

# Bacterial Diseases of Fish — Where Do We Go from Here?

## Recent advances in understanding the virulence mechanisms of fish pathogenic bacteria

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Andrew C Barnes\* and Anthony E Ellis

*Marine Laboratory*

*Aberdeen AB11 9DB, Scotland, UK*

### Introduction

Virulence mechanisms of pathogenic bacteria are important to understand because therein lies the nature of the host–pathogen interaction and knowledge of this allows progress to be made in developing control measures and especially vaccines. In this chapter, a critical overview of the available information regarding the virulence mechanisms of some of the major bacterial pathogens of farmed fish is presented.

### Bacterial Diseases of Cold Water Fish Species

#### *Renibacterium salmoninarum* (*R. sal*)

This Gram-positive bacterium is the etiological agent of bacterial kidney disease (BKD) in salmonid fish. A key aspect of virulence of *R. sal* is its ability to enter, survive and multiply in host macrophages.<sup>1,2</sup> There have been quite a few studies on the interaction between the bacterium and macrophages *in vitro* but one of the problems with these data is the questionable status of the virulence of the bacteria used in the

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\*Present address: Centre for Marine Studies, University of Queensland, Brisbane QLD 4072, Australia.

studies. While all isolates were able to infect and survive for some days in macrophage cultures, they were not all virulent *in vivo* but this has not frequently been tested. In one study, several strains were used for *in vitro* and *in vivo* experiments. Only one strain produced BKD with mortalities and only this strain of *R. sal* was resistant to killing by chemically generated (SNAP) nitric oxide (NO).<sup>3,4</sup> This correlated quite well with the *in vivo* production of NO (measured as serum nitrate) following infection of rainbow trout.<sup>3</sup> Infection with the avirulent strains resulted in slightly raised nitrate levels from days 4–12. No fish died in this group over a sampling period of 34 days. On the other hand, infection with the virulent strain resulted in nitrate levels increasing on day 8 and then progressively increasing to very high levels until day 21 when all the fish in this group had died from BKD. This suggests that the NO response may have cleared the avirulent strains while having no effect on the virulent strain, though the delay in the NO response to the virulent compared with the avirulent strains is interesting. None of the strains used in this study were killed by peroxyntirite.<sup>4</sup>

In most other studies, the virulence of the strains has not been determined at the time of the experiments. With this in mind, it has been shown that photochemically generated  $O_2^-$  reduced *R. sal* viability, even in the presence of superoxide dismutase (SOD), but not in the presence of catalase, suggesting that hydrogen peroxide ( $H_2O_2$ ) was responsible for the killing of strains with undetermined virulence.<sup>5</sup> In the same study, this strain was not killed by normal macrophage cultures but it was killed by macrophage activating factor (MAF)-activated macrophages, even in the presence of an inducible nitric oxide synthase (iNOS) inhibitor, suggesting that reactive oxygen species (ROS) rather than reactive nitrogen intermediates (RNI) were responsible.

The bacterium activates the alternative complement system which allows adherence to macrophages within one hour of contact and by two hours the opsonized bacteria are phagocytosed.<sup>6</sup> Even non-opsonized *R. sal* (of known virulence) are phagocytosed rapidly and have escaped from phagolysosomes into the cytoplasm by 4.5 hours after infection of macrophage cultures.<sup>2</sup>

Following infection of macrophage cultures with *R. sal*, activation of the respiratory burst and production of  $O_2^-$  is rapidly detected (within

30 minutes). The production of  $O_2^-$  is enhanced if the bacteria have been previously exposed to normal rainbow trout serum (fresh or heat-inactivated).<sup>7</sup> Exposure to normal and especially immune serum containing antibodies to the p57 protein also enhance intracellular survival and growth of *R. sal* in macrophage cultures.<sup>8</sup> This may explain reports that induction of antibodies to *R. sal* can actually increase susceptibility to BKD.<sup>9,10</sup>

Thus, very soon after infection with *R. sal*, macrophages go into respiratory burst (RB). However, at one, three and six days after infection, macrophages are unable to produce ROS on stimulation with phorbol myristate acetate (PMA) indicating that the RB is either exhausted by the bacterium or that further production of  $O_2^-$  is blocked.<sup>11</sup> The same lack of RB induction by PMA occurs 18 hours after exposing macrophages to the major soluble antigen (MSA) also known as the p57 surface protein<sup>12</sup> but the ability of this protein to induce the RB at earlier times, resulting in exhaustion of the response at the time of testing was not investigated. While the same reduction in RB was observed in activated macrophages it was not to the same extent as in normal macrophages, and while three days after infection the RB of infected normal macrophages could no longer be elicited by PMA, the activated infected macrophages were still capable of  $O_2^-$  production.<sup>5</sup> Moreover, the activated macrophages were able to kill the bacteria, and while this was only partial at one day following infection, it was virtually complete after seven days. Furthermore, the killing was completely inhibited if catalase was added to the cultures suggesting that  $H_2O_2$  was responsible. Over the same period the bacteria grew in the non-activated macrophages.<sup>5</sup> As mentioned above, the precise degree of virulence of the strain used in these experiments was not determined at the time so it is not known definitely if virulent strains of *R. sal* can be killed by activated macrophages because of their ability to produce higher levels of  $H_2O_2$ .

Nevertheless, the available information indicates that *R. sal* is susceptible to being killed by  $H_2O_2$  and while its own catalase may be able to protect against this bactericidal mechanism in normal macrophages, it is not sufficiently protective in activated macrophages. In the normal host, it appears that *R. sal* becomes opsonized by serum

factors including alternative complement, and rapidly adheres to macrophages inducing the RB perhaps before phagocytosis has begun. The role of the MSA might be important here as it is released by the bacterium in large amounts *in vivo*<sup>9</sup> and may induce macrophages to go into RB at a distance so that the RB is completely exhausted before the bacterium can be phagocytosed. The phagocytic capacity of macrophages exposed to MSA was not inhibited.<sup>12</sup> The presence of opsonizing antibodies would be expected to increase the rate at which the macrophage RB is exhausted. Then the bacterium is taken up by the macrophage and the resilient cell wall components protect the bacterium from the hydrolytic enzymes in the phagolysosomes from where it rapidly escapes into the cytoplasm<sup>2</sup> and where it is presumably safe and can continue to multiply. But how does it achieve this escape process? Following infection *in vivo*, iNOS is expressed and NO is produced. While avirulent strains of *R. sal* can be killed by NO, virulent strains are resistant but the mechanism of this resistance is not understood.

Attempts to investigate the molecular events that occur following infection of macrophages *in vitro* have disclosed some interesting features.<sup>13</sup> There is a rapid abrogation of tumor necrosis factor (TNF)- $\alpha$  gene expression and concomitant stimulation of the expression of iNOS and other proinflammatory cytokine genes like interleukin (IL)-1b and cyclooxygenase (COX)-2 after two hours. This is somewhat paradoxical as expression of these cytokine genes is known to be up-regulated by TNF- $\alpha$  in rainbow trout.<sup>14</sup> Moreover, one day after infection, the expression of the TNF- $\alpha$  gene is markedly stimulated and the iNOS gene expression is switched off. Thus, *R. sal* appears to be modulating cytokine gene expression by the infected macrophages in a curious fashion, and more information of this nature should be helpful in understanding how this pathogen subverts the bactericidal mechanisms of the host phagocytes.

Vaccination with standard bacterins has not been very successful. Recently, vaccination with recombinant metalloprotease has induced good protection while recombinant p57 vaccines made the fish more susceptible.<sup>10</sup>

## *Aeromonas salmonicida* subsp. *salmonicida* (*A. sal*)

*A. sal* causes furunculosis in salmonid fish and prior to 1990 caused devastating losses in Atlantic salmon farming but since then has been very effectively controlled by vaccination.

In salmonids, this pathogen is highly virulent and has a multitude of mechanisms for infecting and surviving in the host. While many of the pathogenic mechanisms are known concerning the acute disease, the bacterium can also cause an asymptomatic carrier state and very little is known concerning the host–pathogen relationship in this form of the infection.<sup>15</sup> The major defence factors of *A. sal* against the host can be summarized as follows. The major surface molecules are the A-protein together with lipopolysaccharide (LPS) which constitute the hydrophobic A-layer. This is involved with adhesion to the host, resistance to complement-mediated killing, resistance to phagocyte-killing mechanisms and iron (heme) uptake.<sup>16</sup> *A. sal* can very rapidly invade the Atlantic salmon (AS) epithelial-like cell line and also primary cultures of rainbow trout enterocytes within five minutes of incubation. Most of the host cells are lysed by a 30-minute incubation.<sup>17</sup> Upon contact with fish cells (RTG-2), *A. sal* produces an ADP-ribosylating toxin (AexT), probably after invasion of the cells, causing cell lysis within two hours.<sup>18</sup> Secretion of this toxin has been shown to be via a type III secretion system.<sup>19</sup>

*In vivo*, a polysaccharide capsule is produced which confers resistance to serum killing and, by covering the hydrophobic A-layer, renders the bacterium less able to auto-aggregate which may assist spreading within the host.<sup>16</sup> Outer membrane proteins (OMPS) include iron-regulated OMPS that serve as receptors for siderophore-iron complexes and constitute a high affinity iron-uptake system.<sup>20</sup> Besides siderophores, other substances are exported from the bacterial cell including toxic enzymes such as proteases and glycerophospholipid: cholesterol acyl transferase (GCAT) which digest host tissues and erythrocyte membranes liberating amino acids, fatty acids and heme for growth.<sup>20</sup> Under iron-restricted conditions and exposure to low levels of H<sub>2</sub>O<sub>2</sub>, the bacterium produces a periplasmic manganese superoxide dismutase (MnSOD), in addition to

the constitutive cytoplasmic iron SOD (FeSOD), as well as an inducible cytoplasmic catalase. These enzymes confer resistance to the bactericidal activity of ROS produced by phagocytes during the respiratory burst.<sup>21,22</sup>

Virulence mechanisms of *A. sal* are certainly multifactorial but it is still unclear which are essential ones. It was considered for a long time that both the secreted serine protease and the GCAT toxin were essential virulence factors but using knockout mutants this was shown not to be the case.<sup>23</sup> Certainly the A-layer structure, constituted of the A-protein and LPS, is an essential virulence factor<sup>16</sup> and maybe the AexT is also as a knockout mutant of this gene displayed no toxicity to RTG-2 cells.<sup>18</sup>

### *Piscirickettsia salmonis* (*P. sal*)

*P. sal* is the etiological agent of salmonid rickettsial septicemia (SRS) in salmonid mariculture. It is a Gram-negative obligate intracellular bacterium and must be cultured in fish tissue culture cell lines.<sup>24</sup> The organism is believed to initially target blood monocytes followed by infection of endothelial cells in all organs.<sup>25</sup> No information is yet available concerning the interaction of this pathogen with the host defence mechanisms but the observation of infected monocytes in blood smears<sup>25</sup> would indicate that *P. sal* can survive within professional phagocytes in a similar manner to *R. sal*. The effect of serum factors including antibodies on the ability of *P. sal* to invade host cells has not yet been investigated.

A few vaccination trials have been conducted using whole cell bacterins but with variable results. Sometimes vaccinated fish were more susceptible to challenge,<sup>26</sup> reminiscent of some *R. sal* vaccine trials. Possibly antibodies to certain epitopes of the pathogen may enhance its ability to invade and survive within host phagocytes. Convalescent coho salmon sera recognizes a number of *P. sal* antigens in Western blots including a 17 kD putative outer surface protein OspA.<sup>27</sup> Vaccines prepared from recombinant OspA alone or fused with T-cell epitopes from tetanus toxin and measles fusion protein have shown good protection, up to 83% Relative Percent Survival (RPS) for the latter.<sup>28</sup> The function of the OspA is not known and the protection induced by these vaccines did not correlate with elevated antibody titers to the

OspA. Obviously much more work is needed to elucidate the mechanisms of virulence and interaction of this pathogen with host defences.

### *Flavobacterium psychrophilum* (*F. psychrophilum*)

*F. psychrophilum* is a Gram-negative filamentous yellow-pigmented rod and the causative agent of rainbow trout fry syndrome (RTFS), a septicemic condition causing large mortalities in fry. Older fish appear to be resistant. The major surface structures and antigens have been characterized.<sup>29,30</sup> The bacterium is enveloped in a loosely attached slime layer comprised of low molecular mass (16 kDa) lipopolysaccharide (LPS) and several proteins. High molecular mass LPS is also present on whole cells and abundantly in culture supernatants. Slime layers are considered important virulence factors of some pathogenic bacteria, often associated with attachment to substrates, resistance to phagocytosis and maintaining degradative enzymes in close contact with substrates.

*F. psychrophilum* can survive in normal and immune serum where it depletes complement but curiously it does not grow.<sup>31</sup> The mechanism of the complement resistance is not known but the observation of a polysaccharide capsule by electron microscopy of bacteria *in vitro*<sup>29</sup> and *in vivo* in infected spleen tissue<sup>32</sup> could explain resistance.

The bacterium produces proteases which may account for its invasiveness and pathogenesis. A metalloprotease with 55 kDa, Fpp1, has been characterized.<sup>33</sup> This enzyme cleaved gelatin, laminin, fibronectin, fibrinogen, collagen type IV, actin and myosin — all basic elements of the fish muscular system. *In vitro*, the protease was produced in early exponential phase and was both calcium and temperature dependent being maximally produced at 12°C and in the presence of 10 mM CaCl<sub>2</sub>. These parameters correspond to those in the natural host during outbreaks of RTFS.

The interaction of *F. psychrophilum* with host macrophages has received some attention recently. *In vivo*, following intraperitoneal injection of the bacterium into 1 g (ten weeks old) and 25 g (20 weeks old) rainbow trout fry, the bacteria were rapidly found in the spleen and later in the kidney only of the 1 g fish. In the latter, the percentage

of macrophages isolated from the spleen which contained intracellular bacteria increased from 12 hours (1.5%) up to three days (10%) and the number of bacteria per phagocytic cell similarly increased from four to ten bacteria, and at six days post-infection the number of bacteria/phagocyte had increased to over 70. Using epifluorescence microscopy, all these bacteria were determined to be viable.<sup>34</sup> Hence it would appear that *E. psychrophilum* is able to resist phagocyte killing mechanisms.

*In vitro*, *E. psychrophilum* rapidly associates with trout macrophages and this can be inhibited in the presence of *N*-acetylneuraminic acid (sialic acid) as well as modification of bacterial surface carbohydrates with Na-metaperiodate.<sup>35</sup> This indicates an opsonin-independent adhesion mechanism. No toxicity to the macrophages was detected over two hours of incubation but bactericidal effects were not measured.

The bacterium and its culture supernatants are able to rapidly induce the respiratory burst in trout phagocytes.<sup>36</sup> Using isolated kidney cells comprising 80% macrophages and 20% neutrophils [in a chemiluminescence (CL) assay using luminol to detect H<sub>2</sub>O<sub>2</sub> production] both bacterial cells and culture supernatant induced a peak response in only three minutes and the response declined to zero in 40 minutes. Bacteria opsonized in normal rainbow trout serum induced two peaks, the first at three minutes and then a prolonged production over 40 minutes with a peak at 25 minutes. Strangely, when Zymosan A was added to the phagocytes two hours after the CL run, a second induction of H<sub>2</sub>O<sub>2</sub> production was observed which was similar to that induced in uninfected phagocytes (peak at 12 minutes, duration two to 45 minutes). This is difficult to explain unless the bacteria induce a respiratory burst only in a subpopulation of the phagocytes, possibly only in the neutrophils as this peak was lower and faster than that induced by the Zymosan A. One might speculate that the bacterium can release surface material which is able to induce and exhaust the respiratory burst in neutrophils at remote sites while the cells can avoid inducing the burst in macrophages during phagocytosis. Obviously, more work is required to elucidate the nature of the interaction between this pathogen and host phagocyte responses. The bacterium is catalase-positive<sup>29</sup> which may confer resistance to H<sub>2</sub>O<sub>2</sub> produced by phagocytes.

Injection vaccination using a formalin-killed bacterin in oil adjuvant is highly protective but immersion or injection vaccination without the adjuvant was not.<sup>37</sup> A vaccine based on a partially purified outer membrane fraction of the bacterium induced protection in rainbow trout by injection without the use of adjuvant but immersion delivery was not studied.<sup>38</sup> The nature of the protective antigens and protective mechanisms is not known. As the disease is mainly in fry which are difficult to inject, more research is required to produce a useful commercial vaccine.

## Bacterial Diseases of Warm Water Fish Species

### Gram-positive Pathogens

Diseases of fish caused by Gram-positive cocci have come to prominence over the last 15–20 years, but early isolations date as far back as the 1970s. They are now ubiquitous in aquaculture with outbreaks recorded in the UK,<sup>39</sup> France,<sup>40</sup> Italy,<sup>41</sup> Spain,<sup>42</sup> Israel,<sup>43</sup> South Africa,<sup>44</sup> Korea,<sup>45</sup> Japan,<sup>46</sup> Australia,<sup>44</sup> Taiwan<sup>47</sup> and USA.<sup>48</sup> Diseases have been caused by a number of different Gram-positive cocci, and the history of recording such outbreaks is characterized by confusion over identity of the pathogens, which to a large degree has now been resolved with the advent of sensitive and highly selective molecular techniques. Whilst there remains a great variety of species causing disease, the most frequently isolated, and the most important economically in warm water aquaculture are *Streptococcus iniae* and *Lactococcus garvieae*, and it is therefore these two species which will be discussed in this section.

#### *Streptococcus iniae* (*S. iniae*)

##### *Background*

Originally isolated from the Amazon freshwater dolphin (*Inia geoffrensis*),<sup>49</sup> *S. iniae* has been associated with outbreaks of disease in many species of farmed and wild freshwater and marine fish, including rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis* spp.), hybrid

striped bass (*M. saxatilis* x *M. chrysops*), red drum (*Sciaenops ocellatus*), European sea bass (*Dicentrarchus labrax*), channel catfish (*Ictalurus punctatus*), barramundi (*Lates calcarifer*), Japanese flounder (*Paralichthys olivaceus*) and yellowtail (*Seriola quinqueradiata*). Distribution appears to be worldwide with serious outbreaks reported in Israel,<sup>50,51</sup> the United States,<sup>48,52</sup> Japan<sup>53</sup> and Australia.<sup>54</sup> *S. iniae* has also been reported to cause invasive infections in humans who have handled diseased fish,<sup>55,56</sup> which has provoked a great deal of research into epidemiology and mechanisms of pathogenicity.

### **Identification**

The phenotypic and genotypic variability of *S. iniae* is a key characteristic which has been associated with its ability to cause invasive infection,<sup>56</sup> whether the isolate is from human or fish origin,<sup>57</sup> vaccine success or failure<sup>58</sup> and marine or freshwater origin.<sup>51</sup>

Shortly after the first isolation of *S. iniae* from freshwater dolphin,<sup>49</sup> the same group isolated a second type, also from freshwater dolphin, which differed in its ability to hydrolyze esculin, and was able to ferment lactose but not salicin.<sup>59</sup> Several phenotypic characteristics of *S. iniae* biotypes are similar to Enterococci, for example the wide permissive temperature range (10–45°C), and this has led to confusion in identifications in the past.<sup>60,61</sup> However, the coupling of molecular typing techniques with biochemical and phenotypic studies has clarified the taxonomic position. Generally, Gram-positive cocci, catalase-negative, β-hemolytic, with inhibition of growth by 6.5% NaCl, pH 9.6 and 40% bile forms the basis of identification along with additional results from biochemical tests given in previous publications,<sup>50,62</sup> but some variability amongst other biochemical tests favors confirmation by molecular methods.<sup>48,62,63</sup> Amplification and sequencing of 16s rRNA from approximate nucleotide positions 50–300 including the variable regions V1 and V2 is useful for confirming identity.<sup>48,63</sup>

### **Epidemiology**

Biochemical differences have been demonstrated between human and fish isolates of *S. iniae* based on pyrrolidonyl arylamidase, arginine

dihydrolase,  $\beta$ -glucuronidase activity and fermentation of ribose and glycogen. In spite of the biochemical differences, the same isolates were identical by randomly amplified polymorphic DNA (RAPD) analysis and repetitive primer polymerase chain reaction (REP PCR).<sup>57</sup>

However, by refining the methodology, sensitive molecular techniques can be employed to distinguish the origin of particular isolates. In Israeli aquaculture, there is some debate over whether *S. iniae* has infected cage-reared fish from a reservoir in the wild population on the Mediterranean coast,<sup>64</sup> or whether the infection was introduced to wild marine populations by import of infected fish for culture into the northern Red Sea region.<sup>51</sup> The isolates from the Mediterranean could not be distinguished from the Red Sea isolates by comparison of 16s rRNA sequences, or by amplified fragment length polymorphism (AFLP) or restriction fragment length polymorphism (RFLP) methods<sup>51</sup> and the resolution to this poses some interesting questions on environmental impact of aquaculture.<sup>65</sup> Eldar *et al.*<sup>66</sup> demonstrated differences between US and Israeli tilapia and trout isolates by analysis of RFLP of ribosomal RNA (ribotyping), effectively ruling out the possibility of an epidemiological link between freshwater isolates in the US and Israel. It was also demonstrated that the type strain from *I. geoffrensis* belonged to a different ribotype compared with these fish isolates.<sup>66</sup> RFLP was also used effectively to demonstrate genotypic differences between two *S. iniae* serotypes isolated from farmed fish in Israel prior to and following vaccination.<sup>58</sup>

Specific genetic differences, determined by pulsed-field gel electrophoresis (PGFE),<sup>55</sup> were found in isolates that were able to cause invasive infections in both fish and humans compared to commensal isolates,<sup>56</sup> with virulent isolates falling into one PFGE clone, whilst isolates from non-diseased fish were genetically diverse. These differences were reflected phenotypically by the formation of a granular precipitate by commensal isolates when in suspension, whilst virulent isolates remained buoyant.<sup>56</sup> More recently, however, Lau *et al.*<sup>67</sup> reported that isolates from infected patients in China differed from those in Canada morphologically, and were genetically unrelated by PGFE profile. Thus it would appear that the virulence factors necessary for causing invasive infections are present on more than one PGFE clone.

## *Host-pathogen interactions, virulence and vaccination*

### *Route of entry*

Rather unusually, much work has been carried out on routes of infection of various species of fish by *S. iniae*. Perera *et al.* indicated that infection was possible by immersion and oral route<sup>68</sup> in tilapia. More specific studies have also been performed. Infection via the nares was shown to be effective in inducing disease at low dose rates in tilapia and hybrid striped bass, while infection via the eye did not result in pathology.<sup>69</sup> More recently, the same laboratory demonstrated infection of striped bass via the gill, although mortality was lower compared to infection with similar doses via the nares.<sup>70</sup> Tissue distribution was rapid when high doses were used, spreading to the olfactory system, brain, blood and kidneys within 12 hours of inoculation with  $5 \times 10^6$  cfu, and 48 hours following inoculation with  $2.6 \times 10^6$  cfu.<sup>70</sup> In barramundi, immersion and oral routes of infection have been demonstrated.<sup>71</sup> The oral route resulted in the chronic form of the disease, displaying symptoms identical to those seen in naturally infected fish. Infection by immersion resulted in more acute symptoms, and infectivity was not affected by salinity or skin trauma.<sup>71</sup> In contrast, in Japanese flounder, only very high doses induced mortality when administered orally, whilst low doses induced high mortality by immersion.<sup>72</sup> Thus, infection may be via gills, nares, or orally, but appears to be somewhat species dependent. Clearly immersion infection will infect via all these routes to a degree and this may account for the generally higher infectivity reported following immersion compared to the specific gill, oral or nares routes. It would appear that, at least in barramundi, cutaneous infection is not a significant cause of mortality as skin trauma prior to immersion challenge did not increase infectivity or morbidity.

### *Capsule*

*S. iniae* is a capsulated bacterium with at least two serotypes.<sup>58,73</sup> Serotypic variation appears to result from capsular variation as protein antigens are conserved and cross-reactive between the serotypes. Both serotypes are resistant to killing by normal and immune trout serum<sup>73</sup> and both express a non-immune immunoglobulin binding protein when

grown in trout serum.<sup>74</sup> This protein is able to bind trout immunoglobulin by the Fc region,<sup>74</sup> and may be effective in reduction of opsonization by the immunoglobulin and subsequent phagocytosis, particularly bearing in mind the evidence for the presence of Fc receptors on fish macrophages.<sup>75</sup> In most Gram-positive coccal pathogens studied to date, increased opsonophagocytosis and killing by macrophages, mediated via anti-capsular polysaccharide antibodies appears to be the major means of defence,<sup>76-78</sup> thus reverse binding of immunoglobulin may indeed make an important contribution to the resistance of *S. iniae* to host defences.

### *Hemolysin*

The hemolysin of *S. iniae* is a functional homologue of streptolysin S (SLS), demonstrated by complementation of an SLS negative group A streptococcus (GAS) mutant and inhibition by trypan blue. The hemolysin is regulated by nine genes with high homology to the GAS *sag* operon. Whilst hemolysin appears to be responsible for soft tissue damage and necrosis, it does not contribute to the establishment of septicemia or resistance to phagocytic clearance.<sup>79</sup> Indeed the relationship between *S. iniae* and host phagocytes and other immunocompetent cells is altogether more interesting and has been further studied.

### *Interaction with immunocompetent cells*

Recent studies have shown that *S. iniae* is able to colonize salmonid macrophage-like cell lines and purified trout head-kidney phagocytes.<sup>80</sup> Indeed, in septicemic fish, approximately 70% of the bacterial load in the blood was found in the phagocytes.<sup>80</sup> However, merely colonizing and surviving within phagocytic cells appears to form only part of the strategy for host colonization and subsequent propagation. Virulent *S. iniae* is capable of inducing apoptotic death of infected macrophages.<sup>80</sup> Cells undergoing apoptosis lack immunocompetence, thus induction of programmed cell death is considered advantageous to the invading pathogen as apoptotic cells are phagocytosed without initiating an inflammatory response, with the consequent activation of antigen presenting cells and non-specific defences.<sup>80,81</sup> In tilapia, *S. iniae* is able to modulate apoptotic death of non-specific cytotoxic cells,<sup>81</sup> and the

authors speculated that those isolates that did not increase apoptotic death may not be virulent in fish.<sup>81</sup> Thus it appears that the ability of *S. iniae* to survive in the phagocytic cells, coupled with the induction of apoptosis, enables it to establish infection and even progress from bloodstream to infection of the central nervous system.<sup>80</sup>

### *Vaccination*

Encouraging results following vaccination of trout with *S. iniae* inactivated bacterins has been reported.<sup>82</sup> The vaccines were autogenous, inactivated cultures without adjuvant and were delivered by intraperitoneal injection. Both laboratory and field trials indicated protection (RPS approx 90%) for up to four months following immunization, with antibody titers reaching 1:20–30 one month after immunization falling to 1:1 after six months.<sup>82</sup> Passive transfer of immunity suggested that the antibody response plays a key role in protection against this disease.<sup>82</sup> Under routine field use in Israeli trout farms, the vaccines reduced mortality from in excess of 50% to less than 5%, and increased weight gain in farmed trout by 20%.<sup>83</sup> However, whilst the success of these early vaccinations was encouraging, their dependence on an antibody response to capsular polysaccharide resulted in vaccine failures after two years of use, following the emergence of a different serotype.<sup>58</sup> The novel variant was readily identified by PCR, using RAPD analysis, and was negative for arginine dihydrolase using the API strep system.<sup>58</sup>

In tilapia, the efficacy of combined isolate and single isolate-containing vaccines were compared. Vaccines were prepared using inactivated cultures supplemented with concentrated extracellular products (ECPs).<sup>84</sup> The combined vaccine containing two isolates (ARS10 and ARS60) of *S. iniae* performed well, regardless of the challenge isolate used. When the vaccines containing the single isolate (ARS10) were used, protection was better against the heterologous challenge (ARS60) than the homologous challenge. In light of recent publications on the pathogenicity mechanisms of *S. iniae* in fish,<sup>80,81</sup> this unusual performance may reflect the nature of the strains used. If the ARS10 strain was able to induce apoptosis in host macrophages,<sup>80,81</sup> then increased opsonization and phagocytosis of the apoptotic factor

resulting from the specific antibody may actually reduce efficacy of the vaccine by depleting the resident macrophage population to a degree. In contrast, strain ARS60 may not possess the apoptotic factor and antibodies only against capsular polysaccharide may be protective. Indeed, this effect may have been exacerbated by the inclusion of concentrated ECPs (and therefore putative apoptotic factor) in the vaccine. Thus, whilst speculative, the relative abilities of these two isolates to induce apoptosis in tilapine macrophages requires further investigation in order to clarify the reason for the reduced efficacy of the ARS10 vaccine against homologous challenge. The positive efficacy recorded in these studies is encouraging, although further work is required to determine the extent of strain and serotypic variation in *S. iniae* before routine vaccination programs can be safely designed.

## *Lactococcus garvieae* (*L. garvieae*)

### *Background*

The genus *Lactococcus* was established as a separate genus from *Streptococcus* in 1985.<sup>85</sup> Generally, the lactococci were not associated with disease in humans or animals, although more recent data suggest otherwise,<sup>86</sup> and the original isolation of the *L. garvieae* was made from bovine mastitis.<sup>87</sup> However, “streptococcal” infections in marine fish had been reported for a number of years, predominantly in Japan,<sup>46</sup> and the isolates responsible were subsequently ascribed to a new species, *Enterococcus seriolicida*, based predominantly on phenotypic characteristics.<sup>61</sup> In 1993, the close similarity of *E. seriolicida* to *L. garvieae* isolated from disease outbreaks in trout showing similar symptoms in Europe (Spain and Italy) was determined by biochemistry and 16s rRNA sequence homology.<sup>42</sup> Subsequently, two groups working independently demonstrated that *E. seriolicida* was a junior synonym of *L. garvieae* based on DNA-DNA hybridization, with similarities of 77% under optimum or high stringency.<sup>41,86</sup>

Whilst *E. seriolicida* and *L. garvieae* were shown to be the same species by DNA-DNA hybridization, there are distinct differences. The Japanese

isolates were almost exclusively from marine fish, with the only freshwater isolates coming from eels. In contrast, the European isolates are almost exclusively freshwater, generally from trout.<sup>41,61</sup> Additionally, they are serotypically different when analyzed with rainbow trout antiserum.<sup>88</sup> All the European freshwater isolates fall into one serotype, whilst all the Japanese fall into a second serotype and this serotypic variation is a result of differing polysaccharide capsules.<sup>88</sup> Previously, two serotypes had been reported in Japan, KG+ and KG-. However, it has subsequently been demonstrated that the KG+ serotypes are capsule deficient<sup>78,89</sup> and avirulent.<sup>90</sup> Capsule deficient Japanese isolates are serotypically identical to European capsule deficient isolates.<sup>88</sup>

More recently, *L. garvieae* has been identified by 16s rRNA sequencing and PCR as a significant pathogen of farmed freshwater prawn (*Macrobrachium rosenbergii*) in Taiwan.<sup>47</sup> The serological relationship of these isolates to the Japanese and European isolates has yet to be determined. There, geographical location would suggest a close relationship to Japanese isolates. However, the fact that they were isolated from freshwater prawns suggests that they may be more akin to the European freshwater isolates.

*L. garvieae* can be divided into three distinct groups, based on RAPD analysis:<sup>91</sup> Spanish, Portuguese, English and Turkish isolates formed one group, French and Italian formed a second, whilst Japanese formed a distinct third group.<sup>91</sup> Generally there is high genetic diversity between *L. garvieae* isolates and further work is required to determine where Australian and Taiwanese isolates fit within the group, along with how the genetic variability translates into physical differences.

### *Host-pathogen interactions, virulence and vaccination*

#### *Interaction with the trout immune system*

Interaction with the rainbow trout immune system is now quite well understood. All isolates of *L. garvieae* fix rainbow trout complement, regardless of serotype, or presence of capsule, though virulent capsulate isolates fix less complement than avirulent capsule-negative isolates.<sup>88</sup> Addition of homologous antibody increases complement fixation by

capsulate isolates, but has no effect on complement fixation by capsule-negative isolates.<sup>88</sup> In spite of complement fixation, virulent isolates are resistant to both normal and immune serum.<sup>78</sup> This may result from inhibition of the membrane attack complex by *L. garvieae*, similar to that reported for some species of *Streptococcus*,<sup>92</sup> or it may simply reflect the inability of the bound complement to penetrate the combined thickness of capsule and cell wall.

Resistance to immune serum *in vitro* does not translate to the situation *in vivo*. Passive immunization of rainbow trout with homologous antiserum results in protection against infection by *L. garvieae*.<sup>78</sup> Protection results from increased phagocytosis and killing of *L. garvieae* by rainbow trout macrophages in the presence of immune serum. Opsonization and phagocytosis are not complement dependent as heat-inactivated antisera are as effective as fresh antisera at increasing bactericidal activity of macrophages.<sup>75</sup> This indicates that there may be Fc receptors on rainbow trout macrophages that can interact directly with the Fc region of the bound antibody. This leads to a further interesting faculty of fish pathogenic *L. garvieae*; they are able to bind trout immunoglobulin in the wrong orientation. Both European and Japanese serotypes express proteins that are capable of binding the Fc region of trout immunoglobulin and this may assist in reducing phagocytosis by trout macrophages by effectively preventing interaction with Fc receptors on the macrophage surface.<sup>75</sup>

#### *Interactions with the immune system of yellowtail*

The interactions of *L. garvieae* with the humoral immune system of yellowtail may be similar to those described for rainbow trout; passive immunization with sera raised against capsulated isolates of *L. garvieae* confers strong protection in yellowtail, coupled with rapid bacterial clearance.<sup>93</sup> Studies on the interaction between *L. garvieae* and yellowtail macrophages revealed that capsule decreased opsonophagocytosis, with capsulated (KG2) strains being phagocytosed at half the rate of non-capsulated (KG1) isolates of *L. garvieae*.<sup>94</sup> However, as the study was conducted by microscopy, there was no indication of the ability of the phagocytes to kill the bacteria.<sup>94</sup> In an earlier study, the same authors

showed opsonization with normal yellowtail serum had no effect on chemiluminescent response of yellowtail macrophages against capsulate isolates, but increased the chemiluminescent response against non-capsulated isolates. However, the authors were using non-immune serum for opsonization and thus looking only at the opsonizing effect of complement, not antibody.<sup>89</sup> Studies in rainbow trout indicated that it is antibody, not complement, which is important for opsonization and increased killing of capsulated *L. garvieae* by rainbow trout macrophages,<sup>75</sup> and maybe this is also the case in yellowtail.

#### *Interaction with the immune system of giant freshwater prawns*

The interaction between *L. garvieae* and the giant freshwater prawn (*M. rosenbergii*) has been studied as a tool to measure the effects of various environmental parameters on the ability of *M. rosenbergii* to clear the pathogen, rather than as direct studies on host-pathogen interactions.<sup>95-98</sup> However, these studies do shed some light on what may be responsible for resistance to *L. garvieae* by *M. rosenbergii*. In essence, factors that decrease the prophenol oxidase system [determined by reduction in phenoloxidase (PO) activity], reduce hemocyte numbers and reduce phagocytic activity decrease the resistance of *M. rosenbergii* to *L. garvieae*. Low levels of copper (0.1 mg/ml) were shown to decrease PO activity over the 96-hour assay period,<sup>95</sup> but this did not decrease the resistance of *M. rosenbergii* to *L. garvieae*. Higher levels of copper had a significant effect on susceptibility with greatly increased mortality recorded at 0.4 mg/ml copper. This level of copper exposure was associated with increase in respiratory burst activity, and decreased PO. Another interesting effect was the response of the hyaline cells. In controls, the hyaline cell count increased by almost 50% over the sampling period, reflected identically in the total hemocyte count (THC). However, no such increase was noted in the *M. rosenbergii* exposed to copper.<sup>95</sup> It may be that the copper reduces the ability to respond to stress through increased THC, rather than having a direct effect on the present numbers of hemocytes. Nitrite had no effect on THC or PO activity, but did reduce phagocytic activity, resulting in increased mortality.<sup>96</sup> Additionally, during A, D1/D2 and D3 stages of the moult cycle, increased susceptibility to *L. garvieae* was attributed

to reduced phagocytic activity.<sup>97</sup> Hypoxia and changes in salinity that reduced phagocytic index also reduced resistance, while high resistance to *L. garvieae* was recorded under conditions which reflected high phagocytic efficiency.<sup>98</sup> Indeed, anything which decreases phagocytic activity of hemocytes appears to increase susceptibility to *L. garvieae*, and suggests that phagocytosis rather than PO is most significant in clearing *L. garvieae*, perhaps indicating a strong parallel between the mode of clearance in crustaceans and teleost fish.

### *Virulence factors*

Whilst the capsule is clearly essential for full virulence, data on other putative virulence factors of *L. garvieae* are relatively scant. Some work was conducted on effects of extracellular products (ECPs) from *Streptococcus* spp. (probably *L. garvieae*) on yellowtail.<sup>99</sup> Crude ECPs pre-injected into yellowtail prior to intramuscular challenge with low virulence isolates greatly increased the mortality and clinical symptoms compared with fish which received the challenge alone.<sup>99</sup> In a later study, ECPs were fractionated by gel filtration chromatography and administered orally or percutaneously to determine toxicity. Whilst none were toxic orally, certain fractions induced exophthalmus and petechiae of the gill opercula when given percutaneously. When one of the fractions was given simultaneously with oral challenge with *Streptococcus*, persistence of bacteria in the gut was increased.<sup>100</sup> There was however, no further characterization of the biochemical nature of these fractions.

More recently, surface appendages similar to fimbriae have been observed. In other bacteria, these are associated with attachment and invasion of host cells. However, strains in which fimbriae were identified had a thinner capsule, and whilst more virulent than capsule-negative isolates, were less virulent than thickly capsulated isolates.<sup>101</sup> The role of these appendages in pathogenicity of *L. garvieae* is therefore yet to be determined. Siderophore production has been reported in *L. garvieae* under certain growth conditions,<sup>102</sup> though the nature of the siderophores was not determined. Expression of siderophore was dependent upon nutrient level rather than iron content, in that siderophores were detected in supernatants from iron-depleted medium, and heme-enriched medium,

but not from nutrient-rich medium.<sup>102</sup> The same authors reported expression of novel proteins during *in vivo* growth, but the precise protein profiles could not be replicated in culture. The function of the *in vivo*-expressed proteins remains to be determined.

### *Vaccination*

In rainbow trout, passive immunization studies have shown that antibodies raised against capsulated isolates of *L. garvieae* confer high protection against subsequent challenge, whereas antibodies against non-capsulated isolates do not, and high agglutinating titers were raised against both capsulated and non-capsulated isolates. This suggests that capsule is the key protective antigen in trout isolates as surface proteins of capsulated and non-capsulated isolates have been shown to be identical.<sup>78</sup> In contrast, in yellowtail, protection was achieved regardless of the presence or absence of capsule.<sup>93</sup> Indeed, higher agglutinating titers were achieved when non-capsulated isolates were used, and cross-protection against capsulated isolates was noted.<sup>93</sup> It would appear, therefore, that the protective antigens in the Japanese serotype isolates are not capsular in nature and are located in the cell wall of both capsule-negative and capsule-positive isolates,<sup>93</sup> with the low agglutinating titers recorded for the capsule positive isolates indicating a degree of masking of the antigens by the capsule. In spite of this major difference between the Japanese yellowtail and European trout isolates, it appears that binding of antibody leading to improved phagocytosis and killing by macrophages is the main means of protection against *L. garvieae* in both yellowtail and rainbow trout. However, the nature of the antigens of importance in Japanese serotypes are yet to be determined.

## Gram-negative Pathogens

### *Photobacterium damsela* subsp. *piscicida* (*Ph. damsela*)

#### *Background*

Pasteurellosis, caused by *Ph. damsela*, continues to be one of the most devastating bacterial diseases in world mariculture. The broad host range, ubiquitous distribution, widespread antibiotic resistance and lack of

reliably effective vaccines combine to ensure that *Ph. damselae* remains a major concern for farmers and vaccine companies.

Formerly known as *Pasteurella piscicida* based predominantly on biochemical characteristics and subsequent numerical taxonomy, the phenotypic characteristics of *Ph. damselae* have been reviewed elsewhere.<sup>103,104</sup> Recent advances in the taxonomy of this organism have focused on molecular techniques. Phylogenetic analyses of small subunit rRNA sequences and whole DNA relatedness lead to the organism being reascribed to the family *Vibrionaceae* as *Ph. damsela* comb. nov.<sup>105</sup> then subsequently renamed *Ph. damselae* (addition of terminal “e”) following general correction of epithets in 1997.<sup>106</sup> *Ph. damselae* subsp. *piscicida* may be readily distinguished from *Ph. damselae* subsp. *damselae* through absence of flagella.<sup>105</sup> Serologically, *Ph. damselae* subsp. *piscicida* is highly homogeneous regardless of strain origin,<sup>107</sup> and fatty acid methyl ester<sup>106</sup> and lipopolysaccharide<sup>107</sup> composition are identical. However, distinctions can be made at the molecular level by AFLP<sup>109</sup> and restriction fragment length polymorphism (RFLP)<sup>108</sup> depending on geographical origin of the isolates, with European isolates falling into one molecular subgroup, whilst Japanese isolates fall into a second.<sup>109,110</sup> More recent AFLP studies have revealed Israeli Mediterranean isolates may be distinguished within the European subcluster.<sup>111</sup>

### *Host–pathogen interactions, virulence and vaccination*

*Interaction with host humoral immunity* In yellowtail, specific antibody to *Ph. damselae* has been recorded in cultured fish with peaks early in the season (June/July) and a secondary response through October/November.<sup>112</sup> The authors of this study speculated that the secondary response was protective as disease was lower during this period. However, it is likely that this probably reflected the increased size of the fish in October/November (277–361 g) compared with June/July (20–29 g). Other studies have shown that sea bass and sea bream, for example, are more resistant to experimental infection at this size.<sup>113,114</sup> Furthermore, studies in our own laboratory have shown no protection of survivors from experimentally infected fish during subsequent re-challenge

experiments (unpublished data). Antibody response to *Ph. damsela*e has also been characterized in sea bass (*Dicentrarchus labrax*).<sup>115,116</sup> Following immunization of juvenile sea bass by intraperitoneal injection with inactivated bacterin, primary antibody secreting cell response in the head kidney and spleen occurred at approximately 528 degree-days post-vaccination.<sup>115</sup> The secondary response was both faster and greater in magnitude, in terms of numbers of antibody secreting cells, occurring approximately 150 degree-days following secondary stimulation.<sup>115</sup> By immersion, the response differs, with the primary organ for antibody secreting cells being the gill.<sup>116</sup> Extremely high numbers of antibody secreting cells were detected in fish of 0.1, 2 and 5 g, but significantly higher numbers occurred in the 2 and 5 g fish compared to the 0.1 g fish.<sup>116</sup> The response was also significantly faster in the older group, (5 g), with peak antibody secreting cells in the gills occurring at about 150 degree-days in 5 g fish compared to in excess of 300 degree-days in the younger groups.<sup>116</sup>

In terms of the antigens recognized following immunization, differences have been reported between those recognized by sea bass, compared to mice and rabbits.<sup>117</sup> Furthermore, the inactivation method, or use of live bacteria also leads to detection of differing components.<sup>117</sup>

#### *Interaction with host macrophages*

There are several studies documenting the interactions of *Ph. damsela*e with fish macrophages. In sea bream, peritoneal exudate cells (PECs) isolated from fish of 20–30 g were able to kill *Ph. damsela*e during the first 24 hours of exposure *ex vivo*.<sup>114</sup> However, in smaller fish (0.5 g), the PECs were not able to kill *Ph. damsela*e and the resulting infection was lethal within five days.<sup>114</sup> Similarly, peritoneal macrophages isolated from 200 g sea bream were rapidly bactericidal against *Ph. damsela*e, regardless of the presence or absence of capsule, although capsulated isolates were phagocytosed at a much lower rate.<sup>118</sup> *Ph. damsela*e was also killed by sea bass and rainbow trout macrophages *in vitro*<sup>119</sup> and this may be explained by reports that *Ph. damsela*e is highly susceptible to killing by reactive oxygen species.<sup>119,120</sup> The observations of susceptibility to phagocytosis and killing by phagocytes indicate that

*Ph. damsela* must avoid phagocytosis in order to cause disease in fish. Whilst the capsule provides some resistance to phagocytosis,<sup>118</sup> and there is now strong evidence that this bacterium is capable of colonizing and surviving within non-phagocytic cells,<sup>121,122</sup> perhaps the most intriguing means of avoiding phagocytosis is the recent report of a secreted product which triggers apoptosis in sea bass macrophages.<sup>123</sup>

#### *Virulence factors*

Protease, phospholipase, lipase and hemolysins have been reported as major toxins of *Ph. damsela*.<sup>103</sup> However, the discovery of a polysaccharide capsule<sup>124</sup> and the confirmation of the facultative intracellular survival of *Ph. damsela* in host non-phagocytic cells<sup>121,122,125</sup> have led to the most significant recent advances in our understanding of the pathogenicity of this bacterium.

*Ph. damsela* expresses a periplasmic iron co-factored superoxide dismutase and a cytoplasmic heme catalase.<sup>120</sup> These are constitutively expressed and do not provide protection against exogenous superoxide anion<sup>120</sup> which would be encountered during phagocytosis by fish macrophages. Fish pathogens that are able to withstand phagocytic attack, such as *A. salmonicida*, have an adaptive antioxidant response that can be upregulated upon exposure to reactive oxygen species.<sup>126</sup>

The lack of such an adaptive antioxidant response in *Ph. damsela* is reflected by the fact that it is unable to withstand killing by sea bass or sea bream macrophages.<sup>113,118</sup> Thus *Ph. damsela* has evolved means of avoiding host macrophages. There is now conclusive evidence that *Ph. damsela* is able to invade and colonize non-phagocytic cells.<sup>121,122,125</sup> Internalization within epithelial cells appears to be a specific process as saturation kinetics have been demonstrated, indicating potential competition for cell-surface receptors. Further evidence of the involvement of specific receptors has been reported, including inhibition of invasion by sugars<sup>121</sup> and specific antibody,<sup>122</sup> and the demonstration that heat killing, but not UV killing of *Ph. damsela* may prevent internalization in fish epithelial cells.<sup>17</sup> It has also been shown that the capsule has no effect on invasion of fish cells.<sup>17</sup>

Identification of surface components which may be involved in the internalization process is of major interest. Incubation of EPC and SBL

cell lines with certain sugars inhibited invasion by *Ph. damsela*,<sup>121</sup> suggesting that carbohydrate structures such as glycoproteins on the surface of *Ph. damsela* may be required for interaction with carbohydrate receptors on the host cell surface. In a more recent study, lectins were used to investigate the structures of carbohydrates/glycoproteins on the surface of *Ph. damsela* that may be required for internalization.<sup>127</sup> Lectins which recognized alpha-linked mannose inhibited invasion of SBL cells by up to 50%.<sup>127</sup> However, the most pronounced inhibition of internalization of *Ph. damsela* by SBL cells was noted with *Sophora japonica* agglutinin, which binds beta-configured N-acetyl galactose. In contrast, *Dolichos biflorus* agglutinin, which binds alpha-configured N-acetyl galactose had no effect on internalization. Sea bass antibodies against *Photobacterium* also inhibited invasion.<sup>122</sup> Lectins and antibody, which inhibited internalization were used to stain Western blots of *Ph. damsela* whole cells, outer membrane protein (OMP) fractions or extracellular products (ECP). This study identified two proteins: a 97 kDa OMP and a 52 kDa ECP protein were stained by antiserum, *S. japonica* agglutinin and Con A, but not by *Dolichos biflorus*, suggesting that these proteins may be involved in internalization.<sup>127</sup>

### *Capsular polysaccharide*

An additional means of avoiding the bactericidal effects of the hosts' macrophages may be the antiopsonic exopolysaccharide capsule which has been shown to reduce phagocytosis, but not killing, by sea bream peritoneal macrophages.<sup>118</sup> Indeed, an increase in capsular expression following growth in polysaccharide-rich media resulted in increases in virulence of *Ph. damsela* of up to 4 log.<sup>124</sup> Electron microscopic studies have revealed that expression of capsule is reduced under iron-limited growth conditions<sup>128</sup> and also decreases with age of the culture.<sup>128</sup> The carbohydrate composition of capsular polysaccharide of *Ph. damsela* will to a degree depend upon the growth conditions. However, it is known to contain sialic acid,<sup>129</sup> which may account for its antiopsonic properties. The capsule may have additional roles besides reduction in phagocytosis and it has also been reported to have a minor role in hemin binding

by *Ph. damselae*.<sup>130</sup> Acquisition of iron by *Ph. damselae* is currently not well understood, and although it has been reported that *Ph. damselae* may use hemin or hemoglobin as its sole iron source,<sup>131</sup> failure to detect siderophores by the authors (unpublished) and other workers<sup>132</sup> suggests that other uptake mechanisms must be involved. Indeed, evidence for cell surface protein receptors has been demonstrated as treatment of whole cells with proteinase K-reduced hemin binding.<sup>130</sup> However, even following protease treatment, *Ph. damselae* retained the ability to bind some hemin and this was demonstrated to be by capsular polysaccharide, though with lower affinity than the surface proteins.<sup>130</sup>

### Vaccination

Many potential vaccine strategies have been proposed for *Ph. damselae* and these have been previously reviewed.<sup>103,133</sup> However, few of these ideas have been carried forward to commercialization. One exception is DI21 vaccine produced by Hypra, Gerona, Spain. This vaccine is based on ECP-supplemented formalin-inactivated cultures of *Ph. damselae* and has been reported to be effective in experimental studies in combination with *Vibrio alginolyticus* bacterin.<sup>134</sup> Other vaccines have been described in a recent workshop,<sup>135,136</sup> most of these are based on ECP-enriched or capsular polysaccharide-enriched formalin-inactivated cultures.<sup>136</sup> Efficacy of these types of vaccine is mixed, and duration of immunity is short.<sup>136,137</sup> However, short term protection may be achieved by immersion and oral delivery against immersion challenge<sup>136</sup> but not against challenge by intraperitoneal injection.<sup>136</sup> This probably reflects the stimulation of antibody secreting cells in the gill by this route of administration.<sup>115,116</sup> As this appears to be a major route of entry of the pathogen,<sup>138</sup> these antibodies may prevent colonization. Indeed, antibodies and lectins that bind proteins expressed in OMP and ECP are capable of blocking entry into fish epithelial cells. Upregulating these during culture and using the inactivated cultures as vaccines was effective. Vaccines expressing the 97 and 52 kDa proteins at levels about four-fold above those expressed in exponential TSB cultures as determined by densitometry of SDS-PAGE gels were quite effective when given by immersion, giving RPS of approximately 50% compared to unvaccinated controls in sea bass, and up to 70% in yellowtail against

challenge by intraperitoneal injection.<sup>127</sup> It may be that the ECP-enriched vaccines, which will clearly contain excess of the 52 kDa protein, work in an identical manner.

## *Edwardsiella ictaluri (E. ictaluri)*

### *Background*

*E. ictaluri* is the causative agent of enteric septicemia of catfish (ESC), and represents the most serious disease in the catfish industry in the US.<sup>138</sup> Isolates of *E. ictaluri* are considered to be highly homogeneous in terms of biochemistry and serology,<sup>139</sup> although different serotypes have been reported outside the US.<sup>141</sup> However, there are no reports of molecular typing methods having been used to try to confirm this homogeneity. Plasmids have been sequenced, but this was to determine function rather than as an epidemiological tool.<sup>140</sup> As *E. ictaluri* has been found outside the US,<sup>141,142</sup> clearly there is a need for a more rigorous molecular screening to clarify the epidemiological situation.

### *Host-pathogen interactions, virulence and vaccination*

#### *Interaction with the immune system of channel catfish*

Antibody response to *E. ictaluri* by channel catfish *Ictalurus punctatus* has been characterized and compared with that of blue catfish *I. furcatus*.<sup>143</sup> A significantly higher antibody response was recorded in channel catfish compared to blue catfish following challenge by intraperitoneal injection. No mortalities were recorded in blue catfish, but significant mortality (>80%) was recorded in channel catfish, in spite of the higher titer.<sup>143</sup> The authors speculated that *E. ictaluri* was not cleared as rapidly from channel catfish as from blue catfish, resulting in the high mortality and also high antibody titers. Rapid clearance from blue catfish would preclude the development of a high circulating antibody titer. Comparison of peripheral blood leukocytes isolated from ESC resistant and susceptible families of channel catfish revealed marginally higher percentages of T-lymphocytes in resistant strains.<sup>144</sup>

Additionally, resistant families displayed more macrophage aggregation in the spleen and posterior kidney throughout the challenge period.<sup>144</sup>

The role of non-specific immunity in resistance to *E. ictaluri* in channel catfish is not clear. Feeding with immunostimulants including killed *S. cerevisiae* and  $\beta$ -1, 3-glucan increased neutrophil migration and phagocytosis, but had no effect on resistance to *E. ictaluri* infection.<sup>145</sup> It is likely that *E. ictaluri*, whilst phagocytosed, was not killed by the neutrophils, indeed this has been previously reported.<sup>146</sup> Corroboration of this observation can also be found in an evaluation of the responses of channel catfish neutrophils to *E. ictaluri*.<sup>147</sup> In this study, neutrophils were clearly demonstrated to phagocytose *E. ictaluri*, however no intracellular killing was detected. In the presence of complement and serum, extracellular killing of *E. ictaluri* was observed, but there was no killing by neutrophils in the absence of these serum components.<sup>147</sup> In contrast, in an earlier study, killing of *E. ictaluri* by channel catfish peripheral blood mononuclear phagocytes *in vitro* was recorded. The killing pathway would appear to be oxidative as addition of superoxide dismutase reduced intracellular killing of *E. ictaluri* by 30–40%.<sup>148</sup> Both antibody and complement appeared to have a role in phagocytosis and activation of oxidative defences by phagocytes as determined by induction of chemiluminescence, in response to *E. ictaluri*.<sup>148</sup> The induction of chemiluminescence was increased by complement in the absence of specific antibody. Peritoneal macrophages would also appear to be bactericidal against *E. ictaluri*. Macrophages from both susceptible and immune catfish were bactericidal, but at high ratios of bacteria to macrophages, cells from immune populations were significantly more lethal.<sup>149</sup> The killing activity of macrophages from immune fish was further enhanced by opsonization with immune serum. In contrast, opsonization with immune serum significantly reduced the killing activity of macrophages from susceptible fish.<sup>149</sup> This may reflect the accelerated rate of phagocytosis of the opsonized bacteria. Whilst the highly bactericidal macrophages from the immune population are able to deal adequately with the higher numbers of phagocytosed bacteria, the less bactericidal macrophages from the susceptible fish may become rapidly over-run.

### *Virulence factors*

*E. ictaluri* expresses long chain O-polysaccharides on its cell surface which is the immunodominant antigen.<sup>150</sup> An isogenic mutant of *E. ictaluri* which was unable to express the O-polysaccharide side-chain was avirulent in catfish<sup>151</sup> and the authors concluded that O-antigen was essential for virulence of *E. ictaluri*. Subsequent characterization of the O-polysaccharide mutant revealed that it was marginally but significantly more susceptible to killing by normal catfish serum,<sup>152</sup> but retained its resistance to killing by neutrophils. The authors also demonstrated that the increased susceptibility was not a result of cationic peptides in the serum as the mutant was no more sensitive to polymyxin B than the wild type.<sup>152</sup>

Significantly, *E. ictaluri* has been shown to be efficiently invasive in a range of mammalian and fish cell lines, and harvested catfish intestinal cells.<sup>153</sup> Mechanisms of invasion were investigated in mammalian cell lines and a role for actin polymerization was proposed based on inhibition of invasion by cytochalasin D to 0.03% compared to untreated controls.<sup>153</sup> Additionally, monodansylcadaverine (MDC), an inhibitor of receptor-mediated endocytosis, significantly reduced invasion (treated 0.04%, compared to 100% in untreated controls).<sup>153</sup> Colchicine, an inhibitor of microtubule formation, had no significant effect on internalization of *E. ictaluri*.<sup>153</sup>

### *Vaccination*

Vaccination of catfish against *E. ictaluri* was reviewed in 1997.<sup>138</sup> This review focused predominantly on killed vaccine and subunits, but highlighted the fact that protection was not correlated with circulating antibody response.<sup>138</sup> The facultative intracellular nature of this pathogen gives credence to the idea that cell-mediated immunity may be more important in pathogen clearance.<sup>138,153</sup> Subsequent to this review, live attenuated vaccine technology has been substantially explored,<sup>154-157</sup> resulting in the use of a licensed product in catfish farms in the US (AQUAVAC-ESC, Intervet). The first attenuated vaccine study in *E. ictaluri* involved use of a *purA* mutant which is auxotrophic for adenine.<sup>154</sup> The mutant, which was 5 log<sub>10</sub> less virulent than the wild type strain, retained its invasive capabilities and was able to persist in

catfish tissues for at least 48 hours. Adenine auxotrophs have not routinely been used in mammalian systems as the extremely low levels of adenine in mammalian tissues preclude the survival of the mutant for sufficient duration to elicit an immune response.<sup>158</sup> However, purine metabolism in fish differs from mammals and may afford the longer survival of the adenine auxotroph observed in the trials.<sup>154</sup> Protection offered by this attenuated vaccine was low, but significant.<sup>154</sup>

A modified live *E. ictaluri* vaccine has shown greater efficacy in catfish.<sup>155–157</sup> The RE-33 mutant is reported to be effective regardless of the catfish family.<sup>155</sup> It has also been used to vaccinate juveniles as young as seven days post-hatch. At this early age, mortalities in non-vaccinates reached 30%–36% following challenge by immersion, about 600 degree-days post-vaccination. In vaccinates, mortalities ranged between 8.1% and 12.8%, equivalent to RPS between 58% and 77%.<sup>156</sup> The same vaccine has also been used to vaccinate eyed-eggs of channel catfish.<sup>157</sup> Incredibly, single immersion vaccination *in ovo* resulted in high relative percent survival (87.9%) when challenged by immersion about 750 degree-days post-vaccination. The immune mechanisms which may be operating at this early age are unknown. Interestingly, boosting the fish vaccinated *in ovo* seven days post-hatch resulted in a significant decrease in survival compared to fish that only received the primary vaccination as eyed eggs.<sup>157</sup>

Subunit vaccines against ESC in catfish have also been investigated.<sup>159</sup> Proteins expressed during infection of catfish by *E. ictaluri* were identified and cloned. These were delivered as recombinant subunits expressed in *E. coli*. However, whilst all vaccines gave high protection against challenge, the empty vector control resulted in the highest protection with an RPS of almost 90%. Thus the efficacy of such vaccines remains in question. Recently, surface proteins, identified by biotinylation and subsequent extraction by affinity chromatography using a streptavidin column, have been investigated for their potential role in internalization.<sup>160</sup> Antibodies raised in rabbits against these proteins excised from SDS-PAGE gels were able to significantly inhibit internalization of *E. ictaluri* in fathead minnow cell lines.<sup>160</sup> It may be that these are good candidates for potential subunit vaccines as antibodies able to block internalization may be protective in the early stages of infection.

Attenuated and subunit vaccines have been explored for their ability to elicit cell-mediated immunity against this facultatively intracellular pathogen. However, the immune response can be enhanced by adjuvanting. Indeed, cholera toxin (CT) and its non-toxic B-subunit (CTB) have been used to increase mucosal immunity.<sup>161</sup> *E. ictaluri* whole cells were conjugated to CT or CTB using N-succinimidyl 3-(2-pyridyldithio) propionate. Conjugation to both CT and CTB significantly increased the antibody response when given intraperitoneally, compared to *E. ictaluri* alone, or conjugated to CT or CTB independently. There was no effect of cholera toxin adjuvant on antibody response when given by the oral or rectal route. Whilst the live attenuated vaccines look promising in the laboratory, further research is required into other types of vaccine and potential adjuvants that may be delivered by immersion or oral route to juvenile catfish.

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