

Streptococcus iniae: serological differences, presence of capsule and resistance to immune serum killing

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ABSTRACT: The biochemical profiles, presence of capsule, outer membrane protein profiles and serological interactions of isolates of *Streptococcus iniae* obtained from different geographical and fish host origins were examined. The isolates had very similar biochemical profiles using API 20 Strep but varied as to whether they were arginine dihydrolase-negative, -positive or -intermediate (AD-ve, AD+ve, AD±ve, respectively). Representatives of each AD type were compared in subsequent experiments. All types possessed a polysaccharide capsule. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of outer membrane proteins or whole cells revealed no difference in banding patterns between isolates. All isolates were resistant to trout normal and specific immune serum and grew well in the presence of added fresh normal serum. Serological analyses of the isolates revealed antigenic differences. Trout antiserum against the AD+ve isolate did not agglutinate the AD-ve or AD±ve isolates, while antisera against the latter 2 types showed low agglutinating activity with all 3 isolates. When whole live cells of AD-ve and AD+ve isolates were dot-blotted, antiserum to the AD+ve isolate did not stain the AD-ve isolate, but antiserum to the AD-ve isolate stained both AD types. However, if the cells were pre-treated with Proteinase K (to remove surface-exposed protein antigens), the AD+ve isolate was stained only by its homologous antiserum. These results suggest that while certain protein antigens of the different AD type strains are immunologically cross-reactive, the capsular antigens appear to be AD type-specific. Furthermore, the results suggest that the cross-reactive antigens on the AD-ve isolate are effectively hidden by the strain-specific capsule, while they are partially exposed on the AD+ve isolate.

KEY WORDS: *Streptococcus iniae* · Serotypes · Capsule · Antiserum resistance · Arginine dihydrolase

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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 fishes (including rainbow trout, tilapia, hybrid striped bass and yellowtail) in Israel, the USA and Japan (Eldar & Ghittino 1999). The organism has also been isolated from diseased humans (Weinstein et al. 1997). Vaccination was successfully used in rainbow trout farms in Israel from 1995 to 1997, but outbreaks resumed thereafter. These were associated

with a variant of the bacterium which, unlike the previous isolates, was arginine dihydrolase negative (AD-ve) (Zlotkin et al. 1998) and could be differentiated using the rapid amplified polymorphic DNA (RAPD) technique (Bachrach et al. 2001). Furthermore, serological analyses using rainbow trout antisera indicated antigenic differences and the earlier isolates (AD+ve) were classified as Serotype I and the later isolates (AD-ve) as Serotype II. Antisera to Serotype I did not agglutinate Serotype II, but some cross-reactivity was observed in the opposite direction (Bachrach et al. 2001).

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Little information is available on the antigenic variability of *Streptococcus iniae* or its virulence factors. While streptococcal pathogens in mammals are well known to produce capsules which play important roles as serotype and virulence determinants (Kasper et al. 1990), no such information is available for *S. iniae*.

The aim of the present study was to compare representative AD–ve and AD+ve isolates of *Streptococcus iniae* with respect to surface antigenic characteristics, presence of a capsule, and resistance to immune serum killing.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptococcus iniae* isolates used in this study were selected according to their arginine dihydrolase (AD) type as determined by the API 20 Strep test (BioMerieux). The source and strain number, as held in the culture collection at the Marine Laboratory, Aberdeen, are shown in Table 1. All isolates were stored in Todd Hewitt broth (THB; Oxoid) containing 20% (v:v) glycerol at -80°C . *S. iniae* was cultured on tryptone soya agar (TSA; Oxoid) at 37°C overnight, or at 22°C for 36 h. Cultures grown on TSA were used to inoculate THB, which was incubated overnight at 30°C , with shaking at 180 rpm. For testing in the API 20 Strep, *S. iniae* was cultured on Columbia agar base containing 5% defibrinated sheep blood.

Biochemical characterisation. Biochemical differences between isolates were determined by API 20 Strep at 37°C (BioMerieux), according to the manufacturers instructions.

Determination of presence of capsule. The presence of a capsule was determined by electron microscopy for *Streptococcus iniae* Strains MT2374, MT2376 and MT2378, using polycationic ferritin and antibody, adapted from the method of Jacques & Foiry (1987). *Lactococcus garvieae* MT2055 was also examined as a capsule-negative control (Barnes et al. 2002). Bacterial suspensions were prepared from overnight cultures on TSA, in phosphate-buffered saline (PBS), to approxi-

mate the opacity of the MacFarland 2 standard. Suspensions were then incubated overnight at 4°C , with heat-inactivated specific antisera from rainbow trout (1:10 antiserum:bacterial suspension for *S. iniae*, 1:500 for *L. garvieae*). Bacteria were harvested by centrifugation at $3000 \times g$ for 10 min before washing twice in 0.07 M cacodylate buffer (pH 7.0). Pellets were resuspended in 0.2 M cacodylate buffer containing 3.6% glutaraldehyde, and incubated for 1 h on ice. Following a further 3 washes and resuspension in 0.07 M cacodylate buffer, suspensions were incubated with polycationic ferritin (Sigma) at a final concentration of 1.0 mg ml^{-1} , for 30 min at 20°C . Suspensions were then washed twice in 0.07 M cacodylate buffer, and osmified (4% osmium tetroxide in 0.07 M cacodylate buffer) for 3 h at 27°C . Cells were dehydrated stepwise in ethanol (10 min at 25, 50, 75, 90, 95 and 100% ethanol), before embedding in embedding resin (medium grade, Taab Laboratories). Ultrathin sections were cut with a Reichert-Jung Ultracut ultramicrotome and mounted on 300-mesh copper grids. Sections were then stained with uranyl acetate (saturated aqueous solution) and lead citrate, and viewed with a Philips 301 transmission electron microscope at 80 kV.

Preparation of protoplasts — mutanolysin-soluble proteins and peripheral membrane proteins. Mutanolysin-soluble proteins and peripheral membrane proteins were prepared as previously described by Yother & White (1994). *Streptococcus iniae* isolates were grown overnight in 50 ml THB in 250 ml Erlenmeyer flasks with shaking at 140 rpm at 30°C . Bacterial cells were harvested by centrifugation at $5000 \times g$ for 10 min at room temperature, and the pellet washed once in double-distilled water (ddH_2O). The pellet was resuspended in 0.5 ml protoplast buffer (20% sucrose, 0.005 M Tris, 0.025 M MgSO_4 , pH 7.4) containing $25 \mu\text{g ml}^{-1}$ mutanolysin (Sigma). After incubation for 40 min at room temperature, protoplasts were removed by centrifugation at $6500 \times g$ for 10 min, and the mutanolysin soluble supernatant was stored at -20°C . To obtain peripheral membrane proteins, pellets were washed once in protoplast buffer and lysed in ddH_2O for 10 min at room temperature before passage ($\times 5$) through a 26-gauge needle. Broken cells and membranes were centrifuged at $10\,000 \times g$ for 10 min, and peripheral membrane proteins were extracted in ice-cold Na_2CO_3 (100 mM, pH 11.2) on ice for 20 min. Samples were then centrifuged at $10\,000 \times g$ for 10 min to remove debris, and the supernatant was stored at -20°C .

Electrophoresis and Western blotting of mutanolysin-soluble proteins and peripheral membrane proteins. Proteins were separated by electrophoresis in SDS-PAGE gels (resolving gel: 10% (w:v) acrylamide; stacking gel: 5% (w:v) acrylamide) using a Hoeffer SE260 system (Amersham Pharmacia Biotech). Proto-

Table 1. *Streptococcus iniae*. Bacterial isolates used in this investigation. Dates given as dd/mm/yy

Isolate	Host	Source	Date isolated
MT2374	Hybrid striped bass (<i>Morone saxatilis</i> \times <i>M. chrysops</i>)	Maine, USA	14/08/96
MT2376	Tilapia (<i>Oreochromis</i> sp.)	Texas, USA	09/09/98
MT2378	Hybrid striped bass	Texas, USA	09/09/98

plast samples were diluted 2-fold with SDS-PAGE sample buffer under non-reducing conditions (i.e. without 2-mercaptoethanol), and 2 to 11 μl of sample was loaded per lane. Following electrophoresis, gels were washed in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), and blotted onto a PVDF (polyvinylidene dichloride) membrane (Immobilon P, Millipore) for 35 min using a semi-dry blotting apparatus (Hoeffer Semi-Phor, Amersham Pharmacia Biotech). Membranes were washed in PBS containing 0.05% Tween, before staining protein bands with Au-rodye (Amersham Pharmacia Biotech) for 15 to 30 min. Approximate molecular weights (MW) were determined using a low MW SDS-PAGE calibration kit (Amersham Pharmacia Biotech).

Preparation and electrophoresis of whole cells.

Whole cells were obtained by centrifuging THB cultures (10 ml) overnight at $3000 \times g$ for 20 min, and washing in ddH₂O with 40 μg glass beads (150 to 212 μm , Sigma). Cells were resuspended in 80 to 100 μl reducing sample buffer (0.125 M Tris, 20% glycerol, 20 $\mu\text{g ml}^{-1}$ bromophenol blue, 2% 2-mercaptoethanol, 4% SDS), and placed in a heat block at 100°C for 20 min. Electrophoresis was carried out as described above, with 25 μl of sample being loaded per lane. Gels were stained with Brilliant Blue R250 (Sigma), for 45 min with gentle agitation, before washing in destain (40% methanol, 10% acetic acid).

Serum. Serum was collected from rainbow trout as follows:

Rainbow trout normal serum: Blood was collected from rainbow trout by caudal venipuncture using Vacutainers (Becton Dickinson Vacutainer Systems). Prior to bleeding, the fish were anaesthetised with MS222 (0.25%, Sigma). 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 (Sakai 1981).

Rainbow trout antiserum to *Streptococcus iniae*: Strains MT2374, MT2376, and MT2378 were cultured overnight in THB and inactivated by addition of formalin at a final concentration of 0.2%. Suspensions were stored at 4°C until required. For immunisation, bacteria were emulsified in an equal volume of Freund's incomplete adjuvant (Sigma). Fish (4 per bacterial strain) were anaesthetised as above and injected intraperitoneally with 100 μl of adjuvanted bacterin. After about 500 degree-days, fish were killed by anaesthetic overdose and bled. Serum was collected, heat-inactivated and stored as described for normal serum above.

Rainbow trout fresh serum: Fresh serum was collected as described above on the day of use, but was not heat-inactivated.

Agglutinating antibody titres. Titres of specific antibody were determined by agglutination tests performed, in duplicate, in a microtitre plate. Specific antiserum (100 μl) was serially 2-fold-diluted in PBS, and a suspension of homologous *Streptococcus iniae* (OD₅₄₀ of 1.0; equivalent to 5.32×10^7 cfu ml⁻¹) was added to each well (50 μl). As a control, heat-inactivated normal serum was also tested. Plates were incubated for 1 h at room temperature and then overnight at 4°C. Titres were taken as the highest dilution of serum to give a positive agglutination reaction. The agglutination test was performed using specific trout antisera obtained from 4 individual fish. Specific antiserum from the individual trout giving the highest agglutinating titres for the homologous strain was selected and used in subsequent experiments.

Bactericidal activity of normal serum and antiserum. *Streptococcus iniae* isolates were cultured overnight in THB and centrifuged at 3700 rpm ($1500 \times g$) for 20 min. Pellets were washed and resuspended in PBS to an OD₅₄₀ of 1.0 (5.32×10^7 cfu ml⁻¹). This was diluted 50-fold in PBS and 50 μl was incubated with an equal volume of heat-inactivated normal serum or antiserum (10% for MT2374 and MT2378; 1% for MT2376 due to the latter's higher agglutinating titre) for 1 h at room temperature. Complement activity was provided by the addition of fresh normal serum (1:4; bacterial suspension/serum:fresh normal serum). As a control, PBS was added to the suspensions in place of fresh normal serum. In replicates of 6, suspensions were incubated in a microtitre plate for 1.5 h at room temperature. Following incubation, suspensions were serially diluted in PBS and spotted (10 μl) onto TSA plates, which were incubated at 22°C for 48 h. Viable colonies were counted, with survival in the heat-inactivated serum+fresh serum being expressed as a percentage of survival in the heat-inactivated serum + PBS controls.

Dot-blot of whole and Proteinase K-treated cells of MT2374 and MT2376. THB cultures were centrifuged overnight, washed in ddH₂O, resuspended in Proteinase K (PK) (Sigma, 100 $\mu\text{g ml}^{-1}$ in ddH₂O) at 37°C for 45 min, and washed in ddH₂O. Whole (washed but untreated cells) and Proteinase K-treated cells were spotted (10 μl), in duplicate onto a nitrocellulose membrane, and left to dry; 5 replicate membranes were prepared. Membranes were washed twice in Tris-buffered saline containing 0.05% Tween (TBST) and blocked with 2% bovine serum albumin (globulin-free, Sigma) for 1 h at room temperature and then overnight at 4°C. After washing in TBST, membranes were incubated in trout antiserum against MT2374 or MT2376, or with normal trout serum. Membranes were washed again and incubated with goat anti-trout Ig alkaline

phosphatase conjugate (Kirkegaard-Perry Laboratories), diluted 1:5000 in TBST, for 1 h at room temperature. After a further 4 washes in TBST, membranes were developed by immersing in nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Kirkegaard-Perry Laboratories) for 30 min.

Statistical analysis. Viable counts of bacteria from the bactericidal assays were checked for Poisson distribution and statistically analysed. Three nested models were fitted and compared using the generalised linear modelling routines in Genstat 5:

Model 1: Viable counts = replicate well + noise; a model that allows the mean viable counts to differ between replicate microtitre wells.

Model 2: Viable counts = test/control + noise; a model which allows difference in mean viable counts between test and control, but assumes no well effect.

Model 3: Viable counts = constant + noise; a model which assumes no difference in mean viable counts between test and control.

Significant differences were determined at $p < 0.05$, and highly significant differences at $p < 0.001$.

RESULTS

API tests

Streptococcus iniae Strains MT2374, MT2376 and MT2378 showed differences in the API 20 Strep test with respect to arginine dihydrolase and ribose utilisation only. MT2374 was negative (AD-ve) and MT2376 was positive (AD+ve) for both these reactions. Replicates of MT2378 were inconsistent (negative or weakly positive) for these reactions and was designated AD±.

Presence of capsule

Isolates MT2374, MT2376 and MT2378 all expressed a capsule, as visualised by an electron-dense layer surrounding the cell membrane (Fig. 1a–c). The capsule-negative control, *Lactococcus garvieae* MT2055 (Fig. 1d), did not exhibit an electron-dense layer on the cell surface.

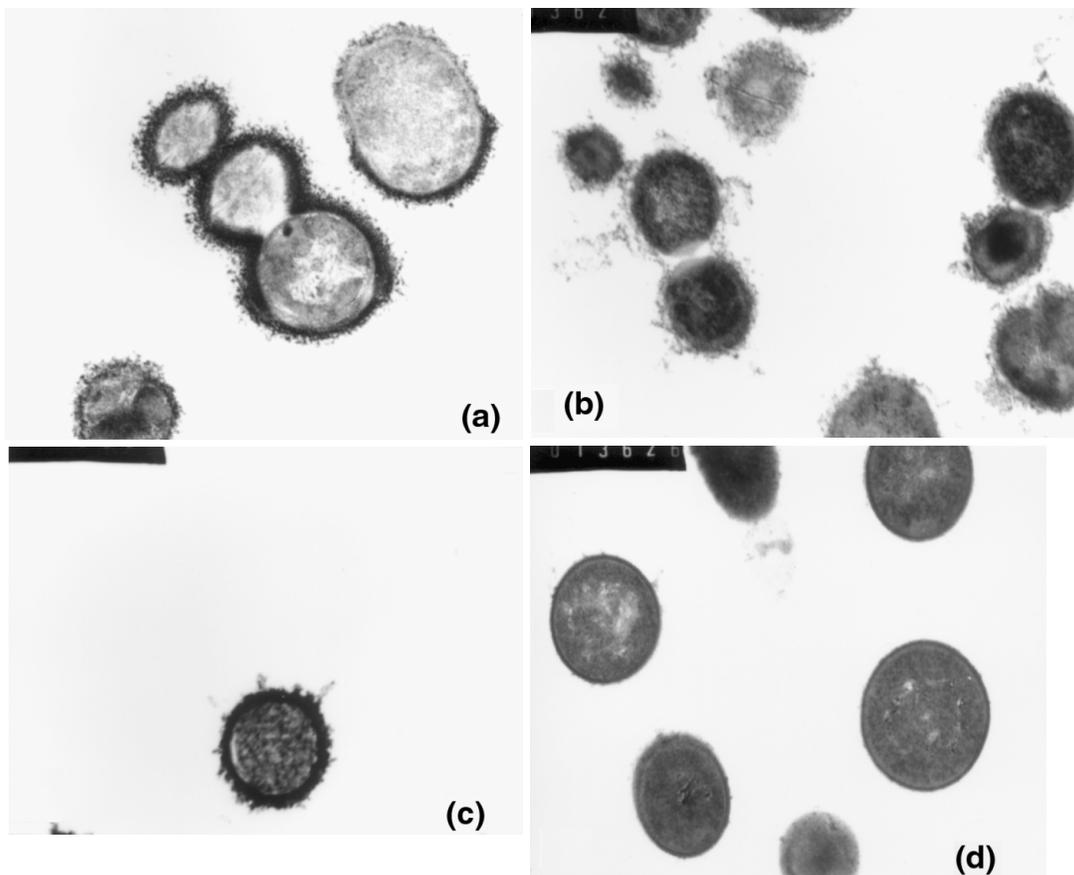


Fig. 1. *Streptococcus iniae* and *Lactococcus garvieae*. Electron micrographs of (a) ferritin-labelled *S. iniae* Strain MT2374, (b) MT2376, (c) MT2378 and of (d) *L. garvieae* (capsule-negative control). Magnification $\times 25\,000$

Antibody agglutination titres

The agglutinating titres of each isolate with each of the trout antisera are shown in Table 2. The antiserum against the AD+ve isolate MT2376 had a very high titre against its homologous strain (and another AD+ve Strain MT2377, not shown) but did not agglutinate any of the other isolates. On the other hand, antisera against the AD-ve or AD±ve strains showed some agglutination of all isolates. However, these antisera had much lower agglutinating titres to their homologous strains than did the antiserum to MT2376. Normal trout serum did not agglutinate any of the isolates.

Survival in normal serum and antiserum

Isolates MT2374, MT2376 and MT2378 demonstrated complete resistance to serum killing by both heat-inactivated normal serum and specific homologous antiserum, as the number of bacteria re-isolated from the wells was similar to the number that had been inoculated. The addition of fresh serum (4 volumes) instead of PBS to the diluted normal or homologous antiserum (1 volume) resulted in enhanced growth of all the bacterial isolates of between 180 and 440% (data not shown). This may simply be related to the amount of nutrients present.

Electrophoretic profiles of whole cells, mutanolysin-soluble proteins and peripheral membrane proteins

No clear differences were seen in protein banding patterns between any of the isolates (data not shown).

Dot-blot of whole and Proteinase K-treated cells

In an attempt to investigate the protein or non-protein nature of cell-surface antigens and their cross-reactivity, whole cells and Proteinase K-treated cells of Strains MT2374 (AD-ve) and MT2376 (AD+ve) were blotted and analysed for antibody-binding using homologous and heterologous antisera. Control blots were probed with normal trout serum instead of antiserum. Whole untreated and Proteinase K-treated cells of isolate MT2374 were stained with its homologous antiserum but not with the heterologous antiserum (data not shown), confirming the serum agglutination data. Untreated cells of MT2376 stained strongly with the homologous trout anti-MT2376, and to a lesser extent with the trout anti-MT2374, confirming that serum raised against MT2374 is cross-reactive with isolate MT2376 in the agglutination assay. However,

Table 2. *Streptococcus iniae*. Agglutinating titres of rainbow trout antisera against homologous and heterologous isolates. AD: arginine dihydrolase

Isolate	AD-type	Antisera to:		
		MT2374	MT2376	MT2378
MT2374	-	8	<2	4
MT2376	+	32	>4096	8
MT2378	±	4	>2	4

Proteinase K-treated cells of MT2376, while still being strongly stained by the homologous antiserum, showed only faint staining with the heterologous antiserum, similar to the background staining seen with normal serum.

DISCUSSION

Outbreaks of streptococcosis in rainbow trout farms in Israel have been reported to be associated with a variant of the pathogen that is arginine dihydrolase-negative (AD-ve) (Zlotkin et al. 1998) and can be differentiated from the previous isolates by the RAPD technique (Bachrach et al. 2001). Furthermore, serological analysis (using the Lancefield technique to compare carbohydrate antigen extracts and bacterial cell agglutination) with rainbow trout antisera has led to the older isolates being termed Serotype I and the newer isolates Serotype II (Bachrach et al. 2001). Trout antisera to the AD+ve Serotype I did not agglutinate the newer AD-ve Serotype II strains, while conversely, antisera to the Serotype II strains showed a slight cross-reactivity to the Serotype I strains (Bachrach et al. 2001). It was postulated by these authors that the massive vaccination programme in Israel might have led to selection and emergence of the Serotype II.

In the present work, several *Streptococcus iniae* isolates from geographically disparate locations in the USA and from different host species (hybrid striped bass or tilapia) were characterised by API 20 Strep, and the AD test showed that some were negative, some positive and some inconsistently and weakly positive (-ve, +ve or ±, respectively). A selection of these variants was used to study serological cross-reactivity and potential virulence factors. Three isolates—an AD-ve isolated from bass in Maine, an AD+ve from tilapia in Texas, and an AD±ve from bass in Texas—were used to produce antisera in rainbow trout. Cross-agglutination tests were performed and the results obtained with the antiserum to the AD+ve strain confirmed those reported by Bachrach et al. (2001) for Israeli strains. Namely, that antiserum to the AD+ve strain only reacted with AD+ve strains, while antiserum to the

AD–ve (and AD±ve) strain cross-reacted with the other AD type strains. However, the homologous agglutinating titres obtained in the anti-AD+ve antisera were much higher than in the anti-AD–ve and anti-AD± antisera, suggesting that the surface antigens of the AD–ve and ±ve isolates are much less immunogenic in rainbow trout than those of the AD+ve isolate.

In order to characterise the potential antigenic profiles, representatives of the different AD types were compared by SDS-PAGE. Preparations of outer membrane proteins (mutanolysin-soluble fraction), peripheral membrane proteins and whole cells showed no differences in the protein banding patterns between the strains, except for minor differences in band intensities. This suggests that there were no differences in the major protein constituents of the strains.

It is well known that streptococcal pathogens in humans possess a polysaccharide capsule, and the antigenic specificity of this capsule provides the basis for the Lancefield grouping system for beta-haemolytic streptococci (Esser et al. 1985). The capsule can be visualised in the electron microscope using ferritin-labelled antisera (Kasper et al. 1990), and it has great importance in virulence mechanisms, whereas protection is related to anti-capsular antibodies (Alonso de Velasco et al. 1995). Furthermore, the results of Bachrach et al. (2001), showing that carbohydrate antigen extracts of the *Streptococcus iniae* reacted with trout antisera, suggested that polysaccharide capsules might be present in *S. iniae*, although Kitao (1993) considered that *Streptococcus* sp. affecting fish were non-capsule-forming, and Fuller et al. (2001) found no evidence for capsule expression. In the present work, the homologous antisera prepared against the 3 AD types of *S. iniae* were used to stain the bacterial cells in combination with cationic ferritin before examination by electron microscopy. All strains were shown to possess a capsule.

In mammals, streptococcal polysaccharide capsules have been associated with the ability to resist killing by normal and immune sera as well as evading phagocytosis. However, immune serum, but not normal serum, acts as an opsonin, promoting phagocytosis and killing through polymorphonuclear phagocytes (Kasper et al. 1990). In the present work, the strains of *Streptococcus iniae* were incubated in normal and homologous antisera with and without fresh serum added as a complement source. The bacteria were all able to grow in the sera, with the excess fresh sera even stimulating growth, presumably because of the extra nutrients available. Further work is necessary to investigate the possible opsonising effect of antisera on this fish bacterial pathogen.

To attempt to elucidate the nature of the specific and cross-reacting antigens which reacted with the different antisera against the 3 AD types grown in THB, the

cells of the AD+ve isolate MT2376 were treated with Proteinase K to digest surface-exposed protein epitopes, and dot blots of whole cells and Proteinase K-treated cells were probed with the antisera to AD–ve or AD+ve isolates. Both antisera stained the whole cells of this bacterial strain, but Proteinase K-treated cells were stained only with the homologous antiserum.

The above data suggest that the cross-reacting antigens between AD–ve and AD+ve isolates are protein antigens, and that the specific, non-cross-reacting antigens are Proteinase K-resistant, possibly carbohydrate, antigens. Furthermore, it is possible that the protein antigens of AD+ve isolates are somewhat exposed, accounting for the ability of antiserum to AD–ve isolates to agglutinate AD+ve isolates. In contrast, the cross-reacting protein antigens on the surface of AD–ve isolates would be hidden by a sero-specific Proteinase K-resistant antigen, the most likely candidate for this being the capsule. This interpretation would imply that not only is the capsule of AD–ve isolates antigenically distinct from AD+ve isolates, but it also provides a more complete coverage of the bacterial cell surface. Thus, the emergence of a capsule with a new antigenic structure and a more effective coverage of cross-reactive antigens is consistent with the emergence of a new Serotype II in Israel, which expresses novel surface antigens and a more invasive systemic pathology while being resistant to the vaccines based on Serotype I (Bachrach et al. 2001). However, the present work, which shows similar serotypic variants amongst isolates from the USA, suggests that these are not confined to Israel, but are more widespread.

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