Hydrophobic and Haemagglutinating Properties of *Renibacterium salmoninarum*

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Twenty-five strains of *Renibacterium salmoninarum*, from diverse geographical locations, were all hydrophobic when tested by ammonium sulphate aggregation and adherence to hydrocarbons. In addition, all strains caused haemagglutination of rabbit erythrocytes, but not of erythrocytes from salmonid fish. The hydrophobic and haemagglutinating properties were further characterized for *R. salmoninarum* ATCC 33209 (the type strain). Treating the bacteria with protease K or trypsin decreased hydrophobicity but had no effect on the ability to haemagglutinate rabbit erythrocytes. Heating the bacteria to 62 or 100 °C reduced hydrophobicity and removed the haemagglutinin from the cell surface. The haemagglutinin may be the heat-stable bacterial antigen extracted from the tissue of infected fish in serodiagnostic procedures.

INTRODUCTION

Renibacterium salmoninarum is the Gram-positive bacterium that causes bacterial kidney disease (BKD) of salmonid fish (Sanders & Fryer, 1980). BKD is transmitted via the eggs of infected adults, and at present no completely successful antibiotic or vaccination regimes are available to cure fish of the disease. Thus, for fish health management, adult brood fish must be screened for the presence of *R. salmoninarum*. The bacterium grows only slowly in culture, requiring about 10 d to grow from heavy inocula and 4–6 weeks from dilute inocula (Fryer & Sanders, 1981); detection methods based on culture are thus impractical and serological methods are routinely used. Many different serological procedures are used, including immunodiffusion (Chen *et al.*, 1974) and counterimmunodiffusion (Cipriano *et al.*, 1985), immunofluorescence (Bullock *et al.*, 1974; Evelyn *et al.*, 1981), co-agglutination (Kimura & Yoshimizu, 1981), and ELISA (Pascho & Mulcahy, 1987; Sakai *et al.*, 1987). However, in these tests, the bacterial antigens being detected are uncharacterized, and cross-reactions with other bacteria have been reported (Austin *et al.*, 1985; Fiedler & Draxl, 1986). These cross-reactions may occur because of serum antigens present in the KDM-2 medium used to grow *R. salmoninarum* (Evelyn, 1977). Such problems can be avoided by using a serum-free charcoal agar, KDM-C (Daly & Stevenson, 1985).

Some observations reported in the literature suggest that the cell surface of *R. salmoninarum* could be hydrophobic. First, the bacteria autoaggregate in normal serum, but heating to 62 °C overcomes this (Evelyn *et al.*, 1973). Second, Smith (1964) reported that the original Dee strain of *R. salmoninarum* formed a pellicle in broth; other authors have also noted a tendency for aggregation, in a variety of broth media (Evelyn *et al.*, 1973; Embley *et al.*, 1982; McCarthy *et al.*, 1984). There would be advantage to *R. salmoninarum* in being hydrophobic. The bacterium is believed to survive in the macrophages of diseased fish (Young & Chapman, 1978), and cells with a hydrophobic surface are more likely to be taken up by macrophages (Van Oss, 1978). In this paper we report the hydrophobic and haemagglutinating properties of 25 strains of *R. salmoninarum* from diverse geographic localities.

Abbreviations: BATH, bacterial adherence to hydrocarbons; SAT, salt aggregation test.

Table 1. *Strains of R. salmoninarum tested for hydrophobicity and haemagglutination activity*

Strain	Isolation origin*	Source†
ATCC 33209 (type strain)	Chinook salmon, Oregon	ATCC
ATCC 33739	Brook trout	ATCC
NCMB 1111	<i>Salmo</i> sp., USA	NCMB
NCMB 1114	Atlantic salmon, Scotland	NCMB
RS22	Atlantic salmon, Nova Scotia	AH
DR-128	Rainbow trout, British Columbia	PBS
MT241	Coho salmon, France	DAFS
JD17	Rainbow trout, Manitoba	FWI
FQ-1	Brook trout, Saskatchewan	FWI
WS-1K-GL	Lake trout, Manitoba	FWI
Sock-3	Sockeye salmon, Manitoba	FWI
Char	Arctic char, Northwest Territories	FWI
2/2/79, 20/2/79, 27/7/78, 26/3/80, 28/2/80	Rainbow trout, England	WRL
JD19	Rainbow trout, Ontario	DMG
NFLD	Atlantic salmon (Ouananiche), Newfoundland	DMG
NB	Atlantic salmon, New Brunswick	DMG
Moc	Brook trout, Ontario	DMG
BC	Coho salmon, British Columbia	DMG
Coho	Coho salmon, Ontario	DMG
503	Brook trout, Ontario	DMG
SP	Rainbow trout, Ontario	DMG

* The Latin names of the fish are as follows: Arctic char, *Salvelinus alpinus*; Atlantic salmon, *Salmo salar*; brook trout, *Salvelinus fontinalis*; chinook salmon, *Oncorhynchus tshawytscha*; coho salmon, *Oncorhynchus kisutch*; lake trout, *Salvelinus namaycush*; rainbow trout, *Salmo gairdneri*; sockeye salmon, *Oncorhynchus nerka*.

† ATCC, American Type Culture Collection, Rockville, Md, USA; NCMB, National Collection of Industrial and Marine Bacteria, Aberdeen, UK; DAFS, D. Bruno, Department of Agriculture and Fisheries for Scotland, Aberdeen, UK; PBS, T. P. T. Evelyn, Department of Fisheries and Oceans, Pacific Biological Station, Nanaimo, Canada; AH, W. D. Paterson, Aqua Health Ltd, Willowdale, Ontario, Canada; FWI, B. Souter, Freshwater Institute, Winnipeg, Manitoba, Canada; WRL, B. Spacey, Wellcome Research Laboratory, Beckenham, Kent, UK; DMG, Department of Microbiology, University of Guelph, Ontario, Canada.

METHODS

Bacterial strains and growth. The 25 strains of *R. salmoninarum* examined and their sources are given in Table 1. They were grown in biphasic culture on slants of serum-free charcoal agar (KDM-C), using 50 ml agar per slant, overlaid with 5 ml 0.1% peptone in 0.85% NaCl (Daly & Stevenson, 1985). Cultures were incubated for 10 d to 2 weeks at 15 °C, until dense growth was present in the liquid phase. The bacteria were washed three times in saline before being analysed or treated.

Heat and enzyme treatments. The type strain of *R. salmoninarum*, ATCC 33209, was suspended in saline such that a 50-fold dilution would give an OD₆₀₀ of 0.16 (Bauch & Lomb, Spectronic 70). The suspension, in a screw-capped test tube, was placed in a steamer for 20 or 40 min, or at 62 °C for 40 min. After heating, the bacteria were centrifuged at 4 °C and resuspended in saline to give the same concentration of bacteria. For enzyme treatments, *R. salmoninarum* ATCC 33209 was suspended in buffer (40 mM-sodium phosphate, 150 mM-sodium chloride, pH 7.2) at a similar density as above. Trypsin or protease K (Sigma) at a concentration of 1 mg ml⁻¹ was added to the bacteria and allowed to react for 3 h. The bacteria were then centrifuged at 4 °C and resuspended in saline to the original cell densities.

Haemagglutination. Rabbit erythrocytes from New Zealand White rabbits were obtained by venipuncture of the ear and collected in Alsever's solution (containing, per litre: 20.5 g glucose, 8.0 g sodium citrate dihydrate, 0.55 g citric acid and 4.2 g NaCl). Blood was taken by caudal venipuncture from brook trout (*Salvelinus fontinalis*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*) and placed in Alsever's solution. All other blood was purchased from Woodlyn Labs (Guelph, Ontario) as suspensions in Alsever's solution. All erythrocytes were washed three times in saline, stored at 4 °C and used within one week.

Strains of *R. salmoninarum* were tested for haemagglutination of rabbit erythrocytes by making a thick suspension of bacteria in saline (approximating an OD₆₀₀ of 5) and reacting 25 µl of this solution with 25 µl 3% (v/v) erythrocytes on a glass slide. The liquid phase of the growth medium was also tested for haemagglutinating activity. Haemagglutination by *R. salmoninarum* ATCC 33209 was quantified in microtitre plates (Dynatech).

Bacteria were diluted in saline such that a 10-fold dilution gave an OD_{600} of 0.56. Doubling serial dilutions of the bacteria were made and 3% erythrocytes in saline were added to the bacterial dilutions. Plates were incubated at 20 °C for 1 h and observed for haemagglutination. The haemagglutination titre was taken as the reciprocal of the last dilution that agglutinated the erythrocytes. Erythrocytes in saline served as the control. Bacteria that had been heat- or enzyme-treated were assayed for haemagglutination of rabbit erythrocytes on a glass slide; the degree of haemagglutination was scored as 0 (negative), 1 (weak), 2 (moderate) or 4 (strong). To test inhibition of haemagglutination by sugars, D-mannose, D-fucose, L-fucose, galactose and N-acetylglucosamine at 100 µg ml⁻¹ were individually incubated for 30 min with bacteria, which were then reacted with 3% rabbit erythrocytes in microtitre plates as above.

Salt aggregation test for hydrophobicity (SAT). The method of Lindahl *et al.* (1981) was used to determine whether *R. salmoninarum* strains were hydrophobic. Briefly, a stock solution of 2 M-ammonium sulphate in 2 mM-sodium phosphate (pH 6.8) was diluted with 2 mM-sodium phosphate. When necessary, the resulting solutions were adjusted to pH 6.8 with 1 M-ammonium hydroxide. The bacteria were suspended in 2 mM-sodium phosphate, such that a 50-fold dilution gave an OD_{600} of 0.16. Equal volumes of 25 µl bacterial solution and ammonium sulphate solution were reacted on a glass slide and gently rocked for 2 min at room temperature. The reaction was considered positive when visible aggregation was seen as compared to bacterial suspensions in 2 mM-sodium phosphate. *R. salmoninarum* ATCC 33209 was tested at final ammonium sulphate concentrations ranging from 0.02 M to 1 M; other strains were tested only at 0.25 M.

Bacterial adherence to hydrocarbons (BATH). Adherence to hydrocarbons was determined essentially according to the methods of Rosenberg *et al.* (1980). Untreated or enzyme-treated bacteria, at the cell density used for heat treatments, were added in 50 µl volumes to tubes containing 2 ml PUM buffer (containing, per litre, 22.2 g K₂HPO₄·3H₂O, 7.2 g KH₂PO₄, 1.8 g urea, 0.2 g MgSO₄·7H₂O; pH 7.1). The bacterial suspensions were then overlaid with 50, 100, 200 or 400 µl hexadecane or *p*-xylene. The tubes were uniformly agitated on a vortex mixer for 1 min, then allowed to sit for 15 min for phase separation. The decrease in the OD_{600} of the lower, aqueous, phase was used as a measure of cell-surface hydrophobicity. A suspension of untreated bacteria was also tested with hexadecane, in the presence of 1 M-ammonium sulphate.

Haemagglutinin hydrophobicity. Cells of *R. salmoninarum* ATCC 33209 were collected from 2-week KDM-C slants and washed three times in saline, then twice in distilled water. The two water extracts were pooled and filtered to remove any residual bacteria (Millipore, 0.45 µm pore diameter). A 5 ml volume of the extract was mixed with 5 ml 2 M-ammonium sulphate and the total sample applied to phenyl-Sepharose CL-4B (Pharmacia) in a minicolumn, which consisted of a 15 mm Pasteur pipette with a 2.2 ml bed volume. The column was washed with, in succession, 10 ml 1 M-ammonium sulphate, 3.6 ml saline, and 6 ml water. The saline and water washes were collected in 200 µl fractions and assayed for their ability to haemagglutinate rabbit erythrocytes in a slide reaction.

RESULTS

All 25 strains of *R. salmoninarum* autoaggregated in the liquid phase of the growth medium, as seen by microscopic examination. Autoaggregation was reversed when the bacteria were resuspended in a solution of low ionic strength, such as 2 mM-sodium phosphate, pH 6.8.

All the strains haemagglutinated rabbit erythrocytes with a 4+ reaction on glass slides. In a quantitative microtitre plate test, *R. salmoninarum* ATCC 33209 gave a haemagglutination titre of 16 for rabbit erythrocytes, and of 4 for pigeon, horse and rat erythrocytes. Goat, sheep, calf, guinea-pig, chicken, rainbow trout, brook trout and coho salmon erythrocytes were not haemagglutinated. Spent growth medium, after removal of the bacteria, also caused haemagglutination of rabbit erythrocytes. The reaction was more pronounced with the supernatant fluid from cultures that were 4 weeks old than with that from 2-week cultures, whereas bacterial cells from the older cultures showed less haemagglutination. None of the sugars tested inhibited haemagglutination. After heating at 62 °C or at 100 °C and resuspension, bacterial cells had reduced haemagglutinating ability (Table 2). However, the supernatant fluids collected from the heated cells did haemagglutinate rabbit erythrocytes. The material from bacteria heated at 100 °C for 40 min was less active than that from bacteria heated for 20 min. Thus, while a haemagglutinin could be extracted from cells by heat treatment, overheating appeared to destroy its activity.

All the strains aggregated readily in 0.25 M-ammonium sulphate, indicative of a hydrophobic surface. *R. salmoninarum* ATCC 33209 adsorbed readily to the hydrophobic hydrocarbon hexadecane (Fig. 1) and equally well to *p*-xylene (data not shown). In the presence of 1 M-

Fig. 1. Partition of *R. salmoninarum* ATCC 33209 to hexadecane and aqueous phases in the BATH test for hydrophobicity. Uniform amounts of bacterial suspensions were mixed with different volumes of hexadecane. The proportion of the cells remaining in the aqueous phase after 15 min was determined by comparing the OD₆₀₀ reading with that of the original suspension. Cell pre-treatments were: ●, none; ▼, trypsin; △, protease K; □, suspension in 1 M-(NH₄)₂SO₄. The test was repeated 3 times; the results shown are from a representative experiment.

Table 2. *Effects of heating and protease treatment on hydrophobicity and haemagglutination activity of R. salmoninarum*

Treatment*	Salt aggregation† [(NH ₄) ₂ SO ₄ , M]	Haemagglutination‡ by:	
		Cells	Culture supernate
None	0.03	4+	0
62 °C, 40 min	> 1	1+	3+
100 °C, 20 min	> 1	0	2+
100 °C, 40 min	> 1	0	1+
Trypsin (1 mg ml ⁻¹)	0.06	4+	0
Protease K (1 mg ml ⁻¹)	0.3	4+	0

* Cell suspensions were pre-treated as indicated. Further details are given in Methods.

† The salt aggregation test (SAT) was as described by Lindahl *et al.* (1981).

‡ Haemagglutination of rabbit erythrocytes was tested by slide agglutination and scored as 0 (negative), 1 (weak), 2 (moderate) or 4 (strong). Cells were separated from their suspending fluid after treatment, and the resuspended cells and the fluid tested independently.

ammonium sulphate, adsorption of the bacteria increased. Heating and enzyme treatment of the bacteria decreased their hydrophobicity (Fig. 1, Table 2).

The haemagglutinating activity could also be extracted from the bacteria by washing with distilled water. In the presence of 1 M-ammonium sulphate, the extracted haemagglutinin bound to phenyl-Sepharose and was eluted from the column at approximately the saline-water interface. This suggests that the haemagglutinin may be, at least in part, responsible for the hydrophobic nature of the surface of *R. salmoninarum*. Bacterial cells that had been treated with heat or protease K, or from which the haemagglutinin had been extracted with water, demonstrated decreased hydrophobicity, staying in suspension while untreated cells readily settled to the bottom of the test tubes.

DISCUSSION

All 25 strains of *R. salmoninarum* tested haemagglutinated rabbit erythrocytes and were hydrophobic, regardless of their geographic or fish species source. Thus, in these characteristics as well as others, isolates of *R. salmoninarum* are relatively homogeneous (Bullock *et al.*, 1974; Bruno & Munro, 1986; Fiedler & Draxl, 1986). The hydrophobic nature of the cell surface explains several previously noted growth phenomena (see Introduction). The decrease in

hydrophobicity after heating the bacteria at 62 °C explains the observation of Evelyn *et al.* (1973), that this pre-treatment prevents autoaggregation.

Binding of the haemagglutinin to the hydrophobic gel phenyl-Sepharose indicates that this active component is partially responsible for the hydrophobic nature of the cell surface. Since it can be removed from the bacteria by placing them in water, it must be non-covalently attached, perhaps by hydrophobic or ionic interactions. The hydrophobicity of the bacteria was reduced by enzyme treatment while the haemagglutinin was not affected, suggesting that different molecules, or different sites on the same molecule, are responsible for these activities. The haemagglutinin may be a bacterial lectin, one of the proteinaceous compounds, such as pili, that bind specifically to sugar receptors (Wadstrom & Trust, 1984). None of the sugars we tested inhibited agglutination of rabbit erythrocytes, but the receptor may contain different simple sugars, or more complex sugars. Transmission electron microscopic examination of cells of *R. salmoninarum* ATCC 33209 negatively stained with ammonium molybdate or uranyl acetate did not reveal any structures, such as pili or a protein surface array, that might account for the hydrophobic nature of the renibacterial surface (data not shown).

The haemagglutinin and hydrophobic surface of *R. salmoninarum* may be important virulence factors. They could facilitate uptake into macrophages, where Young & Chapman (1978) have suggested *R. salmoninarum* survives and possibly replicates. Such surface features could also be involved in the invasion of salmonid ova, allowing for the vertical transmission of bacterial kidney disease, as described by Evelyn *et al.* (1984). Austin & Rayment (1985) have suggested that *R. salmoninarum* may survive in the environment, external to salmonid fish, for short periods. The hydrophobic nature of the bacterium would cause it to leave the water column and attach to surfaces.

A significant aspect of the reported work is the relationship of the haemagglutinin of *R. salmoninarum* to antigens used in serodiagnostics. The extraction of the haemagglutinin from *R. salmoninarum* at 100 °C is reminiscent of the extraction procedure used to obtain an antigen from infected tissues. The PAP technique (Sakai *et al.*, 1987), ELISA technique (Pascho & Mulcahy, 1987), and immunodiffusion (Chen *et al.*, 1974), coagglutination (Kimura & Yoshimizu, 1981) and counter-immunoelectrophoresis (Cipriano *et al.*, 1985) procedures all similarly extract the bacterial antigen from infected tissue by boiling. Getchell *et al.* (1985) described a soluble antigen that was found on the surface of *R. salmoninarum*, designated the F-antigen. It was heat-stable and could be concentrated by its exclusion by a membrane filter with a 100 kDa cutoff. The haemagglutinin examined here is also heat stable and can be similarly concentrated (data not shown), suggesting that it may be the F-antigen. The extraction of the haemagglutinin in water provides a simple way of obtaining this molecule for purification and immunological studies. We are currently purifying the haemagglutinin of *R. salmoninarum*, in order to determine whether it is indeed the antigen being detected in the various serodiagnostic procedures.

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