cell walls. The authors concluded that the surface of the cell is formed mainly by this unique polysaccharide. Their study also revealed that antigenic

proteins were attached to the cell wall. We have recently shown that *R. salmoninarum* is encapsulated (Dubreuil *et al.*, 1990), which might explain the presence of an unusual amount of polysaccharide in the cell wall preparation.

Daly & Stevenson (1987), studying 25 isolates from diverse geographical locations, showed that *R. salmoninarum* possessed a hydrophobic cell surface. The hydrophobicity was shown to be due to the presence of a proteinaceous haemagglutinin/spermagglutinin (Daly & Stevenson, 1989, 1990). This protein could be removed from the cell surface by washing the bacteria with distilled water and had a molecular mass of 57 kDa. Recently, Bruno (1990) showed saline extracts of virulent strains to contain a 57 kDa protein which was not present in extracts of low-virulence strains. The study related the hydrophobic nature and the autoaggregating property of virulent isolates with the presence of this saline-extractable protein.

Haemagglutinating activity has been reported for *R. salmoninarum* for rabbit, pigeon, horse and rat erythrocytes (Daly & Stevenson, 1987). This activity was resistant to mannose, D- and L-fucose, galactose and *N*-acetylglucosamine. Up to now, no surface appendages

such as fimbriae have been revealed by transmission electron microscopy. However, it has been noticed that *R. salmoninarum* forms a pellicle in broth and has a tendency to autoaggregate, two properties often related to the presence of fimbriae (Smith, 1964; Daly & Stevenson, 1987; Bruno, 1988).

This study is the first to show that *R. salmoninarum* isolates possess fimbriae. These proteinaceous structures were isolated and the fimbrin subunits purified and biochemically characterized.

Methods

Bacterial strains and growth medium. *Renibacterium salmoninarum* strain FMV 84-01 was isolated from the kidney of a diseased speckled trout, *Salvelinus fontinalis*. Many vials of the strain were freeze-dried immediately after isolation and identification. Bacteria were grown on charcoal agar plates (Daly & Stevenson, 1985) at 16 °C for 12–15 d. The type strain ATCC 33209^T was also used for electron microscopical characterization of the cell surface.

Extraction and purification of fimbrial protein. Cultures (12–15 d old) of *R. salmoninarum* FMV 84-01 were harvested and suspended in sterile distilled water (3 g in 100 ml) containing 5 mM-phenylmethylsulphonyl fluoride (PMSF, Boehringer Mannheim) as a protease inhibitor. The suspension was stirred vigorously for 20 min at room temperature. Whole cells were then removed by centrifugation at 12000 g for 15 min. The supernatant was filter-sterilized (0.22 µm pore-size filter, Millipore) and the solution was then lyophilized. The sample was rehydrated in one-twentieth the initial volume of potassium phosphate buffer (0.02 M; pH 7.4). Since the protein of interest was insoluble in this buffer, the concentrated sample was centrifuged at 12000 g for 15 min. The precipitate was then solubilized in phosphate buffer containing 6 M-urea (BDH) and centrifuged again for 15 min at 12000 g. The supernatant was purified to apparent homogeneity using a Fast Pressure Liquid Chromatography (FPLC) system (Pharmacia) coupled to a Superose 12 column equilibrated in the same buffer. The system was run at a flow rate of 0.5 ml min⁻¹. The A₂₈₀ of the effluent was monitored with a UV-M detector.

Antibody production. Antiserum was raised in two adult New Zealand White rabbits by intramuscular injections. One rabbit was immunized with a formalin-killed bacterial suspension of 10⁹ cells ml⁻¹ of a 15-d-old culture of *R. salmoninarum* FMV 84-01: 1 ml of this suspension was emulsified with an equal volume of Freund's complete adjuvant. The other rabbit was injected with 50 µg of FPLC-purified protein in Freund's complete adjuvant. A booster dose was given in Freund's incomplete adjuvant to each rabbit on days 14 and 28. On day 42 the rabbits were exsanguinated and the serum collected and stored at -20 °C. Control non-immune serum was obtained before the first injection.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (1970) in a mini-slab gel apparatus (Hoefer). Proteins solubilized in sample buffer were stacked in 4.5% (w/v) acrylamide (100 V; constant voltage) and separated by using 12.5% acrylamide (200 V; constant voltage). Proteins were stained with Coomassie blue, and glycoconjugate compounds were stained by the periodate-Schiff procedure (Fairbanks *et al.*, 1971).

For electroblotting, separated proteins were transferred from the slab gel to nitrocellulose paper by the methanol/Tris/glycine system described by Towbin *et al.* (1979). Electroblotting was performed in a Hoefer Transblot apparatus for 18 h at 60 V. Non-denaturing isoelectrofocusing (IEF) gels were run in a mini slab gel apparatus, in a

pH 3.5–10 ampholine gradient, according to the method of Robertson *et al.* (1987) and were stained with Coomassie blue according to the Bio-Rad IEF standards instruction sheet.

Western blotting. After electroblotting, unreacted sites on the nitrocellulose paper (NCP) were blocked with a 2% (w/v) solution of casein in 10 mM-Tris/HCl, 0.9% NaCl (pH 7.4) (CTS) for 1 h at room temperature. The NCP was then incubated with an appropriate dilution of antiserum in the same buffer for 2 h. The NCP was washed five times with Tris/saline (10 mM-Tris/HCl, 0.9% NaCl, pH 7.4). Then goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase (Caltag Labs) was added in CTS buffer and incubated for 1 h at room temperature. After incubation, the NCP was washed five times in Tris/saline. The reactive bands were visualized as described by Blake *et al.* (1984) using 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim) as the alkaline phosphatase substrate and nitroblue tetrazolium (Sigma) as the colour development reagent.

Amino acid composition. The purified protein was electroblotted on Immobilon transfer membrane (Millipore) following the method of Legendre & Matsudaira (1988). The blotted protein was stained with Ponceau S (Sigma) following the method of Salinovich & Montelaro (1986). The sample was subjected to gas-phase hydrolysis in 6 M-HCl for 1 h at 165 °C. Amino acid composition was determined on a 420A derivatizer-analyser system (Applied Biosystems), using the standard program.

Immuno-electron microscopy. Bacteria were harvested, and washed once in 0.05 M-phosphate buffered saline (pH 7.2) (PBS). Single drops of bacterial suspension were placed on Formvar-coated grids and allowed to partially air-dry. The grids were then placed sequentially on drops of PBS containing 5% (w/v) bovine serum albumin for 5 min and a suitable dilution of rabbit antiserum for 30 min. They were then washed in distilled water and placed on drops of a 1:10 dilution of colloidal gold particles (5 nm) conjugated to goat anti-rabbit immunoglobulin G (E-Y Labs) for 30 min. After a final wash in distilled water, they were stained with 2% (w/v) phosphotungstic acid (pH 7.0), and examined with a Philips 201 electron microscope at an accelerating voltage of 60 kV.

Haemagglutination test. This was done as previously described (Fortin & Jacques, 1987). Rabbit blood was collected in Alsever's solution. The erythrocytes were washed twice in PBS and suspended in PBS to obtain a final concentration of 3% (v/v). One drop of a bacterial suspension (~10¹⁰ cells) or purified fimbrin (1 mg) was added to one drop of erythrocyte suspension in the circular depression of a glass slide. The slide was rocked, at room temperature, for 15 min. In one experiment the bacterial suspension was incubated for 1 h with an equal volume of antiserum raised against fimbriae before doing the haemagglutination test.

Results

Electron microscopy using negative staining suggested the presence of short, flexible fimbriae on the cell surface of *R. salmoninarum* strains FMV 84-01 and ATCC 33209^T. These structures had an apparent diameter of less than 2 nm, were most often seen in bundles, and were distributed peritrichously. Due to their small diameter, they were hard to observe unless labelled with antibodies and gold particles as described below. Our electron microscopic study did not reveal the presence of a protein surface array.

Fig. 1. FPLC elution profile of *R. salmoninarum* fimbrial protein using Superose 12 gel filtration column chromatography. The arrow indicates the elution peak containing the fimbrin.

Fig. 2. SDS-PAGE and immunoblot of *R. salmoninarum* FMV 84-01. Lanes: 1, molecular mass markers; 2, whole-cell lysate; 3, purified fimbrial protein; 4, immunoblot of whole-cell lysate using rabbit antiserum raised against homologous whole cells.

Fimbriae were isolated from cultures of strain FMV 84-01 as described in Methods, and purified to apparent homogeneity using an FPLC system coupled to a Superose 12 column. The fimbrin eluted after approximately 17 min in a single peak that represented the major component of the chromatographed sample (Fig. 1).

Table 1. Amino acid composition of the 57 kDa surface protein from *R. salmoninarum*

Amino acid	No. of residues mol ⁻¹
Asx	83
Thr	47
Ser	38
Glx	36
Pro	28
Gly	107
Ala	51
Val	54
Met	0
Ile	12
Leu	21
Tyr	12
Phe	25
His	10
Lys	37
Arg	11
Cys	ND
Trp	ND
Total no. of residues mol ⁻¹	572
Apparent mol. mass	57.2 kDa
Hydrophobic residues (%)	33.4

ND, Not determined.

* Val, Met, Ile, Leu, Ala, Phe, Trp and Pro.

SDS-PAGE of the material in this peak revealed a single 57 kDa protein as assessed by Coomassie blue staining (Fig. 2, lane 3). Electrophoresis of the whole-cell preparation indicated the 57 kDa protein band to be a major constituent of the cell (Fig. 2, lane 2). Schiff staining of the FPLC-purified protein gave a negative result (data not shown).

The analysis of amino acid composition revealed that the protein was rich in glycine (18.7% of all residues), Asx (asparagine and aspartic acid), valine and alanine, whereas methionine was absent (Table 1). The molecule, comprising approximately 572 residues, was acidic, with Asx (asparagine and aspartic acid) and Glx (glutamine and glutamic acid) representing 20.8% of the residues, and 10.1% basic residues (lysine, histidine, arginine). It contained 33.4% hydrophobic residues. The isoelectric point was determined to be 4.8 under non-denaturing conditions.

Immunoblotting using serum raised against whole cells indicated that the 57 kDa protein was surface-exposed and constituted the immunodominant antigen (Fig. 2, lane 4). Using the rabbit antiserum raised against the purified protein and the immunogold technique we showed that the antibodies labelled the native fimbriae on strain FMV 84-01 (Fig. 3). This technique showed alignment of gold particles along some fimbriae. Broken fimbriae were labelled and appeared as aligned gold

Fig. 3. Transmission electron micrographs of *R. salmoninarum* FMV 84-01 labelled by the immunogold technique. Cells were incubated with rabbit anti-57 kDa protein serum and then with goat anti-rabbit serum labelled with 5 nm colloidal gold. (a) Note the alignment of gold particles (arrows) suggesting the labelling of short fimbriae either on the cell surface or free in the surrounding medium. (b) Higher magnification of another preparation. Note the layer of capsular material (*) and the thin fimbriae radiating from the cell. Gold particles are seen along the length of some fimbriae (arrows). Bars, 100 nm.

particles surrounding the cell. A capsular material layer of approximately 50–60 nm was also observed covering the cells.

Whole *R. salmoninarum* cells agglutinated rabbit erythrocytes. However, when the bacterial cells were treated with the antifimbrial antiserum, haemagglutination was inhibited. The purified 57 kDa protein preparation could by itself agglutinate rabbit erythrocytes.

Discussion

Our study is the first report of the presence of fimbriae on the type strain, ATCC 33209^T, and one of our laboratory strains (FMV 84-01) of *R. salmoninarum*. The original description of *R. salmoninarum* did not include the presence of fimbriae (Sanders & Fryer, 1986). The study

of Daly & Stevenson (1987), in which cells of strain ATCC 33209^T negatively stained with ammonium molybdate or uranyl acetate were examined by transmission electron microscopy, did not reveal any surface structures. Using the same technique we observed short and flexible fimbriae, less than 2 nm in diameter. However, due to their small size their observation was rather difficult.

Removal of the fimbriae by washing the cells with distilled water suggests that they are easily broken. This hypothesis is likely as the material extracted with water was non-soluble (structured) and could be precipitated by low-speed centrifugation. PMSF was added as a protease inhibitor as Bruno & Munro (1986) showed *R. salmoninarum* to produce different proteolytic activities. Omitting PMSF resulted in severe degradation of the fimbrial protein subunit. The protocol we

designed resulted in purification to homogeneity of a single polypeptide with a subunit size of 57 kDa by SDS-PAGE. Although this subunit size is higher than what is known for *Escherichia coli* or *Salmonella*, it is not unique. For example, the fimbriae of *Actinomyces viscosus*, another Gram-positive bacterium, are composed of 64 kDa subunits (Masuda *et al.*, 1981). The amino acid composition and isoelectric point were not markedly different from other fimbrins, with 33.4% hydrophobic residues and an acidic pI of 4.8. However, the hydrophobic amino acid residues in fimbrin of *E. coli*, for example, could represent as much as 50%. In general, this high percentage of hydrophobic residues has been used in assigning hydrophobic properties to fimbriae (Isaacson, 1985). Sugars have been shown to be associated with different fimbriae, such as those of *E. coli* 987P (Isaacson & Richter, 1981) and *N. gonorrhoeae* (Gubish *et al.*, 1982). However, the purified 57 kDa protein from *R. salmoninarum* was not stained by the Schiff method, suggesting that it is probably not a glycoconjugate.

Using immunoelectron microscopy, we have recently shown *R. salmoninarum* to be encapsulated (Dubreuil *et al.*, 1990), and the present study confirmed the presence of a 50–60 nm thick capsule. However, the presence of this structure is not compatible with the demonstration of a hydrophobic cell surface by Daly & Stevenson (1987), as the capsule is composed of polysaccharide. The presence of fimbriae radiating from the cell surface through the capsule could explain the hydrophobic nature of the cell surface. Fimbriae were seen projecting through the capsule (50–60 nm) and extending beyond it. However, the fimbriae were rather short. The length of *E. coli* fimbriae, for example, is variable but may be as much as 4 µm (Ottow, 1975). However, we believe that the fimbriae of *R. salmoninarum* are longer but may be easily broken. Broken fimbriae, visualized as aligned gold particles, were present around the cells.

Immunoelectron microscopy showed antibodies binding along the length of the native fimbrial structures, confirming that the protein which had been purified was the fimbrial subunit and further showing that the epitopes recognized by the antibodies were surface-exposed. Immunoblotting using whole-cell antiserum showed the 57 kDa protein to be the immunodominant antigen on the surface of *R. salmoninarum*. Similarly, a study by Turaga *et al.* (1987) showed that the most prominent antigen found during the course of an *R. salmoninarum* infection had a molecular mass of approximately 60 kDa.

The fimbriae of *R. salmoninarum* may be an important virulence factor by increasing the hydrophobicity of the bacterial cells, which can be taken up more readily by macrophages, where they survive and are believed to

replicate (Young & Chapman, 1978; Bruno, 1986). Daly & Stevenson (1987) showed that water-extracted haemagglutinin bound to phenyl-Sepharose and that the haemagglutinin was, at least in part, responsible for the hydrophobic character of the cell surface. Bacterial cells treated with heat or protease K, or from which the haemagglutinin had been extracted with water, demonstrated decreased hydrophobicity, indicating that a protein was probably responsible for the hydrophobicity and also that the hydrophobicity was associated with the haemagglutinating activity. Bruno (1988) showed that hydrophobicity and virulence in experimentally infected rainbow trout were linked. The presence of the 57 kDa protein in saline extracts of virulent cells and its absence in extracts of avirulent cells suggested that it was contributing to, or associated with virulence (Bruno, 1990).

The ability of antifimbrial antiserum to inhibit haemagglutination, together with the ability of purified fimbriae to induce it, provide strong evidence that haemagglutination is mediated by the fimbriae of *R. salmoninarum*. Since the fimbriae are composed of the 57 kDa protein previously shown to be the haemagglutinin, we speculate that the presence of fimbriae is probably a ubiquitous characteristic of virulent *R. salmoninarum* strains. To our knowledge, all virulent strains so far examined possess the 57 kDa protein, are haemagglutinating and have a hydrophobic surface. Our study has demonstrated that fimbriae are present on the surface of two strains of *R. salmoninarum*, and that these appendages are responsible for the haemagglutinating activity. Further studies will focus on the elucidation of the role of these fimbriae in *R. salmoninarum* infections.

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