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Characterization of the *Renibacterium salmoninarum* haemagglutinin

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Water-extracted proteins from nine geographically diverse strains of *Renibacterium salmoninarum*, all of which agglutinated rabbit erythrocytes and rainbow trout spermatozoa, were compared by SDS-PAGE. Extracts from eight strains, including the type strain, ATCC 33209, were similar, containing a major protein of 57 kDa and a minor protein of 58 kDa. The SDS-PAGE protein profile of the Char strain did not contain the 58 kDa protein. A non-agglutinating strain, MT-239, which was also non-hydrophobic, did not produce any water-extractable protein. Immunoblot reactions with rabbit antiserum prepared against whole cells of the type strain demonstrated that the water-extracted haemagglutinins from the various strains were antigenically related. When purified by polyacrylamide gel zone electrophoresis, the haemagglutinin from *R. salmoninarum* ATCC 33209 formed a doublet band with molecular masses of 57 and 58 kDa, similar to the previously described F antigen. The water-extracted haemagglutinin agglutinated salmonid spermatozoa, was degraded by protease K and trypsin, and was shown to self-assemble onto the cell surface.

Introduction

Renibacterium salmoninarum is the Gram-positive bacterium that causes bacterial kidney disease of salmonid fish (Sanders & Fryer, 1980). It grows very slowly on artificial media, requiring up to 10 d to grow from heavy inocula and 4–6 weeks from dilute inocula (Fryer & Sanders, 1981). Thus, serological methods are preferred over culture for its detection. Serological techniques are based on the detection of a heat-stable surface antigen (Pascho & Mulcahy, 1987) of unknown function. Getchell *et al.* (1985) described and partially purified the cell-surface and heat-stable F antigen, believed to be the antigen detected in various serological reactions. In this study, we report that antigen F is responsible for the haemagglutinating (Daly & Stevenson, 1987) and sperm-agglutinating (Daly & Stevenson, 1989) properties of *R. salmoninarum*.

Methods

Bacterial strains and growth. The following strains of *R. salmoninarum* have been previously described (Daly & Stevenson, 1987): ATCC 33209 (the type strain), ATCC 33739, NCMB 1111, NCMB 1114, Char, Coho (Ontario), RS22, MT241 and 2/2/79. *R. salmoninarum* MT239 was kindly supplied by Dr D. Bruno, Department of Agriculture and Fisheries for Scotland, Aberdeen, UK. The bacteria were grown biphasically for 10 d to 2 weeks on charcoal agar (KDM-C) slants at 15 °C, until dense growth was present in the liquid phase (Daly & Stevenson, 1985).

Haemagglutinin extraction. After 2 weeks of growth, the bacteria were removed from the biphasic agar slants and washed three times with saline (0·85%, w/v, NaCl). The haemagglutinin was removed from the bacterial surface with two washes of distilled water, pH 5·5. This pH was found to result in high yields of the haemagglutinin. This water-extract was filtered through a 0·45 μm membrane (Millipore) and then lyophilized.

Haemagglutinin purification. The lyophilized water-extracted haemagglutinin was rehydrated at 2 mg ml^{-1} with 2 mM-sodium phosphate buffer, pH 6·8, and mixed with an equal volume of 20% (v/v) glycerol and bromophenol blue. The mixture was applied to a 15 cm vertical polyacrylamide gel for zone electrophoresis, using a 3% (w/v) stacking gel and a 5% (w/v) separating gel, in 0·05 M-Tris/0·38 M-glycine buffer, pH 8·3. The sample was electrophoresed at 60 V through the stacking gel and at 120 V through the separating gel until the tracking dye ran off the bottom of the gel. Vertical strips from each side of the gel were stained with Coomassie brilliant blue R250 to locate a single band which was then excised from the gel. This gel strip was placed in a dialysis sac with a molecular mass cut-off of 6000–8000 Da and 5 ml of distilled water was added. The contents of the sac were then dialysed against two changes of 2 litres of distilled water at 4 °C for 24 h. The liquid from the dialysis sac was removed and lyophilized; the lyophilized material was rehydrated with saline and assayed for haemagglutinating and spermagglutinating activity.

Agglutination and hydrophobicity assays. Haemagglutination was assayed with 3% (v/v) rabbit erythrocytes in saline, on a glass slide, as previously described (Daly & Stevenson, 1987). For agglutination of

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Abbreviation: SAT, salt aggregation test (for hydrophobicity).

spermatozoa, cells of *R. salmoninarum* were washed three times in saline and resuspended in saline such that a 50-fold dilution gave an OD_{520} of 0·10. Spermatozoa from three mature, spawning rainbow trout (*Salmo gairdneri*) were pooled and, before use, were diluted in saline until the optical density approximated that of the bacteria. Equal volumes of bacteria and spermatozoa were allowed to react on a glass slide and observed for agglutination as previously described (Daly & Stevenson, 1989). Hydrophobicity of strains was examined using the salt aggregation test (SAT) as previously described (Daly & Stevenson, 1987).

Rabbit antiserum production. *R. salmoninarum* ATCC 33209 was grown biphasically on charcoal agar for 2 weeks at 15 °C. The cells were collected from the aqueous phase, washed three times in saline, then resuspended in saline to an OD_{600} of 0·55. This suspension was mixed 1:1 with incomplete Freund's adjuvant (Calbiochem). A New Zealand White rabbit was injected with 1 ml of the mixture, divided equally between three sites (two intramuscular, one subcutaneous). A second injection was given intravenously 5 weeks later; this consisted of washed bacteria resuspended in saline to an OD_{600} of 0·37. A similar injection was given after a further 1 week, and 4 weeks after that the rabbit was bled. The blood was allowed to clot and the serum removed and frozen at −20 °C.

Affinity-purified rabbit antiserum. Lyophilized water-extracted haemagglutinin from *R. salmoninarum* Char was rehydrated with water to a concentration of 2 mg ml^{-1}, mixed 1:1 with electrophoresis sample buffer and 150 µl applied to an SDS-polyacrylamide gel (10%, w/v, acrylamide) (minigel). After electrophoresis the gel was electroblotted onto nitrocellulose. The protein bands were located by amido black staining of nitrocellulose strips from both sides of the blot. The nitrocellulose was blocked with 3% (w/v) gelatin and then allowed to react with the rabbit antiserum made against whole cells of *R. salmoninarum* ATCC 33209. The amido-black-stained strips were aligned with the blot and the portion containing the haemagglutinin was identified. This strip was excised with a sharp scalpel and cut into small pieces. These were transferred to a test tube and incubated with 1 ml 100 mM-glycine (pH 2·5) for 10 min. The buffer was removed, neutralized with 0·1 vol. 1 M-Tris/HCl, pH 8·0, and sodium azide was added to a final concentration of 0·02% (w/v).

Indirect fluorescent antibody staining. Rabbit antiserum against whole cells of *R. salmoninarum* was diluted 1/100 with PBS, pH 7·2 (Bacto FA buffer, Difco), and reacted with heat-fixed bacteria on a slide for 30 min. The slides were rinsed in PBS for 10 min and then reacted with goat anti-rabbit serum labelled with fluorescein isothiocyanate (FITC, Gibco) for 30 min and rinsed for 10 min in PBS. Slides were observed with a Zeiss standard microscope, utilizing a IV Fl epi-fluorescent condenser with a high-pressure mercury light source and a standard FITC filter set.

SDS-PAGE. This was performed on 10% (w/v) gels with 4% (w/v) stacking gels, as described by Laemmli (1970). The proteins were stained with Coomassie brilliant blue R250 and the molecular masses determined by comparison with a set of low molecular mass calibration markers (Pharmacia).

Immunoblot analysis. The water-extracted proteins were subjected to SDS-PAGE (10%, w/v resolving gel), as described by Laemmli (1970). After electrophoresis, the proteins were electroblotted from the gel onto nitrocellulose paper (0·45 µm, Schleicher and Schuell) using modifications (Burnette, 1981) of the method of Towbin *et al.* (1979). Immunoblots were developed using alkaline-phosphatase-coupled goat anti-rabbit IgG (Sigma) and *p*-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt for colour development.

Enzyme treatment. Two milligrams of lyophilized water-extracted haemagglutinin from *R. salmoninarum* ATCC 33209 were rehydrated with 2 ml buffer (40 mM-sodium phosphate, 150 mM-sodium chloride, pH 7·2). Either 2·5 mg proteinase K (Sigma) or 2·5 mg trypsin (Sigma) was added to 0·5 ml aliquots of this solution. A control contained no enzyme. The three mixtures were left at 20 °C for 1 h and then tested for agglutination of rabbit erythrocytes or rainbow trout spermatozoa. The mixtures were also examined by SDS-PAGE to determine whether the 57 kDa protein was degraded.

Reassembly of haemagglutinin onto R. salmoninarum. Cells of *R. salmoninarum* ATCC 33209 were grown for 2 weeks, then washed twice with saline and three times with distilled water until they did not haemagglutinate rabbit erythrocytes or rainbow trout spermatozoa. The optical density of the bacteria was adjusted such that a 1/100 dilution had an OD_{520} of 0·075. Three milligrams of lyophilized, water-extracted haemagglutinin was rehydrated with 1 ml distilled water and 0·5 ml of this was mixed with 1 ml of washed bacteria and placed in a sterile dialysis membrane sac with a molecular mass exclusion of 10000–12000 Da (Spectrum Medical Industries). The control dialysis sac contained bacteria and no added haemagglutinin. The sacs were dialysed overnight at 4 °C against saline. After dialysis, the cells were collected, washed three times with saline and reacted with rabbit erythrocytes. The hydrophobicity of both bacterial suspensions was measured by the salt aggregation test.

Results

Hydrophobic and agglutinating activities of R. salmoninarum

Nine of the ten strains of *R. salmoninarum* were hydrophobic and readily agglutinated rabbit erythrocytes and rainbow trout spermatozoa. In contrast, strain MT239 was not hydrophobic, giving a SAT value of greater than 2·0, and it also failed to agglutinate rabbit erythrocytes and rainbow trout spermatozoa.

SDS-PAGE profiles of water-extracts

When the water-extraction procedure for removal of the haemagglutinin of *R. salmoninarum* was employed with strain MT239, no solid material was detected after lyophilization. Using similar amounts of the other strains, milligram amounts of material were extracted. The water-extracted haemagglutinins from these nine strains were compared by SDS-PAGE (Fig. 1 *a*, *b*). The protein profiles of eight strains, including the type strain (ATCC 33209), were very similar, showing a major protein of 57 kDa with a minor protein of 58 kDa. Other proteins of lower molecular mass were also evident. In contrast, the Char strain had a protein of similar intensity to the 57 kDa protein but did not have a 58 kDa protein (Fig. 1 *b*, lane 6).

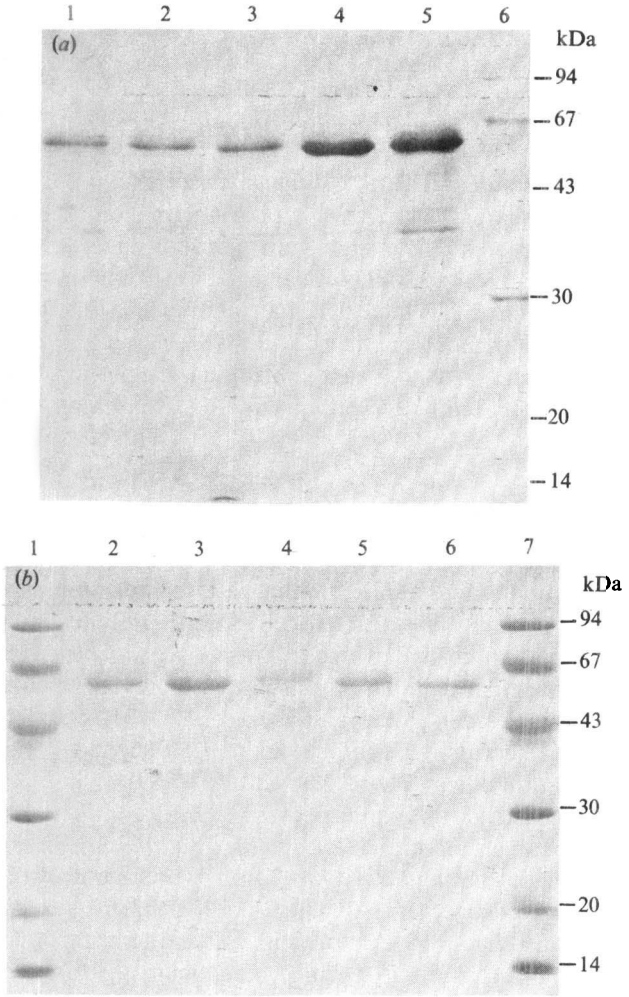

Fig. 1. SDS-PAGE (10% acrylamide) of water-extracted proteins from various *R. salmoninarum* strains. (*a*) Lanes: 1, 2/2/79; 2, RS22; 3, MT241; 4, NCMB 1111; 5, ATCC 33209; 6, molecular mass standards. (*b*) Lanes: 1, 7, molecular mass standards; 2, ATCC 33209; 3, Coho (Ontario); 4, NCMB 1114; 5, ATCC 33739; 6, Char. The Pharmacia kit standards are phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean inhibitor (20 kDa) and α-lactalbumin (14 kDa).

Haemagglutinin purification and characterization

The haemagglutinating proteins from ATCC 33209 and the Char strain were chosen for further purification because of their slightly different profiles on SDS-PAGE. After zone electrophoresis in a 5% non-denaturing gel, the water-extracted haemagglutinin from both strains showed a single intense band upon Coomassie blue staining. After being excised and subjected to SDS-PAGE, the proteins from the two strains were found to have slightly different molecular masses (Fig. 2*a*, *b*). The haemagglutinin of the type strain, ATCC 33209, consisted of a doublet, with bands of molecular masses of 57 and 58 kDa (Fig. 2*a*). In some

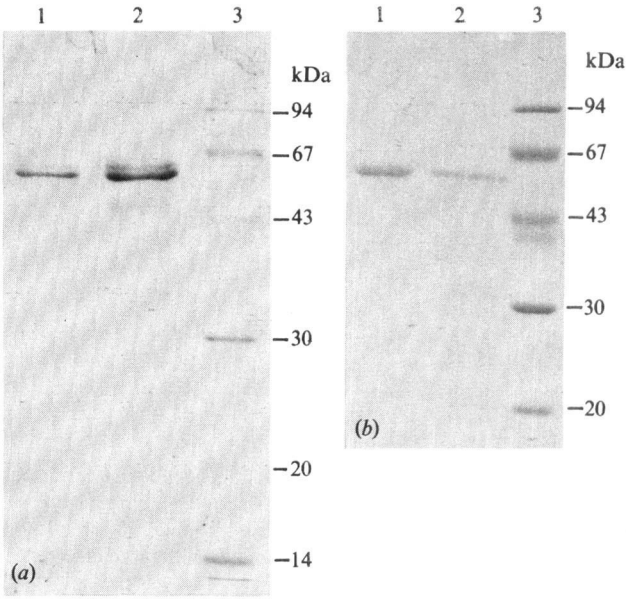

Fig. 2. SDS-PAGE (10% acrylamide) of zone electrophoresis purified haemagglutinins from *R. salmoninarum* ATCC 33209 and Char. (*a*) Lanes: 1, haemagglutinin purified from ATCC 33209; 2, water-extracted proteins from ATCC 33209; 3, molecular mass standards, as in Fig. 1. (*b*) Lanes: 1, haemagglutinin purified from Char; 2, water-extracted proteins from Char; 3, molecular mass standards.

preparations, the 58 kDa protein was less evident. The haemagglutinin of the Char strain consisted of a single band of molecular mass 57 kDa (Fig. 2*b*). The protein from either ATCC 33209 or Char agglutinated both rabbit erythrocytes and rainbow trout spermatozoa, thus demonstrating that the same protein was responsible for both activities.

The 57–58 kDa bands of *R. salmoninarum* ATCC 33209 were degraded by both protease K and trypsin (Fig. 3); this enzyme treatment also destroyed both haemagglutinating and spermagglutinating activities. The SDS-PAGE profile of the water-extracted proteins was identical if β-mercaptoethanol was omitted from the electrophoresis sample buffer (Fig. 3), indicating that subunits are not held together by disulphide bridges.

Immunoblot reactions and immunofluorescence microscopy

In immunoblots of the water-extracted proteins separated by SDS-PAGE, numerous proteins, including the haemagglutinin, reacted with the antiserum produced against whole cells of *R. salmoninarum* ATCC 33209 (Fig. 4*a*, *b*). The Char strain, which did not produce the 58 kDa protein, was used to affinity-purify antibody towards the haemagglutinin from the whole-cell antiserum. When whole-cell antiserum was used as the first antibody for indirect fluorescent antibody staining, cells

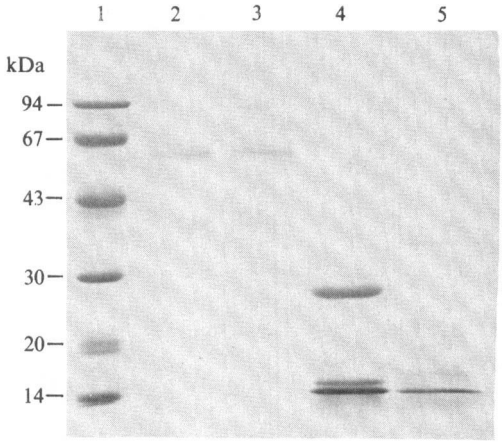

Fig. 3. β-Mercaptoethanol and enzyme treatment of water-extracted proteins from *R. salmoninarum* ATCC 33209. Lanes: 1, molecular mass standards; 2, untreated sample; 3, untreated sample but without β-mercaptoethanol in the sample buffer; 4, treated with proteinase K; 5, treated with trypsin.

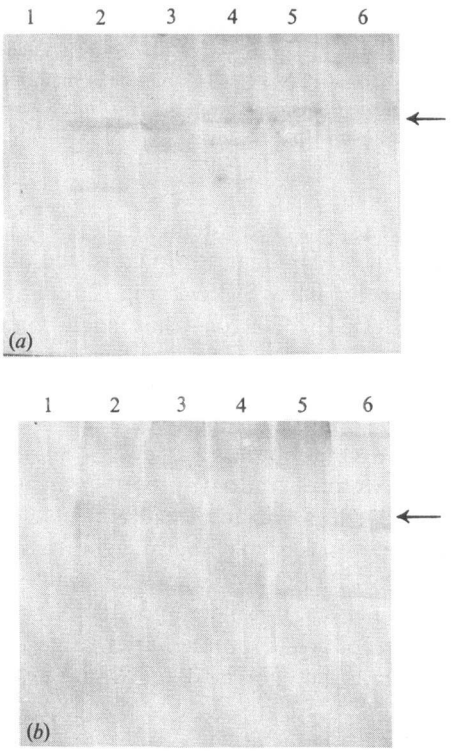

Fig. 4. Western immunoblot of the water-extracted proteins from *R. salmoninarum* isolates, using rabbit antiserum raised against whole cells of *R. salmoninarum* ATCC 33209. (*a*) Lanes: 1, unstained molecular mass standards; 2, Char; 3, ATCC 33739; 4, NCMB 1114; 5, Coho (Ontario); 6, ATCC 33209. (*b*) Lanes: 1, unstained molecular mass standards; 2, 2/2/79; 3, RS22; 4, MT241; 5, NCMB 1111; 6, ATCC 33209. The arrows indicate the position of haemagglutinin.

of ATCC 33209 and MT239 both fluoresced, but when the affinity-purified rabbit anti-haemagglutinin serum was used, only ATCC 33209 fluoresced. This further suggested that MT239 did not produce the haemagglutinin on its surface.

Re-assembly of haemagglutinin onto cells

Water-extracted cells of *R. salmoninarum* ATCC 33209 dialysed against saline in the presence of homologous water-extracted haemagglutinin readily agglutinated rabbit erythrocytes. These bacteria were much more hydrophobic (SAT value 0·3) than control cells that were incubated without haemagglutinin (SAT value >2·0). The bacteria with the reassembled haemagglutinin also auto-aggregated, as observed by phase-contrast microscopy.

Discussion

The similar SDS-PAGE protein patterns and immunoblots of the water-extracted cell surface proteins show that geographically diverse strains of *R. salmoninarum* are serologically similar, consistent with previous results using whole-cell serology (Bullock *et al.*, 1974), counter-immunoelectrophoresis (Getchell *et al.*, 1985) and monoclonal antibodies (Weins & Kaattari, 1989).

The basis for this similarity is the 57 kDa antigen that was found in all strains except MT239, which did not have agglutinating activity and from which no material was water-extracted. All agglutinating strains, except Char, also had a 58 kDa protein. When the haemagglutinating activity was purified, the haemagglutinin of ATCC 33209 consisted of a doublet containing both the 57 and 58 kDa proteins, whereas the Char haemagglutinin contained only the 57 kDa protein. Thus, only the 57 kDa protein is necessary for the haemagglutinating activity for the Char strain, whereas haemagglutinating activity may be associated with both the 57 and 58 kDa proteins of the other strains. The partially purified antigen F described by Getchell *et al.* (1985) has a major band of 57 kDa, thus the haemagglutinin is presumably antigen F. Recently, Weins & Kaattari (1989) described monoclonal antibodies that react with both the 57 and 58 kDa proteins, indicating that they are antigenically related. Presumably the proteins of lower molecular mass than the 57 and 58 kDa doublet are breakdown products of the haemagglutinin. Weins & Kaattari (1989) also demonstrated that both the 57 and 58 kDa proteins were present in the sera of fish infected with bacterial kidney disease, but the lower molecular mass proteins were not evident.

The non-hydrophobic (hydrophilic) nature of *R. salmoninarum* MT239 was previously reported by Bruno (1988). In this study, we were unable to extract haemagglutinin from the surface of this strain, to detect

haemagglutinating or spermagglutinating activity, or to observe staining of MT239 cells using affinity-purified antibody to the haemagglutinin and fluorescence microscopy. Previously we showed that the water-extracted haemagglutinin was hydrophobic (Daly & Stevenson, 1987); hence the lack of the haemagglutinin on the surface of MT239 may be responsible for making its cell surface less hydrophobic.

The hydrophobic nature of the haemagglutinin (Daly & Stevenson, 1987) would account for the observation that the haemagglutinin reassembled onto the bacterial surface. Previously, we had found that protease enzymes had little or no effect on the haemagglutinating activity of whole bacteria (Daly & Stevenson, 1987). As these enzymes are able to degrade the water-extracted haemagglutinin (Fig. 3), this suggests that in its native form, it must be folded in such a way that the sites that are labile to trypsin and proteinase K are hidden.

The present results demonstrate that F antigen or soluble antigen, haemagglutinin and spermagglutinin are all the same molecule, usually seen as a 57 and 58 kDa doublet protein on SDS-PAGE. It is important to clarify and summarize the literature in regard to this important antigen of *R. salmoninarum*. This hydrophobic protein, designated antigen F as one of seven antigens described by Getchell *et al.* (1985), is found both on the cell surface and in broth supernatant fluid of cultured cells (Getchell *et al.*, 1985; Daly & Stevenson, 1987). It agglutinates rabbit erythrocytes and salmonid spermatozoa (Daly & Stevenson, 1987, 1989). Antigen F can self-assemble onto the renibacterial cell surface, with which it is loosely associated, and it can be extracted by distilled water or heat (this report; Daly & Stevenson, 1987). This latter property and the heat stability of the protein have been used to detect antigen F and *R. salmoninarum* in infected fish by ELISA (Pascho & Mulcahy, 1987) and coagglutination (Kimura & Yoshimizu, 1981).

The function of F antigen in the disease process is unknown, although its hydrophobic nature may allow the bacteria to bind to specific tissues and gain entrance to salmonid ova (Daly & Stevenson, 1987). Bruno (1988) found that the non-hydrophobic strain MT239 was less virulent than hydrophobic strains of *R. salmoninarum*. As our study has shown that MT239 lacks the F antigen, it may be an important virulence factor. Turaga *et al.* (1987) found that soluble antigens, containing the F antigen, suppressed *in vitro* antibody responses of coho salmon lymphocytes; perhaps F antigen is responsible for this effect.

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