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ScienceDirect
Veterinary Microbiology 131 (2008) 145–153
veterinary microbiology
www.elsevier.com/locate/vetmic

Strain-associated virulence factors of *Streptococcus iniae* in hybrid-striped bass

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Received 31 July 2007; received in revised form 24 February 2008; accepted 26 February 2008

Abstract

Streptococcus iniae is a major fish pathogen producing invasive infections that result in economic losses in aquaculture. Development of in vitro models of *S. iniae* virulence may provide insight to the pathogenesis of infection in vivo. Three *S. iniae* strains (K288, 94-426, and 29178) were tested for virulence in a hybrid-striped bass (HSB) model using intraperitoneal injection. *S. iniae* strains K288 and 94-426 caused high levels of mortality in HSB (lethal dose 2×10^5 CFU) while strain 29178 was avirulent even upon IP challenge with 1000-fold higher inocula. In vitro assays were developed to test for the presence of characteristics previously associated with virulence in other species of pathogenic *Streptococcus* in animals and humans. In vitro differences relevant to virulence were not detected for beta-hemolysin activity, sensitivity to antimicrobial peptides, or adherence and invasion of epithelial cell layers. However, in whole-blood killing assays, the pathogenic strains were resistant to blood clearance, while 29178 was cleared ($P < 0.001$) and more sensitive to complement ($P < 0.001$). The avirulent strain 29178 was most efficiently phagocytosed and was most susceptible to intracellular killing ($P < 0.01$) by the carp leukocyte cell line (CLC). When exposed to reactive oxygen species, strain 29178 was most susceptible. When the oxidative burst of CLC cells was inhibited, intracellular survival of 29178 was rescued fivefold, while no significant enhancement in survival of K288 or 94-426 was detected. Our results indicate that resistance to phagocytosis, oxidative killing, and associated phagocytic clearance is a significant factor in *S. iniae* virulence.

Keywords: *Streptococcus iniae*; Virulence; Hybrid-striped bass; Oxidative killing

1. Introduction

Disease outbreaks have become a significant hurdle to the profitable culture of fish and shellfish. Streptococcal infections in fish, in particular those

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doi:10.1016/j.vetmic.2008.02.027

produced by the pathogen *Streptococcus iniae*, have increased markedly with intensification of aquaculture practices. *S. iniae* is associated with large-scale mortality in a wide variety of marine and freshwater cultured and wild fish species (Bercovier et al., 1997; Agnew and Barnes, 2007). Clinical symptoms of *S. iniae* infection in fish include loss of orientation, lethargy, ulcers, and exophthalmia, culminating in a fatal meningoencephalitis (Agnew and Barnes, 2007). Occasionally, *S. iniae* can cause serious zoonotic infections in humans injured while handling infected fish (Lau et al., 2006; Weinstein et al., 1997). Compared to strains isolated from diseased fish, the type strain of *S. iniae* (Pier and Madin, 1976) has not been linked to disease in fish.

As in higher vertebrates, the initial response to bacterial infection in fish is through the innate immune response (Ellis, 2001). Both humoral and cellular components of fish innate immunity are largely shared with higher vertebrates (Neumann et al., 2001; Magnadottir, 2006). Environmental factors can influence the activity of innate immune parameters in fish (Magnadottir, 2006), rendering fish in intensive culture systems susceptible to the rapid spread of infection. For human streptococcal pathogens, identification of reliable in vitro assays recapitulating key steps in disease pathogenesis and the innate immune response has helped elucidate and rank a complex array of virulence factors unique to each species. Similar assays may help elucidate virulence determinants of *S. iniae*.

In this work, we examine three strains of *S. iniae* with varying levels of virulence in hybrid-striped bass. We develop in vitro assays reflecting several steps in *S. iniae* disease progression and apply these assays to characterize the three strains. This analysis allows us to draw inferences regarding the virulence characteristics of *S. iniae* important for development of fish infection, and implicates resistance to phagocytic killing as a component of *S. iniae* pathogenicity.

2. Methods

2.1. Bacterial strains and growth conditions

The three *S. iniae* strains compared were K288, obtained from the brain of a diseased hybrid-striped bass, strain 94-426, obtained from the brain of a diseased tilapia, and the ATTC type strain 29178, originally isolated from a fresh-water dolphin abscess. Unless otherwise noted, all *S. iniae* strains were cultured overnight at 30 °C in Todd Hewitt broth (THB) (Hardy Diagnostics, Santa Maria, CA), then diluted 1:20 in THB and grown to mid-logarithmic phase ($OD_{600} = 0.4$ or $\sim 3 \times 10^8$ CFU/ml). For each assay, the number of viable bacteria was verified by drop-plating serial dilutions on THB agar (THA) and enumerating CFU after overnight incubation at 30 °C.

2.2. Fish challenge

Comparative in vivo virulence analysis of strains K288, 29178, and 94426 was performed using an infection challenge of juvenile (~30 g) hybrid-striped bass (*Morone chrysops* × *Morone saxatilis*) (HSB). Fish were maintained at 25 °C in ~75 l flow-through tanks. Mid-log-phase bacteria were pelleted, resuspended and diluted in Ca^{++} and Mg^{++}-free phosphate buffered saline (PBS) (Invitrogen, Carlsbad, CA). Groups of 20 fish were injected intraperitoneally (i.p.) with either PBS alone (control) or 3×10^5 CFU of each *S. iniae* strain in a 100-μl volume. In the same experiment, groups of 20 fish were injected i.p. with strain 29178 at higher inocula from 3×10^6 to 3×10^8 CFU. *S. iniae* were cultured from the brain of all moribund or dead fish, confirming *S. iniae* infection of the central nervous system. Survival was monitored for 14 days. All fish challenges were carried out in at the ALAAC certified Kent SeaTech research facility following IACUC approved protocols.

2.3. Cell lines and culture conditions

The adherent carp (*Cyprinus carpio*) monocytic/macrophage cell line CLC (European Collection of Cell Cultures no. 95070628) (Faisal and Ahne, 1990) is a well-characterized cell line (Weyts et al., 1997) and was used to investigate the interaction of the *S. iniae* strains with phagocytes, The white bass (*M. chrysops*) embryonic epithelial cell line (WBE27) (Shimizu et al., 2003) was used to investigate the ability of the *S. iniae* strains to interact with epithelial layers. Both cell lines were maintained at 28 °C and

5% CO_2 using high glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen).

2.4. Hemolysin assay

To measure relative hemolytic activity (Fuller et al., 2002), *S iniae* strains were grown to log phage, serially diluted in THB in a 96-well plate, and mixed with a 2% solution of PBS-washed sheep erythrocytes in a final volume of 200 μl. After 1 h at 30 °C, the plate was incubated at 4 °C for 30 min and centrifuged at 3000 rpm for 5 min. One hundred microliters of the supernatant was transferred to a new 96-well plate and absorbance at 590 nm measured as an indicator of the relative amount free hemoglobin released upon erythrocyte lysis.

2.5. Adherence and invasion assays

Adherence and intracellular invasion studies were carried out in 96-well tissue culture plates (Costar, Lowell, MA) coated with collagen prior to seeding WBE27 cells at 1×10^5 cells per well at 30 °C. Cells were allowed to adhere overnight. For invasion assays, medium was replaced with high glucose DMEM with 2% heat-inactivated FBS and the bacteria were added at multiplicity of infection (MOI) = 10 bacteria/tissue culture cell. Samples were centrifuged to bring bacteria in contact with the fish cell monolayer, and the plates incubated for 30 min at 28 °C with 5% CO_2. The fish cells were then washed twice with PBS and incubated in fresh medium plus bacteriocidal concentrations of antibiotics (20 μg/ml penicillin + 200 μg/ml gentamicin) for 2 h to kill extracellular bacteria. The fish cells were then washed twice with PBS and lysed using 100 μl of 0.01% Triton X-100 (Sigma–Aldrich, St. Louis, MO). Dilutions were plated on THA to determine the number of intracellular CFU. Adherence assays were carried out in a similar manner, but the antibiotic treatment step was omitted. Additionally, monolayers were washed five times with PBS at the 30 min timepoint before lysing the fish cells and plating to enumerate total cell-associated bacteria. Triton X-100 (0.01%) had no effect on the viability of strains K288, 94-426, or 29178.

2.6. Blood survival assay

Blood survival assays were similar to a previously published study (Buchanan et al., 2005). Mid-log-phase *S. iniae* suspensions of ~100 CFU in 100 μl PBS were added to 300 μl of fresh heparinized (sodium heparin, Sigma–Aldrich, St. Louis, MO) blood from three different HSB in 2-ml siliconized plastic tubes and incubated for 1 h at 30 °C on an orbital shaker. After 1.5 h, 100 μl aliquots were taken from each sample in duplicate and plated on THA for enumeration of surviving bacteria.

2.7. Antimicrobial peptide assay

Antimicrobial peptide assays were based on a previously published study (Buchanan et al., 2005). In individual wells of a 96-well microtiter plate, *S. iniae* strains were grown to log-phase, and 2×10^5 CFU in THB were exposed to final concentrations of 1.5 μM moronecidin or 80 μg/ml polymixin B (Sigma–Aldrich, St. Louis, MO) in quadruplicate at 30 °C. Serial dilutions of the well contents were plated at various timepoints (0–120 min) to enumerate surviving bacterial CFU. All three strains were tested against both antimicrobial peptides.

2.8. Complement-mediated killing assay

To measure the ability of bass complement to kill *S. iniae* strains, 3 ml of blood was collected from each of four HSB, allowed to clot at 4 °C for 1.5 h and centrifuged at 3000 × *g* at 4 °C for 10 min. The serum was collected and centrifuged again. Half of the serum sample was heat inactivated at 60 °C for 30 min. 1×10^5 CFU of *S. iniae* were washed, resuspended in PBS and added to 150 μl of active or heat-inactivated serum. Samples were mixed and incubated at 22 °C for 2 h and aliquots plated for enumeration of surviving CFU.

2.9. Intracellular survival assays

Intracellular survival assays were carried out in a similar manner to invasion assays. Diphenyleneiodonium chloride (DPI) (Sigma–Aldrich, St. Louis, MO) was used to block the oxidative killing mechanisms of leukocytes (Liu et al., 2005). CLCs were seeded at a

density of 10^5 cells per well in DMEM with 2% heat-inactivated FBS at 30 °C. Ninety minutes prior to addition of bacteria to the fish cells, the medium was replaced in half of the wells with DMEM + 2% heat-inactivated FBS ± 10 μM DPI. Log-phase *S. iniae* were added to the wells at MOI = 10, plates centrifuged, then incubated for 30 min at 28 °C in 5% CO_2. Wells were then washed with PBS and the medium replaced with DMEM medium +2% heat-inactivated FBS + 20 μg/ml penicillin +200 μg/ml gentamicin, maintaining the presence or absence of 10 μM DPI. Wells were incubated at 28 °C with 5% CO_2 for 5 h. At 5 h, cells were washed twice with PBS and lysed with 100 μl of 0.1% Triton X-100. Well contents were plated on THA to enumerate the number of intracellular surviving bacteria.

2.10. Fluorescence assay for rate of phagocytosis

CLCs were seeded on collagen-coated 96-well plates at a density of 10^5 cells per well at 30 °C. *S. iniae* strains were grown to log-phase and incubated with a final concentration of 100 μg/ml of fluorescein isothiocyanate (FITC) (Invitrogen, Carlsbad, CA) for 1 h. Bacteria were resuspended in DMEM +2% FBS ± 10 μM DPI and added to the cells at MOI = 10. Samples were centrifuged as above and incubated for 20 min. The medium was then replaced with DMEM +2% FBS supplemented with 4 μM moronecidin (to kill extracellular bacteria), incubated for an additional 10 min at 28 °C, washed with PBS, the medium replaced with DMEM +2% FBS +0.5 μM Sytox Orange (Invitrogen, Carlsbad, CA), followed by incubation at 28 °C in the dark × 10 min. Wells were visualized with a Zeiss Axiovert inverted microscope at 320× using standard rhodamine and FITC filter sets. Images were captured with an integrated CCD camera (Carl Zeiss, Thornwood, NY) and the Axiovision software package (Carl Zeiss). Using this protocol, phagocytosed intracellular bacteria retained their FITC label and appeared green. Remaining extracellular bacteria were permeabilized with moronecidin, labeled with DNA stain Sytox Orange, and appeared red. For counts of number of phagocytosed bacteria, the same protocol was followed without FITC-labeled bacteria or use of Sytox Orange. After 30 min incubation, cells were washed twice with PBS and lysed with 100 μl of 0.1% Triton X-100. Well contents were plated on THA to enumerate the number of intracellular bacteria.

2.11. Reactive oxygen assays

Tests for susceptibility to oxidants were performed in PBS. 2×10^6 bacteria were incubated for 2 h in 0.03% hydrogen peroxide (H_2O_2) (Sigma–Aldrich, St. Louis, MO) at 30 °C. One thousand U/ml of catalase (Sigma–Aldrich, St. Louis, MO) were added to quench H_2O_2 at the end of the assay. Dilutions were plated on THA to determine the number of surviving CFU. To measure sensitivity to singlet oxygen, 1×10^7 bacteria were incubated in individual wells of a 24-well plate in the presence or absence of 2 μg/ml methylene blue and placed exactly 20 cm from a 100-W light source (Liu et al., 2005). Bacterial viability was assessed after 1 h by plating dilutions on THA. Control plates handled identically but wrapped in foil or exposed to light in the absence of methylene blue did not show evidence of bacterial killing.

2.12. Statistical analysis

Data analyses were performed using the statistical tools included with GraphPad Prism 5 (GraphPad Software, San Diego, CA). In vitro assay data were analyzed using ANOVA followed by Tukey post hoc tests for differences between strains. Fish survival after bacterial challenge was analyzed using a Logrank Test. For all tests, $P < 0.05$ was considered statistically significant. In vitro assays were set up in quadruplicate for each treatment, and the assay was repeated three times to confirm results; data presented (mean ± standard error of the mean, S.E.M.) are from a single representative assay.

3. Results

3.1. S. iniae strains vary markedly in fish virulence

An established HSB challenge model (Buchanan et al., 2005) was used to compare virulence of three stains of *S. iniae* at an i.p. challenge dose of 3×10^5 CFU. Strain 94426 was rapidly lethal, producing 100% mortality by day 3. Strain K288

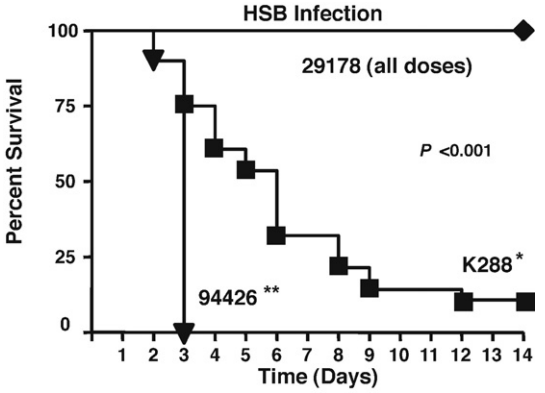

Fig. 1. Strain specific differences in virulence. Hybrid-striped bass (groups of 20) were injected intraperitoneally with 3×10^5 CFU of strains K288 or 94426, or with 3×10^5, 10^6, or 10^7, or 10^8 of strain 29178. Kaplan–Meier survival plot indicates in vivo attenuation of the strain 29178 ($P < 0.001$). Treatments sharing an asterisk were not significantly different ($P < 0.05$).

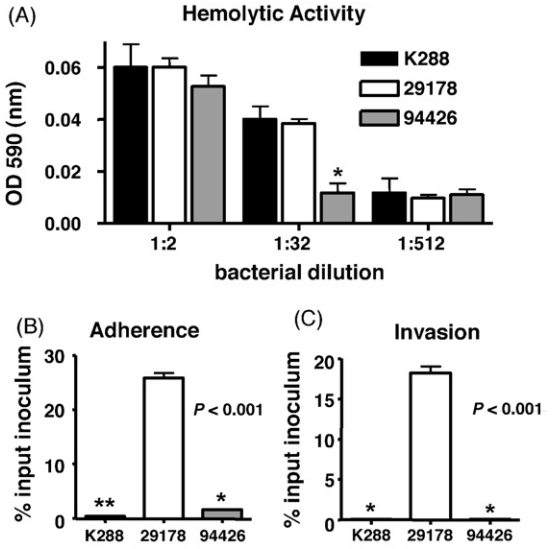

Fig. 2. (A) Hemolytic activity of *S. iniae* strains K288, 29178, and 94426. Values reported are mean hemoglobin release from sheep blood measured at 590 nm by spectrophotometer ± S.E.M. (B) Strain 29178 is more adherent and (C) invasive of fish epithelial cell line WBE27. Values reported are percentage of bacterial cells that have adhered to or invaded WBE27 cells ± S.E.M. Treatments sharing an asterisk were not significantly different ($P < 0.05$).

was also highly virulent, but fatal meningoencephalitis developed more slowly, with 50% lethality by day 5 and 90% lethality at day 14. In striking contrast, no mortality was seen in fish challenged with 29178, the type strain of this pathogen (Pier and Madin, 1976), at the equivalent challenge dose or doses up to 1000-fold greater, throughout the 14-day observation period ($P < 0.001$, Fig. 1). No mortalities occurred in PBS injected controls.

3.2. Loss of virulence in strain 29178 is not associated with decreased hemolytic activity, cellular adherence, or intracellular invasion

Production of a pore-forming β-hemolytic cytotoxin is a feature *S. iniae* shares with the human pathogens *Streptococcus pyogenes* and *Streptococcus agalactiae*, and isogenic mutants of each species lacking toxin production show attenuated virulence in animal models of infection (Fuller et al., 2002; Liu et al., 2004; Datta et al., 2005; Locke et al., 2007b). However, we found avirulent *S. iniae* strain 29178 was no less hemolytic than the virulent strains K288 and 94426 (Fig. 2A). Another phenotype correlated with diminished virulence in clinical isolates of *S. pyogenes* and *S. agalactiae* is the reduced ability for adherence and intracellular invasion of host epithelial cells (LaPenta et al., 1994; Valentin-Weigand et al., 1997; Tyrrell et al., 2002). Using a cell culture model, we found the avirulent *S. iniae* strain 29178 was significantly more adherent and invasive to fish epithelial cells than the two virulent strains (Fig. 2B and C). Thus, the inability of strain 29178 to cause disease in fish does not appear to be related to deficiencies in toxin production or the ability to adhere to or invade fish cells.

3.3. Avirulent strain 29178 is readily killed in fish blood and serum and susceptible to phagocytic uptake

Gene deletion strains of *S. iniae*, *S. pyogenes* or *S. agalactiae* with diminished ability to survive in fresh blood show reduced animal virulence (Liu et al., 2004; Datta et al., 2005; Buchanan et al., 2005; Locke et al., 2007a). Using ex vivo assays, we found that the avirulent strain 29178 is remarkably sensitive to killing in whole blood ($P < 0.001$) compared to virulent strains (Fig. 3A). Whole blood killing reflects combined antibacterial activities of serum and circulating phagocytic cells types such as neutrophils or macrophages. Similarly, while virulent strains were

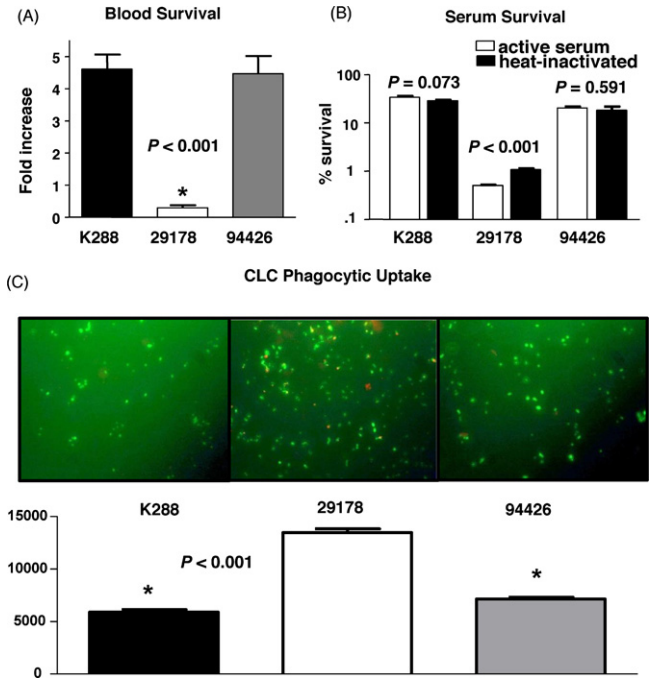

Fig. 3. (A) Survival of strain 29178 is significantly decreased compared to strains K288 and 94426 following 1 h incubation in whole fish blood. Values reported for A–C are mean ± S.E.M. (B) Significant differences in survival in heat-inactivated serum compared to active serum were detected only for strain 29178. (C) The rate of phagocytosis as measured by incubating *S. iniae* strains for 30 min on a monolayer of a carp macrophage cell line (CLC) at an MOI of 10. Fluorescence imaging (400×) shows phagocytosed intracellular bacteria labeled with FITC (green) and adherent extracellular bacteria labeled with Sytox Orange (red). The greater number of green-labeled bacteria for strain 29178 indicates more bacteria from this strain were phagocytosed. Results were equivalent with and without the addition of diphenyleneiodinum chloride (DPI); pictures presented here are with DPI. Wells were prepared in the same manner for counts of the number of bacteria phagocytosed after 30 min. Treatments sharing an asterisk were not significantly different ($P < 0.05$).

resistant to complement-mediated killing, increased susceptibility was observed in the avirulent strain ($P < 0.001$, Fig. 3B). Finally, fluorescence-based analysis and quantitative counts revealed avirulent strain 29178 to be significantly ($P < 0.001$) more susceptible to phagocytic uptake by the CLC fish macrophage cell line than either of the virulent strains K288 and 94426 (Fig. 3C). Thus, increased blood survival of virulent *S. iniae* strains may reflect relative resistance to both complement and phagocyte mediated clearance mechanisms.

3.4. Avirulent strain 29178 is susceptible to reactive oxygen species and rapidly killed within macrophages

Lastly, we sought to determine whether strain differences were apparent in the response to intracellular effectors of phagocyte killing. Cationic antimicrobial peptides produced in phagocytes are an important part of the innate immune system of higher animals, but, interestingly, the avirulent strain 29178 was more resistant to the HSB defense peptide moronecidin than the two virulent *S. iniae* isolates (Fig. 4A); similar results were found with the cationic antimicrobial peptide of bacterial origin, polymyxin B (Fig. 4A). Another key mechanism of phagocyte intracellular killing is the generation of reactive oxygen species (“oxidative burst”). The avirulent strain 29178 was found to be markedly more susceptible to killing by hydrogen peroxide ($P < 0.001$) and singlet oxygen ($P = 0.0281$) than the two virulent strains K288 and 94426 (Fig. 4B). The rapidity of killing within CLC macrophages was assessed by calculating the percent surviving intracellular CFU 5 h after phagocytosis. Strain 29178 was phagocytosed at a high rate (Fig. 3C)

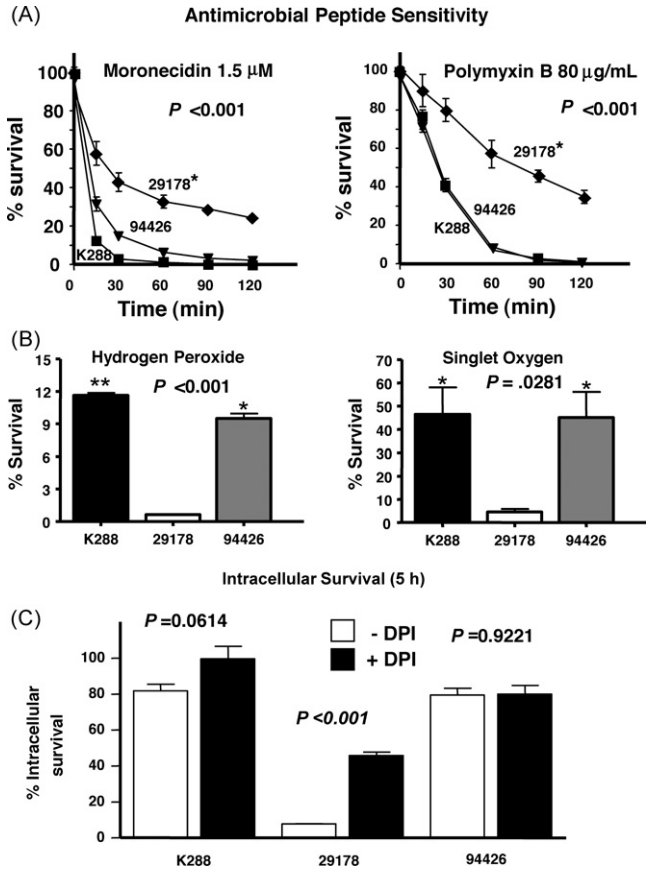

Fig. 4. (A) Antimicrobial peptide kinetic killing profiles of 1.5 μM moronecidin and 80 μM polymyxin B indicate decreased sensitivity to AMPs for strain 29178. Values reported for A–C are mean ± S.E.M. (B) Strain 29178 was significantly more sensitive to hydrogen peroxide (2 h exposure) and singlet oxygen (1 h exposure) compared to strains K288 and 94426. (C) Intracellular survival of phagocytosed bacteria in a carp macrophage cell line (CLC) was determined after 5 h incubation. Values reported are the percentage of surviving bacteria after 5 h compared to the number of bacteria phagocytosed measured after 30 min. The addition of DPI significantly increased survival of only strain 29178. Treatments sharing an asterisk were not significantly different ($P < 0.05$).

and found to be rapidly killed intracellularly (95% clearance) compared to the two pathogenic strains (~20% clearance) (Fig. 4C). The intracellular killing of strain 29178 was significantly reduced by addition of the oxidative burst inhibitor DPI ($P < 0.001$). Thus, resistance to oxidative killing appears to be a significant contributor to *S. iniae* intracellular survival, resistance to phagocytic killing, and virulence.

4. Discussion

Although *S. iniae* is a pathogen of economic importance, relatively little is known regarding mechanisms of disease pathogenesis and few specific virulence determinants have been established. These include the enzyme phosphoglucomutase which contributes to cell wall integrity and resistance to antimicrobial peptides (Buchanan et al., 2005); the polysaccharide surface capsule which impairs phagocytic uptake (Locke et al., 2007a; Miller and Neely, 2005), and the pore-forming cytolysin streptolysin S (Locke et al., 2007b). Correlative studies also suggest the ability to invade host cells, an affinity for macrophage uptake, and the ability to persist within macrophages can be linked with increased *S. iniae* virulence (Zlotkin et al., 2003). Here, we report findings supporting relative resistance to phagocytic

uptake and oxidative killing as important components of virulence for *S. iniae*.

In a survey of possible differences between virulent and avirulent strains of *S. iniae*, we found avirulent strain 29178 had equivalent streptolysin S activity, enhanced resistance to antimicrobial peptides, and increased epithelial cell adherence and invasion compared to two virulent strains. Thus the proven or suspected virulence phenotypes of cytolysin production (Locke et al., 2007b), antimicrobial peptide resistance (Buchanan et al., 2005) and cellular invasion (Zlotkin et al., 2003) were not implicated in the marked virulence attenuation of strain 29178, which cannot kill fish even at challenge doses 1000-fold greater than the LD90 for the pathogenic strains. Rather, our in vitro model systems suggest deficiencies in the ability of strain 29178 to resist reactive oxygen species, complement killing, and phagocytic uptake translate to diminished blood survival and marked virulence attenuation.

Increased susceptibility to complement-mediated killing potentially explains a portion of the decreased virulence in strain 29178. However, most striking was the sensitivity of the avirulent strain to oxidative killing and reduced ability to avoid uptake by phagocytic cells. Oxidative killing through generation of reactive oxygen or nitrogen species is a key method utilized by phagocytic cells such as macrophages and neutrophils for neutralization of bacterial pathogens (Ellis, 2001; Neumann et al., 2001). The virulence of some strains of bacteria is linked to the ability to resist oxidative killing, allowing survival inside the phagocyte and enhanced pathogenesis (Liu et al., 2004; Liu et al., 2005). Given the approximately 10-fold increased susceptibility to reactive oxygen species in the avirulent strain, the capacity of virulent *S. iniae* to survive in host cells may provide a mechanism whereby the organism can gain access to deep tissues, blood, or the central nervous system.

In addition to resistance to oxidative killing, avoidance of phagocytosis is a well-described mechanism by which bacteria escape immune clearance (Segura et al., 2004; Finlay and McFadden, 2006), In *S. iniae*, capsule deficient isogenic mutants have been shown to have dramatically reduced ability to avoid phagocytosis and decreased virulence in fish (Locke et al., 2007a; Lowe et al., 2007). The ability of the virulent strains studied in this work to avoid phagocytic uptake compared to the avirulent strain supports the role of avoidance of phagocytosis as an important component of virulence in *S. iniae*.

Our observation that resistance to phagocytosis is an important component of virulence in *S. iniae* does not agree with previously reported observations. Past studies have suggested that increased affinity for uptake by macrophages is an important component of *S. iniae* pathogenesis, allowing bacteria to be transported intracellularly into the central nervous system (Zlotkin et al., 2003). However, Zlotkin et al. (2003) also considered intracellular survival of the pathogen to be a key component of pathogenicity, conferring an advantage in the establishment of menigoencephalitis. This result is consistent with our findings that the ability to survive within phagocytes, and specifically to resist oxidative killing, are important factors in *S. iniae* virulence. It may be that during initial infection, general resistance to phagocytosis and the killing mechanisms of phagocytes allows the establishment of a sustained bacteremia. The *S. iniae* cells which are phagocytosed during this bacteremia then survive well enough intracellularly to be transported across the blood–brain barrier and infect the central nervous system, as suggested by Zlotkin et al. (2003). Thus, resistance to phagocytosis and the ability to survive within phagocytes may be important in *S. iniae* virulence.

In summary, the pathogenesis of disease caused by *S. iniae* is still being elucidated. Development of in vitro assays of *S. iniae* to assess virulence factors can provide insight into the pathogenesis of infection in vivo. Adherence and invasion of cells or resistance to antimicrobial peptides does not appear to represent a critical step in invasive *S. iniae* disease; rather, the capacity to avoid phagocytic clearance and oxidative killing appears to be a critical component of virulence.

Acknowledgements

This work was supported in part by the National Sea Grant College Program under Grant #NA04OAR4170038, project #59-A-N, through the California Sea Grant College Program, and through the USDA CSREES National Research Initiative (grant number 2002-35204-11623). The views expressed herein do not necessarily reflect the views

of any of those organizations. We thank Kent SeaTech Corporation for providing fish and use of their challenge facilities.

References

Agnew, W., Barnes, A.C., 2007. *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. Vet. Microbiol. 122, 1–15.

Bercovier, H., Ghittino, C., Eldar, A., 1997. Immunization with bacterial antigens: infections with streptococci and related organisms. Dev. Biol. Stand. 90, 153–160.

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E., Nizet, V., 2005. *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. Infect. Immun. 73, 6935–6944.

Datta, V., Myskowski, S.M., Kwinn, L.A., Chiem, D.N., Varki, N., Kansal, R.G., Kotb, M., Nizet, V., 2005. Mutational analysis of the group A streptococcal operon encoding streptolysin S and its virulence role in invasive infection. Mol. Microbiol. 56, 681–695.

Ellis, A.E., 2001. Innate host defense mechanisms of fish against viruses and bacteria. Dev. Comp. Immunol. 25, 827–839.

Faisal, M., Ahne, W., 1990. A cell line (CLC) of adherent peripheral blood mononuclear leucocytes of normal common carp *Cyprinus carpio*. Dev. Comp. Immunol. 14, 255–260.

Finlay, B.B., McFadden, G., 2006. Anti-immunology: evasion of the host immune system by bacterial and viral pathogens. Cell 124, 767–782.

Fuller, J.D., Camus, A.C., Duncan, C.L., Nizet, V., Bast, D.J., Thune, R.L., Low, D.E., De Azavedo, J.C., 2002. Identification of a streptolysin S-associated gene cluster and its role in the pathogenesis of *Streptococcus iniae* disease. Infect. Immun. 70, 5730–5739.

LaPenta, D., Rubens, C., Chi, E., Cleary, P.P., 1994. Group A streptococci efficiently invade human respiratory epithelial cells. Proc. Natl. Acad. Sci. U.S.A. 91, 12115–12119.

Lau, S.K.P., Woo, P.C.Y., Luk, W.-K., Fung, A.M.Y., Hui, W.-T., Fong, A.H.C., Chow, C.-W., Wong, S.S.Y., Yuen, K.-Y., 2006. Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and beta-hemolytic than those from North America. Diagn. Microbiol. Infect. Dis. 54, 177.

Liu, G.Y., Doran, K.S., Lawrence, T., Turkson, N., Puliti, M., Tissi, L., Nizet, V., 2004. Sword and shield: linked group B streptococcal β-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. Proc. Natl. Acad. Sci. U.S.A. 101, 14491–14496.

Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J., Nizet, V., 2005. *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. J. Exp. Med. 202, 209–215.

Locke, J.B., Colvin, K.M., Datta, A.K., Patel, S.K., Naidu, N.N., Neely, M.N., Nizet, V., Buchanan, J.T., 2007a. *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. J. Bacteriol. 189, 1279–1287.

Locke, J.B., Colvin, K.M., Varki, N., Vicknair, M.R., Nizet, V., Buchanan, J.T., 2007b. *Streptococcus iniae* β-hemolysin streptolysin S is a virulence factor in fish infection. Dis. Aquat. Org. 76, 17–26.

Lowe, B.A., Miller, J.D., Neely, M.N., 2007. Analysis of the polysaccharide capsule of the systemic pathogen *Streptococcus iniae* and its implications in virulence. Infect. Immun. 75, 1255–1264.

Magnadottir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20, 137–151.

Miller, J.D., Neely, M.N., 2005. Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. Infect. Immun. 73, 921–934.

Neumann, N.F., Stafford, J.L., Barreda, D., Ainsworth, A.J., Belosevic, M., 2001. Antimicrobial mechanisms of fish phagocytes and their role in host defense. Dev. Comp. Immunol. 25, 807.

Pier, G.B., Madin, S.H., 1976. *Streptococcus iniae* sp. nov., a beta-hemolytic streptococcus isolated from an Amazon freshwater dolphin, *Inia geoffrensis*. Int. J. Syst. Bacteriol. 26, 545–553.

Segura, M., Gottschalk, M., Olivier, M., 2004. Encapsulated *Streptococcus suis* inhibits activation of signaling pathways involved in phagocytosis. Infect. Immun. 72, 5322–5330.

Shimizu, C., Shike, H., Malicki, D.M., Breisch, E., Westerman, M., Buchanan, J., Ligman, H.R., Phillips, R.B., Carlberg, J.M., Van Olst, J., Burns, J.C., 2003. Characterization of a white bass (*Morone chrysops*) embryonic cell line with epithelial features. In Vitro Cell Dev. Biol. Anim. 39, 29–35.

Tyrrell, G.J., Kennedy, A., Shokoples, S.E., Sherburne, R.K., 2002. Binding and invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. Microbiology 148, 3921–3931.

Valentin-Weigand, P., Jungnitz, H., Zock, A., Rohde, M., Chhatwal, G.S., 1997. Characterization of group B streptococcal invasion in HEp-2 epithelial cells. FEMS Microbiol. Lett. 147, 69.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B.M., Borczyk, A., Low, D.E., 1997. Invasive infections due to a fish pathogen, *Streptococcus iniae*. N. Engl. J. Med. 337, 589–594.

Weyts, F.A.A., Rombout, J.H.W.M., Flik, G., Verburg-Van Kemenade, B.M.L., 1997. A common carp (*Cyprinus carpio* L.) leucocytes cell line shares morphological and functional characteristics with macrophages. Fish Shellfish Immunol. 7, 123–133.

Zlotkin, A., Chilmonczyk, S., Eyngor, M., Hurvitz, A., Ghittino, C., Eldar, A., 2003. Trojan horse effect: phagocyte-mediated *Streptococcus iniae* infection of fish. Infect. Immun. 71, 2318–2325.