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# *Streptococcus iniae* expresses a cell surface non-immune trout immunoglobulin-binding factor when grown in normal trout serum

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**Abstract**

Three capsulated isolates of *S. iniae* representing serotype I and II and being arginine dihydrolase positive, negative or variable (AD+ve, AD − ve, AD+ − ve) were investigated for their ability to bind rainbow trout serum immunoglobulin by the Fc region. Using a coagglutination assay with bacteria grown in Todd–Hewitt broth (THB), no evidence of non-specific Fc-binding of trout immunoglobulin (Ig) was obtained. However, when grown in normal trout serum, all isolates produced similar protein patterns in SDS–PAGE, but they were markedly different from the patterns of the bacteria grown in THB. Some bands with MW 70 kDa and over 100 kDa were very intense in the profiles of the serum-grown isolates. In Western blots, these bands of all isolates were immunostained with the conjugated goat antiserum to trout Ig, after blocking with normal goat serum, demonstrating that the bacteria had bound the trout Ig during growth in the serum. When the isolates were grown overnight in trout antiserum against *Lactococcus garvieae* they coagglutinated with *L. garvieae* cells but *S. iniae* isolates grown in normal trout serum did not. These data indicate that *S. iniae* grown in serum express surface factors which can bind trout Ig by the Fc-region.


*Keywords:* *Streptococcus iniae*; Rainbow trout; Antibody; Fc-binding proteins

## 1. Introduction

Over the past few years, the Gram-positive bacterium *Streptococcus iniae* has been associated with outbreaks of disease in several species of farmed freshwater and marine fish (including rainbow trout, tilapia and yellowtail) in Japan, Israel and the United States [1]. The organism has also been isolated from

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Table 1
Bacterial isolates of *S. iniae* used in this investigation

| Marine laboratory strain no. | Host | Location | Date isolated |
|---|---|---|---|
| MT2374 | Hybrid striped bass | Maine | 14/08/96 |
| MT2376 | Tilapia | Texas | 09/09/98 |
| MT2378 | Hybrid striped bass | Texas | 09/09/98 |

diseased humans [2]. Furthermore, serological analyses using rainbow trout antisera indicated antigenic differences and the earlier isolates were classified as serotype I and the later isolates as serotype II. Antisera to serotype I did not agglutinate serotype II but some cross-reactivity was observed in the opposite direction [3].

Little information is available on the antigenic variability of *S. iniae* or its virulence factors. Streptococcal pathogens in mammals are well known to produce immunoglobulin Fc-binding proteins [4], which play important roles in virulence, but no such information is available for *S. iniae*. In a recent paper, it was demonstrated that *S iniae* produce a polysaccharide capsule which probably accounts for the serological differences [5].

The aim of the present study was to investigate the ability of some geographically distinct isolates of *S. iniae* from different species of fish to produce immunoglobulin Fc-binding factors.

## 2. Materials and methods

### *2.1. Bacterial strains and culture conditions*

*S. iniae* isolates used in this study are shown in Table 1. MT codes represent the individual catalogue number for each isolate, held in the culture collection at the Aberdeen Marine Laboratory. All isolates were stored in Todd Hewitt Broth (THB; Oxoid, Basingstoke, UK) containing 20% glycerol at −80 °C. *S. iniae* was cultured on Tryptone Soya Agar (TSA, Oxoid, UK) at 37 °C. Cultures grown on TSA were used to inoculate THB, which was incubated overnight at 30 °C, with shaking at 180 rpm. The identity of each isolate was confirmed by API 20Strep (BioMerieux, Marcy-l'Etoile, France), according to the manufacturers instructions. *Lactococcus garvieae* strain MT2055, used in the coagglutination assay, was cultured on TSA at 22 °C.

### *2.2. Growth in rainbow trout normal serum*

Blood was collected from rainbow trout by caudal venipuncture using Vacutainers (Becton Dickinson Vacutainer Systems, Meylan, France). Prior to bleeding, fish were anaesthetised with MS222 (0.25%, Sigma, Poole, UK). Blood was allowed to clot overnight at 4 °C, before serum was collected by centrifugation at $3000 \times g$ for 20 min. Complement was heat-inactivated by placing sera in a water bath at 44 °C for 20 min [6]. *S. iniae* grown on TSA were suspended in phosphate buffered saline (PBS) to approx. $5.32 \times 10^7$ cfu $ml^{-1}$ and 100 μl was inoculated into 1 ml of normal trout serum and grown overnight at 37 °C.

### *2.3. Preparation and electrophoresis of whole cells*

Suspensions of bacterial isolates (approx $5.32 \times 10^7$ cfu $ml^{-1}$) were inoculated (100 μl) into 1 ml cultures of either Todd–Hewitt Broth or heat-inactivated normal trout serum, and grown overnight at 37 °C. Whole cell extracts were obtained by centrifuging at $10{,}000 \times g$ for 5 min, and washed three times in 50 mM sodium

phosphate buffer, pH 7.4. Cells were resuspended in 50 μl reducing sample buffer (0.125 M Tris, 20% glycerol, 20 μg/ml bromophenol blue, 2% 2-mercaptoethanol, 4% SDS). The resulting suspensions were vortexed and placed in a heat block at 100 °C for 5 min. The lysates in sample buffer were then cleared by centrifugation at 18,000 × *g* for 5 min. Electrophoresis was carried out in SDS–PAGE gels (stacking gel: 5% acrylamide; resolving gel 10% acrylamide), using a Hoeffer SE260 system (Amersham Biosciences, Little Chalfont, UK), with 25 μl of sample being loaded per lane. Gels were stained with Brilliant Blue R250 (Sigma, Poole, UK), for 45 min with gentle agitation, before washing in destain (40% methanol, 10% acetic acid).

### *2.4. Non-specific binding of trout immunoglobulin (Ig)*

#### *2.4.1. Western blot*

Following electrophoresis, gels were washed in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) and blotted on to PVDF membranes (Immobilon P, Millipore) for 35 min using a semi-dry blotting apparatus (Hoeffer Semi-Phor, Amersham Biosciences, Little Chalfont, UK).

Blots were blocked by the addition of normal goat serum (Diagnostics Scotland, Edinburgh, UK), to a final concentration of 2% in Tris-buffered saline containing 0.05% Tween (TBST), for 1 h at room temperature and then overnight at 4 °C. Membranes were washed in TBST. Membranes were then washed twice in TBST before incubating with goat anti-trout Ig alkaline phosphatase conjugate (Kirkegaard–Perry Laboratories, Gaithersberg MD, USA), diluted 1:5000 in TBST, for 1 h at room temperature. After a further four washes in TBST, membranes were developed by immersing in NBT/BCIP phosphatase substrate (Kirkegaard–Perry Laboratories, USA) for 30 min.

To examine whether the Ig binding proteins could bind trout Ig in situ, duplicate Western blots, prepared as described above, were blocked with 2% bovine serum albumen in TBST overnight at 4 °C. After washing in TBST, blots were incubated for 1 h at room temperature with normal trout serum (2% in TBST) or TBST alone as a control. After further washes in TBST, the blots were blocked with normal goat serum and stained with goat anti-trout Ig conjugate as described above.

#### *2.4.2. Coagglutination*

*S. iniae* were cultured overnight in either THB, normal trout serum or trout antiserum to *L. garvieae*. Bacterial cells were harvested by centrifugation and washed in physiological phosphate buffered saline (PBS). Suspensions of *S. iniae* grown in THB were made in PBS to an $OD_{540}$ of 1.5, and incubated for 30 min at room temperature with an equal volume of rainbow trout antiserum against *L. garvieae* [7]. Cells were removed by centrifugation at 13,000 × *g* for 10 min, washed twice in PBS, and resuspended to the original density. Serial twofold dilutions of all the bacterial cultures were made across microtitre plates up to 1 in 128, and 10 μl of a suspension of *L. garvieae* (approx. $10^{10}$ cfu $ml^{-1}$ in PBS) was added to each well. Microtitre plates were then incubated for 4 h at room temperature, then overnight at 4 °C, and examined for agglutination.

#### *2.4.3. Elution of trout Ig from S. iniae grown in trout serum*

Following overnight growth in normal trout serum, the *S. iniae* were washed in cold phosphate buffer (50 mM pH 7.4) and resuspended to their original cell density. The resulting suspension was split into three tubes each containing 300 μl of cell suspension. Cells were pelleted by centrifugation and resuspended in 50 μl of either 50 mM phosphate buffer, pH 7.4, 2 M NaCl or 0.1 M glycine pH 4, for 10 min at room temperature. The bacterial cells were centrifuged and the cell pellets resuspended in 50 μl reducing sample buffer. The supernatants were desalted on Sephadex G25 microspin columns (Amersham Biosciences, Little Chalfont, UK) and diluted in 50 μl reducing sample buffer. After boiling for 5 min and centrifugation at 18,000 × *g* for 5 min, cell pellets (10 μl per lane) and the supernatants (15 μl per lane) were analysed by electrophoresis and Western-blot as described above. Low range molecular weight SDS calibration markers

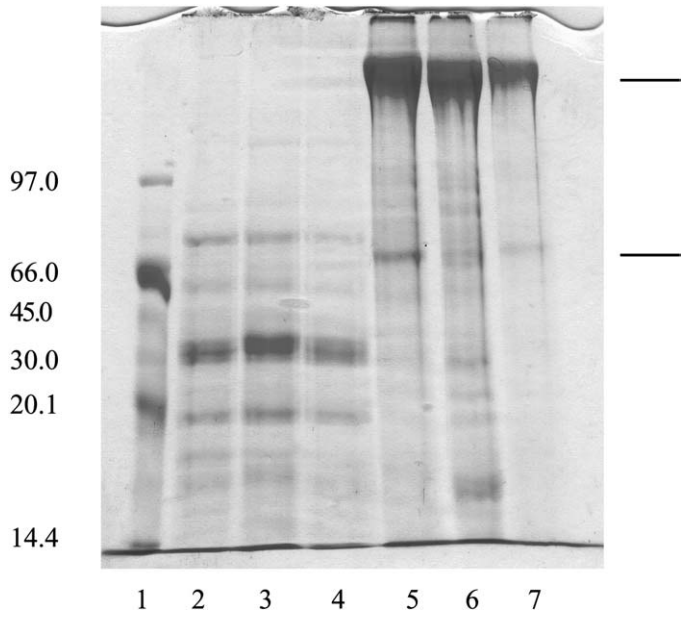


Fig. 1. SDS PAGE gel (10%) stained with Coomassie brilliant blue R250 showing: Lane 1, LMW SDS–PAGE Calibration kit; lane 2, MT2374 grown in Todd–Hewitt broth (THB); lane 3, MT2376 grown in THB; lane 4, MT2378 grown in THB; lane 5, MT2374 grown in normal trout serum (NS); lane 6 MT2376 grown in NS; lane 7 MT2378 grown in NS.

(Amersham Biosciences, Little Chalfont, UK) and normal trout serum (1:4 in reducing sample buffer, 2 μl per lane) were also applied to the gels for reference. Blots were probed with the conjugated goat anti-trout Ig antiserum after blocking in normal goat serum, as described above.

## 3. Results

### *3.1. Electrophoretic profiles of whole cells grown in Todd–Hewitt broth or normal trout serum*

Clear differences were seen between whole cell profiles comparing the isolates (MT2374, MT2376 and MT2378) when grown in THB with the isolates grown in normal trout serum (Fig. 1). Three bands were more prominent in isolates grown in THB, with molecular weights of 43, 52 and 73 kDa respectively. Two bands were more prominent in isolates grown in normal trout serum; one with a MW of 70 kDa and another with a relatively high MW of over 100 kDa. No clear differences were seen between the profiles of MT2374, MT2376 and MT2378, comparing the isolates grown in THB, or comparing the isolates grown in normal serum, although in the latter case, MT2376 appeared to have an extra band at about 35 kDa.

### *3.2. Binding of trout immunoglobulin (Ig)*

#### *3.2.1. Western blot of whole cells grown in THB or normal trout serum*

Western blots of whole cells of isolates MT2374, MT2376 and MT2378 were analysed for Ig-binding using normal trout serum (Fig. 2A). No immunostaining of bands was observed with cells grown in THB (not shown). However, the prominent bands seen in the protein-stained blot (MW 70 and >100 kDa) of all 3 bacterial strains grown in serum were stained (Fig. 2A). In addition to these bands, another band with MW of about 60 kDa was also stained with the goat anti-trout Ig antiserum. All three bands

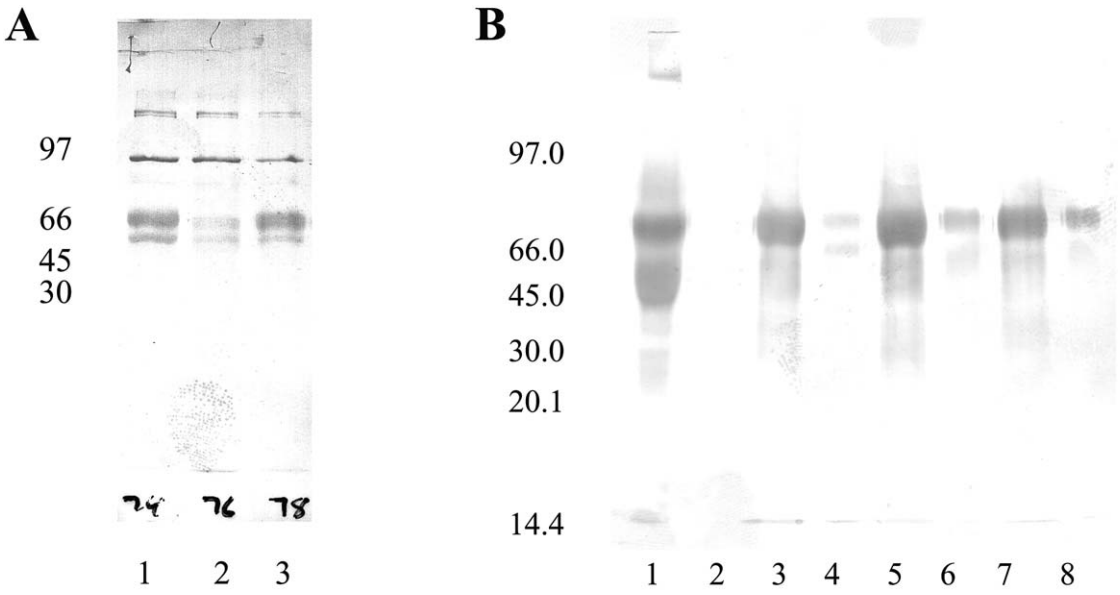


Fig. 2. (A) Western blot showing whole cell extracts of *S. iniae* MT2374, MT2376 and MT2378 grown in rainbow trout normal serum, blocked with 2% goat serum and probed with goat anti trout Ig alkaline phosphatase conjugate. (B) Western blot showing normal trout serum (lane 1), and *S. iniae* MT2376 whole cell extracts (lanes 3, 5 and 7), and supernatants used to elute trout Ig: phosphate buffer pH 7.4 (lane 4), 2 M NaCl (lane 6) and 0.1 M glycine pH 4 (lane 8). Blot was blocked with normal goat serum (2%) and probed with goat anti-trout alkaline phosphatase conjugate.

(with MW of about 60, 70 and over 100 kDa) matched with stained bands in blots of normal trout serum (Fig. 2B, lane 1).

*3.2.2. Coagglutination of S. iniae grown in THB*

Coagglutination assays were performed to investigate the ability of trout Ig to non-specifically bind to *S. iniae* isolates MT2374 and MT2378 using specific antiserum from trout directed against *L. garvieae*. Microbial antigens may bind Ig either non-specifically, by the Fc component of Ig, or specifically, by the Fab component. It therefore follows that if the Fc component is bound by *S. iniae*, then the antibody-coated *S. iniae* should be able to specifically bind *L. garvieae* by the exposed Fab component, resulting in agglutination. However, no such agglutination was observed with *S. iniae* isolates MT2374 and MT2378 grown in THB.

*3.2.3. Co-agglutination assay with S. iniae grown in trout serum*

Bacteria were grown overnight in normal trout serum or trout antiserum to *L. garvieae*. The cells were washed and mixed with *L. garvieae* cells. The *S. iniae* grown in normal serum did not agglutinate on mixing with *L. garvieae* but those grown in anti-serum to *L. garvieae* did.

*3.2.4. Elution of trout Ig from S. iniae grown in trout serum*

The three immunoreactive bands were present in the cell fraction of cells washed in phosphate buffer and a small amount was present in the supernatant. Following the wash in 2 M NaCl, both the cell fraction and the supernatant showed quite strong staining of the bands, particularly the 70 kDa band. However, the

strongest staining of the 70 kDa band appeared in the supernatant from cells washed in 0.1 M glycine indicating that the bound trout Ig could be eluted with the glycine (Fig. 2B).

## 4. Discussion

Streptococcal pathogens in mammals are well known to produce Protein G with the ability to bind the Fc region of Ig molecules. Fc-binding is able to block the region of Ig molecules responsible for the binding and activation of complement and opsonisation, thereby avoiding these major defence mechanisms [4]. Thus, the ability of different serotypes of *S. iniae* with different arginine dihydrolase (AD) phenotypes, to bind rainbow trout Ig was investigated in the present study using Western blotting of whole cells and co-agglutination using living cells. When the bacteria were grown in THB, no evidence of non-specific binding of Ig was found.

However, SDS–PAGE analysis of whole cells, following growth in normal trout serum as compared to the Todd–Hewitt broth, revealed changes in the protein-banding patterns. All three strains showed similar changes in profiles though MT2376 showed an extra band with MW of about 35 kDa only when grown in serum. Some bands disappeared following growth in serum and some, particularly with MW of 70 kDa and over 100 kDa, became much more pronounced or were even novel.

Western blots of whole cells grown in THB or in normal trout serum were probed for non-specific binding of trout Ig by incubating the blots with normal trout serum and then staining with a conjugated goat anti-trout Ig antiserum. While there was little staining of the cells grown in THB, the extra bands of all three strains grown in serum stained well along with another band of about 60 kDa. However, this staining was not due to incubating the blot with trout serum as the control blots, incubated in the absence of trout serum with only the conjugated goat anti-trout Ig antiserum after blocking with normal goat serum, stained identically. This suggested that the stained bands were due to the presence of trout Ig which had been non-specifically bound by the bacteria during their growth in the trout serum. This was corroborated by immunoblots of normal trout serum stained with the conjugated goat anti-trout Ig antiserum, where bands of 70 and 60 kDa were prominently stained. Rainbow trout Ig heavy chains have MWs of 70 and 60 kDa [8]. The identity of the high MW (>100 kDa) band which stained with the goat antiserum probably represents trout Ig heavy and light chain polymers or undissociated tetramers and this band was also present in the Western blot of trout serum. It is noteworthy that all the extra bands seen in the protein stained SDS–PAGE gels of the serum-grown bacteria, correspond to the MW of trout Ig. In salmonid fish, Ig comprises less than 6% of the total serum protein [9] and as there was no sign of other serum protein bands in the gels, this suggests that the binding of serum proteins during growth in the serum was Ig-specific.

It is also interesting that the immunostained bands in the serum-grown bacterial samples corresponded to the trout Ig bands in the trout serum, indicating the Ig had been dissociated from the bacterial cell membrane proteins by the electrophoresis procedure. However, the *S. iniae* Ig-binding proteins evidently could not then bind more Ig as no extra bands were observed in blots incubated with trout normal serum before staining with the conjugated goat anti-trout Ig (not shown). This is in contrast to streptococcal group C and G Fc-binding proteins which are capable of binding human IgG following Western blotting under reducing conditions [4].

Attempts were made to dissociate the trout Ig from the bacterial cells grown in serum by washing in PBS or 0.1 M glycine pH 4. The washed cells and the supernatants were blotted and probed with the goat anti-trout Ig antiserum. The phosphate buffer and 2 M NaCl supernatants contained only small amounts of the trout Ig, while the 0.1 M glycine, pH 4, supernatant contained more strongly staining immunoreactive bands. These results indicate that the binding of the trout Ig by the *S. iniae* cells resembles the Fc-binding of Ig by streptococcal protein G which is dissociated at low pH [4]. Furthermore, the fact that the *S. iniae* grown in normal trout serum did not agglutinate yet bound the trout Ig, indicates the binding

of the Ig was not by the Fab region as trout Ig has 8 Fab antigen-binding sites and is very efficient at agglutinating bacteria [10].

The coagglutination experiments to detect non-immune Fc-binding of trout Ig were repeated on the *S. iniae* grown in normal trout serum or trout antiserum specific for *L. garvieae*. When mixed with cells of the latter, the *S. iniae* grown in normal serum did not agglutinate with the *L. garvieae* while *S. iniae* grown in the specific antiserum did. These results confirm the above, that when grown in trout serum, *S. iniae* produce a cell surface Fc-binding factor for trout Ig.

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