Role of a Novel Deacetylase Homologue (Pdi) in Virulence of the Aquatic Pathogen

*Streptococcus iniae*

A Thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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2008
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2008
DEDICATION

I would like to dedicate my work to my family and close friends, for all of the love, support, and wisdom they continue to provide me. It has served as the fuel of my learning during this tremendous experience. Without them I would not have been who I was before, nor will I be able to reach my full potential without them in the future.
Courage doesn't always roar. Sometimes courage is the little voice at the end of the day that says I'll try again tomorrow.

~ Mary Anne Radmacher

Courage is not the absence of fear, but rather the judgment that something else is more important than fear.

~ Ambrose Redmoon

What lies behind us and what lies before us are tiny matters compared to what lies within us.

~ Ralph Waldo Emerson
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ABSTRACT OF THESIS

Role of a Novel Deacetylase Homologue (Pdi) in Virulence of the Aquatic Pathogen

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Professor Victor Nizet, Chair
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*Streptococcus iniae* is an aquatic zoonotic pathogen that causes hundreds of millions of dollars in losses annually to the world-wide aquaculture industry and poses a significant
risk to humans who handle raw fish. In vivo screening of a randomly generated bacterial transposon mutant library created from the wild-type *S. iniae* strain K288, led to the discovery of an attenuated transposon mutant with a disruption in a gene region near a putative polysaccharide deacetylase we named *pdi*. Here I describe a proposed role of the novel *pdi* gene in *S. iniae* virulence, and compare it to a paralogous virulence gene (*pgdA*) found in several other pathogenic species. Following the creation of the isogenic Δ*pdi* mutant we observed its attenuation in an in vivo HSB virulence model, as well as decreased survival in whole fish blood. We also observed an impairment of the Δ*pdi* mutant in its ability to adhere and invade fish epithelial cells, a virulence mechanism that until now, has been speculated, but not yet associated with virulence in vivo or with a specific virulence gene.
INTRODUCTION

Global significance of aquaculture and the bacterial pathogen *Streptococcus iniae*

The contribution of aquaculture to the global food fish supply has increased from < 4% of total production in 1970, to what will be greater than 50% of the annual supply in 2007 (United Nations Report, 2007). At the same time, a recent study has warned against the possible disappearance of all the planet’s wild capture fisheries by the year 2048 (Worm et al., 2006), serving to underscore aquaculture’s crucial role in meeting the ever-increasing seafood demands of a growing global population.

Economic pressure has led to more intensive aquaculture husbandry practices due to strong competition of other forms of land and water use. Systemic infections have emerged as a serious fish health and economic problem in intensive aquaculture operations, with bacterial infections being the most serious and widespread (Plumb, 1999). In the past few decades, diseases produced by the bacterial pathogen *Streptococcus iniae* have emerged as a major hindrance and threat to aquaculture operations worldwide. The most notable feature of *S. iniae* pathogenicity in finfish is fatal meningoencephalitis associated with large-scale mortalities in a wide variety of marine and freshwater cultured species. Affected species include trout (Eldar & Ghittino, 1999), salmon (Eldar et al., 1994), tilapia (Press et al., 1998), hybrid striped bass (HSB) (Zlotkin et al., 1998), ayu (Ugagin, 1981), yellowtail (Kitao, 1982), European seabass (Bercovier et al., 1997), barramundi (Bromage et al., 1999), red drum (Eldar et al.,...
1999), gilthead sea bream (Zlotkin et al., 1998), olive flounder (Nguyen & Kanai, 1999),
and ornamental cichlids (Eldar et al., 1995). Economic losses due to S. iniae infection
have been known to exceed $150 million/yr (Shoemaker & Klesius, 1997), thus
threatening the existence of U.S. HSB and tilapia aquaculture.

In addition S. iniae has established itself as a zoonotic risk for human infection,
especially in areas of the world that preferably prepare and consume raw fish (Lau et al.,
2003). To date, at least 25 human cases of invasive streptococcal infection attributed to
S. iniae have been confirmed in the United States, Canada, China and Taiwan (Agnew &
Barnes, 2007; Sun et al., 2007; Weinstein et al., 1997), and since there is currently no
prospective epidemiologic surveillance for human S. iniae infections, the true number
may be much higher (Facklam et al., 2005; Lau et al., 2006).

**Clinicopathologic and epidemiologic features of S. iniae infection**

The clinicopathologic manifestations of S. iniae infection are generally shared across fish
species. Symptoms of infection include lethargy, anorexia, erratic swimming, loss of
orientation (Bercovier et al., 1997), and protrusion of the eye globe with clouding of the
cornea resulting from severe hemorrhagic suppurative panophthalmitis, or "popeye
disease" (Bercovier et al., 1997; Sano & Fukuda, 1987). S. iniae infection can be
experimentally reproduced in HSB and tilapia by direct inoculation (Evans et al., 2000)
or placement of fish in tanks containing infected fish or water (Perera et al., 1997;
Robinson & Meyer, 1966). Streptococcal infection is thought to be facilitated by gill or
skin injury (Rasheed & Plumb, 1984; Sano & Fukuda, 1987) and is increased with higher water temperatures (>15°C for trout and >21°C for tilapia) (Bercovier et al., 1997). Stressors encountered in intensive aquaculture such as crowding and decreased water quality enhance fish predisposition to bacterial disease. Abnormalities in leukocyte count, depopulation of splenic and renal lymphoid tissues, and delayed leukocyte infiltration can be seen in association with increased levels of ACTH or cortisone in fish that have experienced stressful conditions (Roberts, 1993).

**Limitations of antibiotic treatment and the need for vaccine development**

Antibiotics, delivered in feed or through injection, are being developed for use in aquaculture to treat streptococcal infections, but are not fully sufficient. Currently, there are only three antibiotics approved for use in food fish: Romet 30® (sulfadimethoxine and ormetoprim), Sulmerazine, used for controlling septicemia and furunculosis, and Terramycin® (oxytetracycline), which is a general treatment towards bacterial diseases. To date, the Food and Drug Administration of the United States has only approved antibiotics for use in salmonids, catfish, and lobsters. As with any agricultural operation, antibiotic use can lead to development of drug resistant pathogens and thus have broader-scale environmental impacts through release of farm effluent into the environment.

The inherent drawbacks of antibiotic usage in aquaculture have led to increased interest in the development of fish vaccines. Large-scale use of vaccines in aquaculture is
uncommon except for a few specific species/disease combinations (Eldar et al., 1997; Newman, 1993). Vaccines are not available for streptococcal infections in fish although research has been conducted on several inactivated “bacterin” preparations. Some success has been achieved in Israel using formalin-killed S. difficile and S. iniae strains for injection vaccination in tilapia and trout, respectively (Eldar et al., 1997; Newman, 1993). Still, this vaccine has lost efficacy with the emergence of new strains of S. iniae and is no longer in commercial use. More recently, mortality was successfully reduced in tilapia vaccinated against S. iniae using an intraperitoneally administered bacterin and concentrated extracellular products (Klesius et al., 1999). This non-adjuvanted USDA vaccine, however, is not commercially available, and studies on production efficiency were deemed negligible in trials using hybrid striped bass (Ostland, 2003). This suggests limitations of this bacterin vaccine in certain intensive aquaculture operations, thus new vaccine development against S. iniae infection in fish is essential. Gaining greater understanding of the molecular bases of S. iniae virulence could prove valuable in this effort.

**Streptococcus iniae pathogenicity factors**

While there are many factors by which pathogenic bacterial species may exhibit virulence potential in a host, general mechanisms such as toxin production, evasion of host immune defenses, and the ability to adhere to and invade host cellular barriers are often key contributors. To better understand molecular aspects of virulence in *Streptococcus iniae*, we have created a library of randomly generated transposon mutants and screened for
virulence attenuation in hybrid striped bass (*Morone chrysops* x *M. saxatilis*, HSB) model of infection. The analysis and study of mutants from this library and associated research has served to identify the only three proven virulence factors known to contribute to *S. iniae* pathogenicity in fish. These are streptolysin S (Fuller *et al.*, 2002; Locke *et al.*, 2007b), phosphoglucomutase (Buchanan *et al.*, 2005), and capsular polysaccharide (Barnes *et al.*, 2003; Locke *et al.*, 2007a). Streptolysin S produces cytotoxicity to host cells, while phosphoglucomutase and capsular polysaccharide have been shown to contribute to *S. iniae* resistance to phagocytosis and innate immune clearance.

Here I report the analysis of the putative cell surface polysaccharide deacetylase gene (*pdi*) identified through our *S. iniae* transposon mutant screen that appears to play a novel role in *S. iniae* virulence in fish by enhancing adherence and invasion of host cells. In this study we observed virulence attenuation associated with isogenic replacement of the *S. iniae pdi* gene through targeted mutagenesis, and characterize its contribution to virulence in a variety of in vivo and in vitro models of disease pathogenesis.
MATERIAL AND METHODS

Bacterial strains, culture, transformations, and DNA techniques

Wild-type (WT) *S. iniae* strain K288 was isolated from the brain of a diseased hybrid striped bass (HSB) at the Kent SeaTech facility in Mecca, CA (Buchanan *et al.*, 2005). K288 was identified as *S. iniae* through biochemical testing and analyses of ribosomal 16S sequence (data not shown) (Buchanan *et al.*, 2005). *Escherichia coli* used in cloning was grown on Luria agar at 37°C with antibiotic selection of 500 µg ml⁻¹ erythromycin (Erm), 100 µg ml⁻¹ spectinomycin, and 20 µg ml⁻¹ chloramphenicol (Cm) when appropriate. Mach 1 chemically competent *E. coli* (Invitrogen) and MC1061 electrocompeent *E. coli* used for plasmid propagation during transformation, were recovered through growth at 30°C, or 37°C, when appropriate, with shaking in S.O.C. medium (Invitrogen). A PureLink Quick plasmid miniprep kit (Invitrogen) was used to isolate plasmids. *S. iniae* was grown in Todd Hewitt broth (THB) without shaking, or on Todd Hewitt agar (THA) plates, at 30°C unless otherwise indicated, with antibiotic selection of 2 µg ml⁻¹ Cm, or 5 µg ml⁻¹ Erm when required. *S. iniae* genomic DNA was isolated using the DNA Easy Tissue Kit (Quiagen). Dilution plating on THA was used for enumeration of CFU for in vitro assays. β-hemolytic activity was assessed on sheep blood agar plates (tryptic soy agar with 5% sheep red blood cells added) as previously described (Locke *et al.*, 2007a). In all assays, overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase (OD₆₀₀ = 0.40), unless otherwise stated.
S. iniae strains were rendered electrocompetent for transformation by culturing bacteria in THB containing 0.6% glycine as previously described (Locke et al., 2007a).

**Cell line and culture conditions**

The WBE27 white bass embryonic epithelial cell line (ATCC CRL-2773) (Shimizu et al., 2003) was grown at 28°C with 5% CO₂. Cells were passaged less than 10 times before use in experiments and were maintained in 125 ml tissue culture flasks in Dulbecco’s Modified Eagle’s Medium (DMEM)(Gibco) containing 10% heat-inactivated fetal bovine serum (FBS)(Gibco).

**Transposon mutagenesis**

Transposon mutagenesis of strain K288 followed procedures previously described using the temperature-sensitive plasmid pTV₁OK containing transposon Tn₉₁₇ (Buchanan et al., 2005). Briefly, individual colonies of K288 transformed with pTV₁OK were inoculated in THB plus kanamycin and grown to an OD₆₀₀ of 0.90 at a permissive temperature (30 °C) for plasmid replication. Cultures were diluted 1:100 in THB with Erm and grown at a nonpermissive temperature (37 °C) to an OD₆₀₀ value of 0.90, then plated on THA with Erm for isolation of candidate insertion mutants. Single random transposon insertions were verified through Southern Blot analysis of a subset of the library mutants (data not shown) (Buchanan et al., 2005).
Sequence assembly and localization of transposon mutants

Contiguous sequences used in Vector NTI (Invitrogen) ORF analysis and bioinformatics, were created from an automated assembly of pyrosequencing (4-5-4 Life Sciences). This assembly was generated by the Phred/Phrap/Consed suite (URL: http://www.phrap.org/phredphrapconsed.html) and resulted in 1865 contigs ranging in size from 51 bp to 22 kb. Without the need of further assembly, we used these contigs to build our S. iniae genome database that we used for BLAST searches, as there is no complete S. iniae genome sequence currently available. Using a local version of BLAST (version 2.2.14) (Altschul, 1997), we searched our S. iniae database and located the contiguous sequences matching the insertion sites of mutants containing the Tn917 transposon mutation.

Identification of S. iniae pdi

The Tn917 insertion sites in attenuated transposon mutants were identified through single primer PCR and direct genomic sequencing. PCR fragments extending out from the end of the transposon were created at random via single primer PCR using the internal forward primer 5’-AATCTGTACCACTAATAACTC-3’. PCR fragments were sequenced using the external forward primer 5’-AATGTACAAAAATAACAGCGA-3’. Chromatogram files were analyzed using 4Peaks software (Mekentosj). The amino acid sequence of Pdi was compared to sequences in the GenBank databases using the BLASTX program (Altschul, 1997). Vector NTI (Invitrogen) was used in ORF
identification and graphic representation of chromosomal positioning of the \textit{pdi} gene and transposon Tn917 insertion.

\textbf{Bioinformatics and phylogenetic analysis of Pdi and its homologous proteins}

To better understand the function of Pdi, we performed signal peptide analysis using Signal P 3.0 (URL: \url{http://www.cbs.dtu.dk/services/SignalP/}) (Bendtsen \textit{et al.}, 2004), as well as protein family analysis using PFAM (URL: \url{http://pfam.sanger.ac.uk/family?acc=PF01522}). We used the tools of Biology Workbench (SDSC, URL: http://workbench.sdsc.edu/) for sequence alignment, phylogenetic analysis, and sequence coloring using Boxshade. Finally, for structure alignment and prediction of conserved residues, we used the ConSurf server (URL: \url{http://consurf.tau.ac.il/}) and the Evolutionary Trace report maker (URL: \url{http://mammoth.bcm.tmc.edu/report_maker/index.html}). The structure used as a “seed” for the analysis was the structure of \textit{S. pneumonniae} peptidoglycan deacetylase (PgdA), submitted in the Protein Data Bank (PDB) under id 2c1g.
Abbreviations used in the alignments and phylogenetic analyses

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Organism</th>
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<tbody>
<tr>
<td>Eco</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>SAu</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>Sep</td>
<td><em>Staphylococcus epidermidis</em></td>
</tr>
<tr>
<td>Spy</td>
<td><em>Streptococcus pyogenes</em></td>
</tr>
<tr>
<td>SPn</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>Sag</td>
<td><em>Streptococcus agalactiae</em></td>
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<td>SEQ</td>
<td><em>Streptococcus equi</em></td>
</tr>
<tr>
<td>STh</td>
<td><em>Streptococcus thermophilus</em></td>
</tr>
<tr>
<td>SMu</td>
<td><em>Streptococcus mutans</em></td>
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<td><em>Streptococcus sanguinis</em></td>
</tr>
<tr>
<td>Sub</td>
<td><em>Streptococcus uberis</em></td>
</tr>
<tr>
<td>BCe</td>
<td><em>Bacillus cereus</em></td>
</tr>
<tr>
<td>BSu_su</td>
<td><em>Bacillus subtilis subsp subtilis</em></td>
</tr>
<tr>
<td>LLa_la</td>
<td><em>Lactococcus lactis subsp. lactis</em></td>
</tr>
<tr>
<td>LMo</td>
<td><em>Listeria monocytogenes</em></td>
</tr>
<tr>
<td>PoDe</td>
<td><em>Polysaccharide deacetylase</em></td>
</tr>
<tr>
<td>PgdA</td>
<td><em>Peptidoglycan deacetylase</em></td>
</tr>
<tr>
<td>PIA</td>
<td><em>Polysaccharide intercellular adhesin (PIA) biosynthesis deacetylase</em></td>
</tr>
<tr>
<td>Pdi</td>
<td><em>Polysaccharide deacetylase of S. iniae</em></td>
</tr>
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</table>

Allelic exchange mutagenesis of the *pdi* locus

Allelic exchange mutagenesis was carried out as previously described (Locke *et al.*, 2007a). Briefly, PCR was used to amplify ~ 400 bp upstream and ~ 200 bp downstream of the targeted *S. iniae* chromosomal gene region. Primers adjacent to the upstream and downstream regions of *pdi* were constructed with 25-bp 5’ extensions corresponding to the 5’ and 3’ ends of the chloramphenicol acetyltransferase (*cat*) gene from pACYC (Nakano *et al.*, 1995). Upstream and downstream products were then combined with a 660-bp amplicon of the *cat* gene using fusion PCR (Buchanan *et al.*, 2006). The fusion product resulting from this PCR contained an in-frame substitution of *pdi* with *cat* and
was then subcloned into the Gateway entry vector pCR8/GW/TOPO. This vector was then used to transform chemically competent Mach 1 *E. coli* (Invitrogen). Plasmid DNA was extracted and the fusion PCR amplicon was transferred into the temperature-sensitive knockout vector pKODestErm (Locke *et al.*, 2007a) via an LR recombination reaction in order to create the knockout plasmid pKOdpi. After propagation in MC1061 *E. coli*, the pKOdpi construct was introduced into WT *S. iniae* through electroporation. Transformants were identified at 30 °C by Erm selection and shifted to 37 °C (a non-permissive temperature for plasmid replication). Using the differential antibiotic selection of Cm<sup>+</sup> and Erm<sup>−</sup> candidates were identified as potential allelic exchange mutants. Targeted in frame replacement was confirmed through PCR documenting the desired insertion of *cat* and absence of the *pdi* sequence in chromosomal DNA isolated from the Δpdi mutant.

**In vivo fish challenges**

Groups of 10 HSB fingerlings (~15 g) were challenged through intraperitoneal (i.p.) injection with either *S. iniae* WT or mutant strain bacteria from mid-log phase cultures. Injection with a 26.5 gauge needle was used to deliver either 1 × 10<sup>5</sup> or 1 × 10<sup>4</sup> CFU in 50 µl volumes of PBS. Fish were held with aeration and flow-through water at 24-27 °C for 7 days post injection and were monitored daily for mortalities.
Whole blood survival

Heparinized blood collected for whole blood survival assays was taken and pooled from 4-6 HSB fingerlings. Fresh blood was collected via caudal vein blood draw. Seventy-five µl of blood was added to plastic 2-ml siliconized centrifuge tubes containing approximately $5 \times 10^2$ CFU of a mid-log phase culture in a 25 µl volume, and incubated at 30°C for 1 h on an orbital shaker (225 rpm). Reactions were done in quadruplicate for each strain tested. 50 µl of each reaction was spread on THA, and a control plate was prepared at initiation of the assay as a reference for starting CFU. Each experiment was repeated three times.

Resistance to oxidative killing

Bacterial strains of the Δpdi mutant and WT S. iniae were grown to mid-log phase and were diluted in PBS such that adding 100 µl of culture to a 96 well plate delivered $2 \times 10^5$ CFU/well. One hundred µl of dilute H$_2$O$_2$ was then added to each well to reach a final concentration of 0.03%, 0.06%, or 0.09%. After incubation at 30°C for 1h, the reaction was quenched by adding 10 µl of a 1000 U ml$^{-1}$ catalase solution in PBS. CFU data from a control plate prepared at the initiation of the assay served as a reference for the 60 min time point. Each experiment was repeated three times.
Antimicrobial peptide susceptibility assays

Mid-log phase cultures of *S. iniae* were diluted in fresh THB, and $1 \times 10^5$ CFU in a total of 180 µl were added to replicate wells of a 96 well plate. Dilutions of antimicrobial peptides (AMPs) were prepared in dH$_2$O and 20 µl was added to experimental wells for a final concentration of 1.5 µM moronecidin (Lauth *et al.*, 2002) or 60 µM polymyxin B (SIGMA). dH$_2$O alone was used as a negative control. To measure antimicrobial killing kinetics, 25 µl aliquots were taken from each well at various time points, were serially diluted in PBS and were plated on THA for surviving CFU determination. Each experiment was performed in triplicate.

Lysozyme sensitivity assays

To ascertain lysozyme sensitivity of log phase *S. iniae*, bacteria were grown to mid log phase ($OD_{600} = 0.40$) and mixed with 80 µg ml$^{-1}$ chicken egg-white Lysozyme (SIGMA). To ascertain lysozyme sensitivity of stationary phase *S. iniae*, bacteria were taken in early stationary phase ($OD_{600} = 0.95$), diluted to $OD_{600} = 0.40$, to ensure similar starting CFU between samples and assays, and used immediately. For both log and stationary phase assays, bacterial samples were washed once in PBS and resuspended and diluted in lysozyme buffer solution (10 mM Tris-HCl, pH 8.0). One hundred-eighty µl of diluted bacteria ($3.6 \times 10^5$ CFU) were added to a 96 well plate and 20 µl of lysozyme solution were added to each well to give a final concentration 80 µg ml$^{-1}$. Control samples
received 20 µl of lysozyme buffer solution alone and were washed and diluted as described above. *Bacillus subtilis* was used as a control strain to test for positive lysozyme activity. At various time points 25 µl aliquots of each strain were serially diluted and plated on THA in order to enumerate surviving CFU.

**Invasion and adherence assays**

Invasion and adherence assays were carried out in 96 well collagenized plates (Nunc). One day prior to the assay, WBE27 cells were seeded in the wells at a density of ~ 1 x 10^5 cells per well in 100 µl DMEM 10% FBS media and were allowed to grow to confluency overnight. Cell culture media was removed, and 100 µl mid-log phase bacterial cultures diluted 1:40 in DMEM 2% FBS media were added to confluent cells at a multiplicity of infection (MOI) of 5 (bacteria to cells). The plate was then centrifuged at 350 × g for 15 min to ensure contact between bacteria and the cell monolayer. At 30 min post-centrifugation, the media was removed and the wells were washed four times with 200 µl DMEM 2% FBS to remove any non-adherent bacteria. The cells were then lysed by titration with 100 µl of 0.01% Triton X-100 and 25 µl removed for serial dilution and plating on THA to enumerate adherent CFU. Invasion assays were carried out in a similar manner except at 1 h post centrifugation cell media was removed, wells were washed once, and then were incubated with 150 µl fresh DMEM containing 300 µg ml⁻¹ gentamicin (Invitrogen) and 30 µg ml⁻¹ penicillin (Invitrogen) for an additional hour. All strains of *S. iniae* were tested to ensure susceptibility to these antibiotic concentrations at 1 h. Cells were then washed three more times and lysed by tituration. Surviving
intracellular bacteria were enumerated by plating serial dilutions of lysed cell supernatant on THA. Bacteria plated at the initiation of the assay and after 1 h in assay medium served as control references for adherence and invasion respectively.

**Growth rate and autolysis analysis**

*S. iniae* cultures were grown to mid-log phase and were diluted 1:10 in THB in a 96 well plate. Eight replicates for each strain were monitored for growth over twelve hours using an EL808 plate reader and Gen5 software (BioTek Instruments Inc.). The incubation temperature was held constant at 30° C, with shaking before each reading every 30 min. Autolysis assays were performed as previously described (Kristian *et al.*, 2005) using the EL808 plate reader and Gen5 software to monitor decreases in optical density.

**Statistical analyses**

Data were analyzed using the statistical tools included in Microsoft Excel (Microsoft). Data resulting from in vitro assays were assessed using two-tailed unpaired *t* tests. A *P* value of < 0.05 was considered to be statistically significant. In vitro assays were repeated at least three times, and the data presented (mean ± standard error) are from single representative assays unless otherwise stated. Statistical values obtained from mortality curves of in vivo challenges were generated from chi-squared tests.
RESULTS

**Identification and bioinformatic analysis of *S. iniae* virulence gene *pdi*.**

Using an HSB infection model, the attenuated transposon mutant TnM7 was identified from the Tn917 chromosomal insertion library of WT *S. iniae* strain K288. TnM7 represents one of approximately forty *S. iniae* transposon mutants that were identified through initial virulence screening (n=3) in HSB and were recognized as interesting based on the observation of no less than thirty percent survival of fish injected with a mutant compared with the complete mortality of fish injected with the WT strain. TnM7 then received additional virulence screening (n =10), using our HSB infection model and maintained a strong degree of attenuation. On this basis, we sought to examine the possible gene or operon that may have been disrupted by transposon insertion in the TnM7 chromosome, using single primer PCR to amplify genomic regions around the transposon insertion site and sequence data obtained from amplified DNA fragments.

BLASTX analysis in GenBank showed that the transposon insertion in mutant TnM7 mapped to a location in the *S. iniae* chromosome adjacent to an ORF encoding a candidate gene with homologies to known polysaccharide deacetylases. With contiguous sequence data of the parent *S. iniae* strain K288 genome generated from pyrosequencing and assembled using the Phred/Phrap package, we again used BLAST to locate the putative polysaccharide deacetylase gene (which we have termed *pdi*) in the *S. iniae* genome. Utilizing VectorNTI for ORF analysis of the *pdi* containing contig helped us to
conclude that TnM7 had a disruption in the intergenic region between pdi and a second open reading frame encoding a candidate gene with homologies to homoserine dehydrogenases (Fig. 1). A full Blast search of the *S. iniae* pdi ORF revealed orthologous genes in other species, which we have aligned in Fig. 2.

**Figure 1.** Chromosomal of map of insertion site in *S. iniae* transposon mutant Tnm7. Graphic representation of the chromosomal region where the transposon insertion of attenuated *S. iniae* transposon mutant TnM7 led to the discovery of pdi, a putative gene encoding a polysaccharide deacetylase. The insertion was found to be in the intergenic region of a putative homoserine dehydrogenase gene and pdi.
Figure 2. Phylogenetic analysis and alignment of *S. iniae* Pdi with related proteins. Panel (A.) shows an unrooted phylogenetic tree of the closest evolutionarily related polysaccharide deacetylases to *S. iniae* Pdi found in the GenBank databases, using the Basic Local Alignment Search Tool (BLAST). (B.) The same relationships in the form of a rooted phylogenetic tree. Both representations depict orthologs of Pdi found in other species as well as paralogous peptidoglycan deacetylase proteins (PgdA) (C.) Amino acid alignment of conserved regions between polysaccharide deacetylases and peptidoglycan deacetylases that are found in multiple species.
To further investigate the predicted protein, which we named Pdi, we performed signalP analysis and found it had signal peptide probability of 0.875 with a lower probability of being cleaved (0.716) suggesting that Pdi is likely to be a membrane bound protein in the Gram-positive cell wall of *S. iniae*. A motif search and protein family analysis using Interpro and PFAM revealed that Pdi, and its orthologs, share a deacetylase motif near the C-terminus (PFAM Id: Polysacc_deac_1 PF01522). It was found that this motif is also shared by the protein PgdA (*Fig. 3*), which is highly conserved in many bacteria and has been well characterized for it’s role in virulence in *Streptococcus pneumonia* (Vollmer & Tomasz, 2000), and *Listeria monocytogenes* (Boneca et al., 2007). PgdA, however, contains a Gram-positive signal peptide, with a high probability (0.8) of being cleaved, which means that it is likely to be secreted. Using PgdA sequence data from *Streptococcus pneumoniae* and *Streptococcus pyogenes* we blasted against contiguous *S. iniae* genomic sequences from pyrosequencing and the Baylor genome project (URL: www.hgsc.bcm.tmc.edu/bcm/blast/microbialblast.cgi?organism=Siniae ) and confirmed the presence of an ortholog to PgdA in *Streptococcus iniae* that is distinct from Pdi (*Fig. 4*), suggesting that these are paralogous proteins. These analyses further imply that while *pdi* is a gene likely responsible for the production of a cell surface deacetylase similar to PgdA, it is in fact a novel gene that has yet to be characterized in any pathogenic species, including *S. iniae*. Thus, any identified roles of the *S. iniae* pdi gene in virulence could be divergent and distinct from those attributed to *pgdA*. 
Figure 3. Three dimensional analysis of conserved residues between *S. iniae* Pdi and *S. pneumoniae* PgdA. (A.) ConSurf analysis results showing the three-dimensional structure of the probable active site of *S. iniae* Pdi based on crystallographic analysis in PgdA (Protein Data Bank ID 2c1g). (B.) Same protein from a different angle that shows different conserved residues, e.g., D275, D276, P366, and G378. (C.) A closer view showing how most conserved residues are located within one region in the enzyme, suggesting involvement in the deacetylase domain.
Figure 4. Analysis of *S. iniae* paralogous deacetylase proteins Pdi and PgdA. Panels (A.) and (B.) show unrooted and rooted phylogenetic trees respectively, of the *Streptococcus iniae* PgdA homologue. Panel (C.) Shows an alignment of the predicted paralogous *S. iniae* Pdi and PgdA proteins. A low degree of similarity exists between these two proteins suggesting that the function of Pdi is likely to be divergent and novel to that of PgdA.
Targeted mutagenesis of the *S. iniae* *pdi* gene

Precise in-frame allelic replacement of *pdi* with chloramphenicol acetyltransferase (*cat*) was achieved in the WT *S. iniae* strain K288 in order to generate an isogenic Δ*pdi* mutant (Fig. 5). In this fashion, we tested whether loss of virulence exhibited by the TnM7 mutant could indeed be attributed to altered expression of the *pdi* gene, rather than a spontaneous mutation elsewhere in the chromosome or polar effects of transposon insertion.

**Figure 5.** Schematic representation of allelic replacement of *S. iniae* *pdi* gene with *cat*. Shown is a representation of the double-crossover event required for precise in-frame replacement of the WT *S. iniae* *pdi* gene with the chloramphenicol acetyltransferase (*cat*) gene. The *S. iniae* Δ*pdi* mutant was created by allelic exchange mutagenesis of *pdi* with *cat* in the WT strain K288 chromosome.
The HSB intraperitoneal (i.p.) challenge model was used to further evaluate the virulence of the newly created Δpdi knockout. Significant attenuation was observed, as there was no mortality observed in fish challenged with the Δpdi knockout at a dose that killed 70% of fish injected with WT (\(P = 0.0011\)), and 30% mortality in fish challenged with the Δpdi knockout at a dose that killed 90% of fish injected with WT (\(P = 0.0031\)) (Fig. 6).

**Figure 6.** Attenuated virulence of the *S. iniae* isogenic Δpdi mutant in a HSB model. The Δpdi mutant or WT bacteria were injected intraperitoneally at two doses (n=10 per treatment). The mutant strain was significantly less virulent than wild type at doses of both \(1 \times 10^4\) (\(P = 0.0011\)) and \(1 \times 10^5\) (\(P = 0.0031\)) CFU.
Effects of \emph{pdi} deletion on \emph{S. iniae} chain length and buoyancy

WT \emph{S. iniae} occur in short chains and are \(\beta\)-hemolytic on sheep blood agar (Figs. 7A & 8B). The \(\Delta\text{pdi}\) allelic exchange mutant was found to loose ability to remain associated in pairs or short chains, as cocci were found predominantly occurring as single cells (Fig. 7B). Expression of the \emph{pdi} gene in the constitutive high expression plasmid pDestErm was used to compliment the \(\Delta\text{pdi}\) knockout mutant and restored chain length to WT levels (Fig 7C).

\textbf{Figure 7. The \(\Delta\text{pdi}\) mutant lacks ability to remain in chains.} Photomicrographs indicate the \emph{Streptococcus iniae} \(\Delta\text{pdi}\) mutant is impaired in its ability to remain in chains. In panel A, (WT) most bacteria appear in short chains, while in panel B (mutant), most bacteria exist as single cocci. In panel C, the mutant is complemented to restore the WT phenotype.

Cultures of the \(\Delta\text{pdi}\) mutant grown in liquid media revealed the mutant to exhibit greater buoyancy than the WT \emph{S. iniae} parent strain (Fig. 8A); the WT phenotype was partially restored in the plasmid-complemented strain. No differences in \(\beta\)-hemolytic activity of
the strains were observed on sheep blood agar plates (Fig. 8B). A similar growth profile was observed between the WT and mutant through log phase ($P = 0.6$); thus no apparent gross defect in viability or fitness is conferred from loss of the $pdi$ gene (Fig. 8C). Phenotypes of impaired chain formation and differences in culture buoyancy of $\Delta pdi$ imply that normal the function of $pdi$ involves alteration of molecules on the cell surface of $S. iniae$.

**Figure 8. Comparison of hemolytic activity, culture buoyancy, and growth analysis.** (A.) The $\Delta pdi$ mutant was more buoyant in liquid culture compared to WT; WT buoyancy could be partially restored by complementation with $pdi$. (B.) Hemolytic activity as observed on sheep blood agar plates was comparable between $S. iniae$ WT, $\Delta pdi$ mutant, and complemented strains. (C) No significant differences ($P = 0.6$) were observed in growth rate between WT (squares) and the $\Delta pdi$ mutant (circles). The complemented mutant (triangles) grew slower growth in early-log phase compared to WT ($P = 10^{-14}$) and the $\Delta pdi$ mutant ($P = 10^{-9}$). Values plotted are mean ± SEM.
Effect of \( \textit{pdi} \) mutation on \( \textit{S. iniae} \) susceptibility to lysozyme killing.

Resistance to cell wall hydrolysis by lysozyme has been reported as a key protective role of peptidoglycan deacetylases (\( \textit{pgdA} \)) in bacterial species such as \textit{Bacillus cereus} (Psylinakis \textit{et al.}, 2005), \textit{Streptococcus pneumoniae} (Vollmer & Tomasz, 2000) and \textit{Listeria monocytogenes} (Boneca \textit{et al.}, 2007). Similarly, we found the \( \textit{S. iniae} \Delta \textit{pdi} \) mutant to be more sensitive to lysozyme compared to WT. Whereas the sensitivity to lysozyme in \( \textit{pgdA} \) mutants of other pathogenic species has been shown to occur specifically in the stationary phase of growth, we have observed lysozyme sensitivity of the \( \textit{S. iniae} \Delta \textit{pdi} \) mutant to be significantly higher than WT in both log (\( P = 0.01 \)) and stationary phases (\( P = 10^{-5} \)) of growth after 20 minutes exposure to 80 \( \mu \text{g ml}^{-1} \) lysozyme treatment (\textit{Fig. 9}).
Figure 9. Lysozyme killing of the *S. iniae* Δ*pdi* mutant vs. WT. Observed differences in the kinetic killing of WT (black bars) and the Δ*pdi* mutant (white bars) by lysozyme show that the mutant is significantly more susceptible at twenty minutes to lysozyme killing in both log and stationary phase, *(P = 0.01)* and ***(P = 10^{-5})*** respectively. Each experiment was performed using 80 µg ml^{-1} lysozyme. Values plotted are mean ± SEM.

**Autolytic potential does not differ between *S. iniae* WT and the Δ*pdi* mutant.**

A peptidoglycan deacetylase in *Lactococcus lactis* has recently been shown to affect chain length morphology, resistance to lysozyme killing and protect against cell autolysis (Meyrand et al., 2007). We recognized the possibility that the differences in attenuation observed in vivo were due to a greater propensity for autolysis in the Δ*pdi* mutant than in WT, however, our experiments found that the rates of autolysis for the two strains were not significantly different *(P = 0.95)*** (Fig. 10).
Autolytic potential is the same in WT and the $\Delta pdi$ mutant. Results of investigation into the autolytic potential of the WT (squares) and $\Delta pdi$ mutant (triangles) show no significant difference ($P = 0.95$) in autolytic potential. This suggests that attenuated virulence of the $\Delta pdi$ mutant observed in vivo is not due to particular autolytic phenomena. Values plotted are mean ± SD.

$S. iniae pdi$ promotes survival in blood.

To investigate additional possible mechanisms for the attenuation of the $\Delta pdi$ mutant observed in vivo, we assessed the ability of the mutant to survive in fresh fish blood. We found that the $\Delta pdi$ mutant was significantly impaired in its ability to survive in whole blood compared to WT ($P < 0.002$) (Fig. 11). The complimented strain showed a significant increase ($P < 0.01$) in its ability to survive in whole blood compared to the mutant, with a restored survival comparable to that of WT. These results suggest a
fitness advantage conferred by the *S. iniae* *pdi* gene against immune clearance by components present in the fish blood circulation.

**Figure 11.** *S. iniae* Δ*pdi* mutant survives significantly less than WT in whole fish blood. The WT and Δ*pdi* mutant were incubated with pooled blood from HSB fingerlings (avg. n =5) to assess possible mechanisms for the observed attenuation by the Δ*pdi* mutant in the in vivo challenge model. The Δ*pdi* mutant showed a significantly reduced ability to survive in whole fish blood compared to WT *(P < 0.002)*, with survival being restored to levels comparable to WT in the complimented strain, which were significantly greater than the Δ*pdi* mutant ***(P < 0.01).*** Values are mean ± SEM.

**S. iniae pdi does not confer resistance to oxidative killing or cationic AMPs.**

Reactive oxygen species and antimicrobial peptides (AMPs) are mechanisms that phagocytic and other immune cell types have evolved to mediate clearance of bacterial invaders in whole blood (Gallo & Nizet, 2003; Kaattari & Piganelli, 1996; Locke *et al.*,...
To further analyze why the Δpdi mutant was less able to survive in whole fish blood, we compared the resistance of WT and the Δpdi mutant to oxidative killing by hydrogen peroxide, as well as the sensitivities of both strains to the HSB derived AMP moronecidin or the bacterially derived cationic AMP polymyxin B. We observed no significant difference (P = 0.8) in the susceptibility to hydrogen peroxide or AMP killing between the two strains (Fig. 12A-C). These studies imply that the increased susceptibility of the Δpdi mutant to whole blood killing is not derived from enhanced sensitivity to the innate immune system’s oxidative killing mechanisms or AMPs.
Figure 12. Resistance to oxidative killing and antimicrobial peptides of WT *S. iniae* and the Δ*pdi* mutant. Reactive oxygen species and AMPs are a common means by which pathogens are neutralized.

(A.) No significant differences (*P* = 0.8) were observed in the resistance of WT (*black*) and the Δ*pdi* mutant (*gray*) to oxidative killing by H$_2$O$_2$. Similarly, WT (*squares*) and the Δ*pdi* mutant (*circles*) showed no significant differences in their rates of kinetic killing by the antimicrobial peptides  (B.) Moronecidin (*P* = 0.3) or  (C.) Polymyxin B (*P* = 0.2). Values are mean ± SEM.
*S. iniae Δpdi* mutant shows an impaired ability to adhere and invade epithelial cells.

Adherence and invasion of innate immunity barriers, such as epithelial cells and the blood brain barrier, are thought to be keys to virulence for many streptococcal species (Locke *et al.*, 2007b; Rajam *et al.*, 2007; Williamson *et al.*, 2007). We found the ability of the Δpdi mutant to both adhere and invade the white bass epithelial cell line WBE27 to be significantly reduced compared to WT (*Fig. 13*).

**Figure 13.** Adherence and invasion of white bass epithelial cell line WBE27 by *S. iniae* WT and Δpdi mutant. Adherence and invasion of innate immunity barriers, such as epithelial cells and the blood brain barrier are thought to be keys for virulence in many streptococcal species. The *S. iniae* Δpdi mutant was observed to have a significantly reduced ability to adhere *(P < 0.02)* and invade ***(P < 0.007)*** the white bass epithelial cell line WBE27 compared to WT. Values are mean ± SEM.

The Δpdi mutant was found to have approximately 2-fold lower capabilities of both adhering *(P < 0.02)* and invading *(P < 0.007)* host epithelial cells. To date, no
association between a cell surface deacetylase of a pathogenic bacterial species and the organism’s ability to bind or enter host cells has been described. This is a potentially significant finding, as experimental evidence suggests that a major contribution to S. iniae pathogenicity derives from its ability to cross cell barriers and evade host defenses (Eyngor et al., 2007; Miller & Neely, 2005).
DISCUSSION

Enzymes shown to deacetylate polysaccharide molecules on the surface of cells have been described as virulence factors for various pathogenic species. *Staphylococcus epidermidis* uses the surface attached polysaccharide deacetylase enzyme IcaB, to deacetylate poly-
\(N\)-acetylglucosamine (GlcNAc) residues of the PIA molecule. The deacetylation of PIA promotes virulence mechanisms of *S. epidermidis* such as biofilm formation, surface colonization, and resistance to human antimicrobial peptides (Vuong et al., 2004). In *Streptococcus pneumoniae* and *Listeria monocytogenes*, cell surface peptidoglycan deacetylases (*pgdA*s) have also been shown to play a role in virulence through mutagenesis studies (Boneca et al., 2007; Vollmer & Tomasz, 2002). These PgdA enzymes have been shown to \(N\)-deacetylate GlcNAc residues of the peptidoglycan cell wall and provide resistance to the activity of lysozyme. Additionally, PgdA has recently been described to promote survival in macrophages, and dampen defenses of the host innate immune response (Boneca et al., 2007).

Through our studies of the *Streptococcus iniae* Tn917 insertion mutant library we discovered the mutant TnM7 with attenuated virulence in a HSB model of meningoencephalitis. Using genomic pyrosequencing data we were able to map the transposon insertion of TnM7 to a location near a putative cell surface polysaccharide deacetylase gene in the *S. iniae* chromosome. After blasting this sequence against the genomes contained in the GenBank databases we termed this putative gene and its
predicted product \( pdi \) and \( Pdi \) respectively. Through phylogenetic and domain analyses we have concluded that \( S. iniae pdi \) is a paralog of the highly conserved Gram-positive \( pgdA \) gene, but that \( Pdi \) and \( PgdA \) are not likely to be functionally redundant. Although the two predicted proteins contain the same motif, differences still exist, as \( PgdA \) is likely a secreted protein and \( Pdi \) is likely anchored to the cell membrane as a surface protein. Moreover, we have discovered that the \( S. iniae \) genome contains a homologous ORF to \( pgdA \) in addition to \( pdi \). Because bacteria rarely maintain paralogs that have the exact same function, this leads us to believe that these two deacetylases may have different substrate specificity as well as different roles in \( S. iniae \) biology and/or virulence.

Observations of morphological differences between the isogenic \( \Delta pdi \) mutant and WT strain provide strong circumstantial evidence that \( pdi \) works on the cell surface of \( S. iniae \). The \( \Delta pdi \) mutant has an impaired ability to form chains characteristic of the WT \( S. iniae \) strain, but rather is predominantly found as singular cocci. We believe that this phenotype contributes to the observed increased buoyancy in liquid media, as longer chains are more likely to settle out of solution. Our results demonstrate opposite coinciding effects to those describe by Locke et al., where the \( Streptococcus iniae \) \( \Delta cpsD \) capsular polysaccharide mutant formed longer chains than WT \( S. iniae \) and showed a sinking phenotype in liquid media.
In our studies we found that the \textit{pdi} gene confers resistance to clearing by innate immune mechanisms as observed through the almost 2 fold decrease in survival of the $\Delta pdi$ mutant in whole blood, while survival was restored to WT levels in the $\Delta pdi + ppdi$ complimented strain. We hypothesize that decreased survival of the \textit{S. iniae} $\Delta pdi$ mutant in whole fish blood may be partially be explained by loss of chain formation, as larger groupings of streptococci may serve to physically exceed the phagocytic uptake capacity of host immune cells. Ongoing studies in our laboratory are examining the potential contribution of the \textit{pdi} gene to delaying the kinetics of phagocytic uptake. Deacetylation of \textit{S. iniae} cell surface molecules may also introduce significant changes in surface expressed molecular patterns that are less easily recognized by the host immune system, thus allowing WT \textit{S. iniae} to survive and persist in vivo (Arancibia \textit{et al.}, 2007; Nigro \textit{et al.}, 2007).

We believe a major correlation of the attenuated virulence of the $\Delta pdi$ mutant observed in the HSB infection model can be ascertained from the mutant’s decreased ability to survive in whole blood. In examining specific potential effectors of phagocytic killing, we have found no difference in the kinetic killing of WT and $\Delta pdi$ by hydrogen peroxide (a component of the oxidative burst) and the cationic HSB antimicrobial peptide moronecidin. As seen with cell surface deacetylases of other pathogenic species, the \textit{S. iniae pdi} gene confers significant resistance to the enzymatic activity of lysozyme, as the \textit{S. iniae} $\Delta pdi$ mutant is significantly more susceptible to lysozyme killing than WT in both log and stationary phase. The differences observed, however, are not nearly of the
same magnitude as the 3-log fold increases in sensitivity observed upon generation of polysaccharide deacetylase mutants of other pathogenic species (Boneca et al., 2007). For this reason, we believe the decreased resistance to lysozyme of the isogenic Δpdi mutant is not likely to fully account for its inhibited survival in whole blood or in its attenuated virulence observed in vivo. It is important to note that theses assays used lysozyme from a non-host species (chicken) and it is possible that different results would be observed in similar experiments performed using fish lysozyme.

The impaired ability of the Δpdi mutant to adhere to and invade host cells may indicate a novel molecular determinant of S. iniae virulence, as to our knowledge all virulence factors of S. iniae described to date have reported on either toxin production or direct impedance of host innate immune mechanisms. It has recently been shown that transcytosis across host cell barriers may be a key virulence mechanism of Streptococcus iniae for which adherence and invasion of host cells plays an integral role (Eyngor et al., 2007). Fish mortality associated with S. iniae infection is most often attributed to fatal meningoencephalitis, or meningitis, (Bercovier et al., 1997; Zlotkin et al., 1998) in which the bacterial agent must cross the blood brain barrier in order to cause the fatal pathogenicity of this disease. The S. iniae Δpdi mutant was observed to have an almost 2-fold decrease in the ability to adhere and invade host epithelial cells compared to that of WT. The impaired ability of the Δpdi mutant to adhere and invade host cells may render the bacteria less able to initiate proper contact needed for colonization and entry into variety of cells types including the blood brain barrier. Exploitation of an
intracellular niche may also allow the pathogen access to privileged sites less accessible
to host immune effectors. Additionally, the reduction of this functional virulence
mechanism in the *S. iniae* Δ*pdi* mutant may restrict the bacteria to extracellular locations
that are more inhospitable and subject to host defense clearance mechanisms (Eyngor *et al.*, 2007), thus contributing largely to the attenuation witnessed in ex vivo and in vivo
experiments.

In summary, we have described a putative cell surface polysaccharide deacetylase gene
(*pdi*) that we propose plays a novel role in *Streptococcus iniae* virulence towards fish.
The *pdi* gene has a predicted amino acid sequence containing similar active regions to
other well studied cell surface deacetylases, and through allelic-replacement mutagenesis
we have shown that *pdi* affects morphological phenotypes of *S. iniae* and plays a
detectable role in resistance to lysozyme killing. The *pdi* gene contributes to the ability
of *S. iniae* to adhere to and invade host cells, and because of its predicted cell surface
anchoring, it itself may function as an adhesin that directly interacts with host cell
receptors. As the true polysaccharide substrate of *pdi* is not known, we propose that in
addition to its own role, it could be involved in the posttranscriptional regulation of other
virulence molecules, which may themselves confer an ability to adhere and invade host
cells. From our data we believe that the Δ*pdi* gene could serve as a target for therapeutic
intervention against *S. iniae* infection or be used to develop attenuated strains for
vaccination programs in the aquaculture industry.
REFERENCES


