The novel polysaccharide deacetylase homologue Pdi contributes to virulence of the aquatic pathogen *Streptococcus iniae*

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INTRODUCTION

In the past few decades, the pathogen *Streptococcus iniae* has emerged as a major hindrance to aquaculture operations worldwide (Agnew & Barnes, 2007), causing economic losses measured in hundreds of millions of dollars annually. In addition, *S. iniae* has established itself as a zoonotic risk, especially in areas of the world that preferentially prepare and consume raw fish. Because little is known about the mechanisms of *S. iniae* pathogenesis or virulence factors, we established a high-throughput system combining whole-genome pyrosequencing and transposon mutagenesis that allowed us to identify virulence proteins, including Pdi, the polysaccharide deacetylase of *S. iniae*, that we describe here. Using bioinformatics tools, we identified a highly conserved signature motif in Pdi that is also conserved in the peptidoglycan deacetylase PgdA protein family. A Δpdi mutant was attenuated for virulence in the hybrid striped bass model and for survival in whole fish blood. Moreover, Pdi was found to promote bacterial resistance to lysozyme killing and the ability to adhere to and invade epithelial cells. On the other hand, there was no difference in the autolytic potential, resistance to oxidative killing or resistance to cationic antimicrobial peptides between *S. iniae* wild-type and Δpdi. In conclusion, we have demonstrated that pdi plays a role in the pathogenesis of *S. iniae*. Identification of Pdi and other *S. iniae* virulence proteins is a necessary initial step towards the development of appropriate preventive and therapeutic measures against diseases and economic losses caused by this pathogen.

Abbreviations: AMP, antimicrobial peptide; HSB, hybrid striped bass, i.p., intraperitoneal; PIA, polysaccharide intercellular adhesin, WT, wild-type.

The GenBank/EMBL/DDBJ accession number for the *pdi* sequence of *Streptococcus iniae* is FJ664396.

Four supplementary figures, showing a schematic representation of allelic replacement of the *S. iniae pdi* gene with cat and the results of comparison of haemolytic activity, culture buoyancy and growth analysis, investigation into the autolytic potential, and resistance to oxidative killing and antimicrobial peptides of WT *S. iniae* and the Δpdi mutant, are available with the online version of this paper.

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**The aquatic zoonotic pathogen *Streptococcus iniae* represents a threat to the worldwide aquaculture industry and poses a risk to humans who handle raw fish. Because little is known about the mechanisms of *S. iniae* pathogenesis or virulence factors, we established a high-throughput system combining whole-genome pyrosequencing and transposon mutagenesis that allowed us to identify virulence proteins, including Pdi, the polysaccharide deacetylase of *S. iniae*, that we describe here. Using bioinformatics tools, we identified a highly conserved signature motif in Pdi that is also conserved in the peptidoglycan deacetylase PgdA protein family. A Δpdi mutant was attenuated for virulence in the hybrid striped bass model and for survival in whole fish blood. Moreover, Pdi was found to promote bacterial resistance to lysozyme killing and the ability to adhere to and invade epithelial cells. On the other hand, there was no difference in the autolytic potential, resistance to oxidative killing or resistance to cationic antimicrobial peptides between *S. iniae* wild-type and Δpdi. In conclusion, we have demonstrated that *pdi* is involved in *S. iniae* adherence and invasion, lysozyme resistance and survival in fish blood, and have shown that *pdi* plays a role in the pathogenesis of *S. iniae*. Identification of Pdi and other *S. iniae* virulence proteins is a necessary initial step towards the development of appropriate preventive and therapeutic measures against diseases and economic losses caused by this pathogen.**
attenuation in hybrid striped bass (HSB; Morone chrysops × Morone saxatilis) (Buchanan et al., 2005). The analysis and study of mutants from this library, along with candidate gene approaches, has previously served to elucidate virulence factors contributing to S. iniae pathogenicity in fish, including phosphoglucomutase (Buchanan et al., 2005), capsular polysaccharide (Barnes et al., 2003; Locke et al., 2007a; and capsular polysaccharide (Barlow et al., 2007; Miller & Neely, 2005). In addition, recent whole-genome pyrosequencing of S. iniae strain K288 has uncovered an nrg-like pathogenicity locus (Locke et al., 2008), which includes an M-like protein virulence factor similar to the fibrinogen-binding proteins discovered in S. iniae strains QMA0076 and QMA0131 (Baiano et al., 2008).

There has been a recent appreciation of the role of bacterial deacetylases in virulence. In Staphylococcus epidermidis, exopolysaccharide deacetylation has been shown to be crucial for biofilm formation, surface colonization, resistance to neutrophil phagocytosis, and resistance to cationic antimicrobial peptides (AMPs) (Vuong et al., 2004). Protection against host defences by peptidoglycan deacetylases has also been reported for Listeria monocytogenes (Boneca et al., 2007) and Streptococcus pneumoniae (Vollmer & Tomasz, 2000, 2002). In both cases, modification of the cell wall by a peptidoglycan deacetylase (PgdA) provides increased resistance to lysozyme and is essential for full virulence of the pathogen in a small animal model of infection.

In this study, transposon mutagenesis coupled with whole-genome pyrosequencing allowed us to identify a polysaccharide deacetylation gene, pdi (polysaccharide deacetylation of S. iniae), implicated in S. iniae virulence. We provide an analysis of the conserved amino acid residues of Pdi homologues in different microbial genomes. By targeted mutagenesis, we confirm a contribution of the pdi gene to S. iniae virulence in a fish infection model, and use in vitro assays to probe potential mechanistic associations of pdi with augmented lysozyme resistance, enhanced blood survival and increased adherence to and invasion of fish epithelial cells.

METHODS

Bacterial strains, culture, transformations, and DNA techniques. Wild-type (WT) S. iniae strain K288 was isolated from the brain of a diseased HSB at the Kent SeaTech facility in Mecca, CA, USA (Buchanan et al., 2005). Escherichia coli used in cloning was grown on Luria agar at 37 °C with antibiotic selection of 500 μg erthyromycin ml⁻¹ (Erm), 100 μg spectinomycin ml⁻¹, and 20 μg chloramphenicol (Cm) ml⁻¹, when appropriate. Mach 1 chemically competent E. coli (Invitrogen) and MC1061 electrocompetent E. coli, used for plasmid propagation during transformation, were recovered through growth at 30 °C or 37 °C, when appropriate, with shaking in Super Optimal Broth with Catabolite Repression (S.O.C.) medium (Invitrogen). A PureLink Quick plasmid miniprep kit (Invitrogen) was used for plasmid isolation. S. iniae was grown in Todd–Hewitt broth (THB) without shaking or in Todd–Hewitt agar (THA) plates at 30 °C, unless otherwise indicated, with antibiotic selection of 2 μg Cm ml⁻¹, or 5 μg Erm ml⁻¹ when required. The DNA Easy Tissue kit (Qiagen) was used for the isolation of S. iniae genomic DNA. Dilution plating on THA was used for enumeration of c.f.u. for in vitro assays. β-haemolytic 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-exponential phase (OD600 0.40), unless otherwise stated. The bacteria were rendered electrically competent for transformation by the addition of 0.6% glycine to THB 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. The temperature-sensitive plasmid pTV10K containing transposon Tn917 was used for transposon mutagenesis of strain K288 as previously described (Buchanan et al., 2005). Briefly, individual colonies of K288 transformed with pTV10K were inoculated in THB plus kanamycin (500 μg ml⁻¹) and grown to OD600 0.90 at a permissive temperature (30 °C) for plasmid replication. Cultures were diluted 1:100 in THB with Erm (5 μg ml⁻¹) and grown at a non-permissive temperature (37 °C) to OD600 0.90, then plated on THA with Erm for the isolation of candidate insertion mutants. Single random transposon insertions were verified through Southern blot analysis of a subset of the library mutants. Over 1000 mutants were screened for attenuation in vivo in an HSB challenge model by intraperitoneal (i.p.) injection. (Buchanan et al., 2005). Screening the transposon library in this manner revealed over 40 attenuated mutants, including mutant TnM7.

Identification of mutated genes. The Tn917 insertion sites were identified in the candidate insertion mutants 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 by the use of the internal forward primer 5'-AATCTGTAACCACTAATAACCTC-3'. The external forward primer 5'-AATGTAACAAATACACGGAG-3' was used for sequencing the PCR fragments.

Sequence assembly and localization of pdi. Contiguous sequences used for ORF determination and bioinformatics analysis were created from an automated assembly of pyrosequencing results (454 Life Sciences, Roche). This primary assembly was generated by the Phred/Phrap/Consed suite (Gordon et al., 1998) and resulted in 1865 contigs ranging in size from 51 bp to 22 kb (Locke et al., 2008). Without further assembly, the 1865 contigs were used for building a local S. iniae K288 BLAST database, and then a local copy of BLAST version 2.2.14 (Altschul et al., 1997) was used for finding the contiguous sequences matching the pdi-deficient transposon mutant, TnM7, in S. iniae K288. Vector NTI (Invitrogen) was used in ORF identification and graphic representation of chromosomal positioning of pdi (GenBank accession number: FJ604396).

Bioinformatics and phylogenetic analysis of Pdi and its homologous proteins. Pdi homologues in different microbial genomes were retrieved from the National Microbial Pathogen Data Resource, (NMPDR; McNell et al., 2007), by the use of the SEED similarity tool (Overbeek et al., 2005). SignalP algorithm (version 3.0) was used to screen the protein sequences for leader peptides ( Bendtsen et al., 2004). Several tools were used for motif finding and protein family analysis, including InterPro (Apweiler et al., 2001),
Pfam (Finn et al., 2008), and FigFam (Aziz et al., 2008). Further sequence analysis was performed on the Biology Workbench analysis platform (Subramaniam, 1998), URL: http://workbench.sdsc.edu). The specific Workbench tools that were used are: CLUSTAL W (Higgins et al., 1994; Thompson et al., 1994) for sequence alignment, PHYLIP (Felsenstein, 1997) for primary phylogenetic analysis, and Boxshade for sequence colouring. For phylogenetic analysis by the maximum-likelihood method, we used the Phylogeny.fr online server (DeCSteph et al., 2008) and we edited the tree using the FigTree software (http://tree.bio.ed.ac.uk/software/figtree). The Baylor S. iniae genome project (http://www.hgsbc.tmc.edu/ BCM/blast/microbialblast/organism =Sinaiae) was utilized for comparative analyses.

**Allelic exchange mutagenesis of the pdi locus.** To confirm that the attenuation of TnsM7 was a result of the inactivation of the pdi gene rather than a polar effect of transposon insertion or spontaneous mutation elsewhere in the chromosome, we generated an isogenic Δpdi mutant by precise in-frame allelic replacement of pdi with chloramphenicol acetyltransferase (cat) in the WT S. iniae strain K288 (see Supplementary Fig. S1 available with the online version of this paper). 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 cat gene from pACYC (Nakano et al., 1995). Fusion PCR was then used to combine upstream and downstream products with a 660 bp ampiclon of the cat gene (Buchanan et al., 2006). The fusion product resulting from this PCR contained an in-frame substitution of pdi with cat and was subcloned into the Gateway entry vector pCR8/GW/TOPO. This vector was then used to transform chemically competent Mach1 E. coli cells (Invitrogen). Plasmid DNA was extracted, and the fusion PCR amplicon was transferred into the temperature-sensitive knock-out vector pKODestErm (Locke et al., 2007a) via an attL→attR (LR) recombination reaction to create the knockout plasmid pKOpdi. After its propagation in MCI1061 E. coli, the pKOpdi 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). Differential antibiotic selection of Cm’ and Erm’ allowed identification of candidate colonies as potential allelic exchange mutants. Targeted in-frame replacement was confirmed through PCR-documenting the desired insertion of cat and the absence of the pdi sequence in chromosomal DNA isolated from the Δpdi mutant.

**In vivo fish challenges.** Groups of 10 HSB fingerlings (~15.8±3.7 g) were challenged through IP injection with either WT or mutant S. iniae bacteria from mid-exponential-phase cultures. Injection with a 26.5 gauge needle was used to deliver either 1×10^6 or 1×10^7 c.f.u. 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 their survival was monitored daily.

**Whole blood survival.** Heparinized blood collected for bacterial survival assays was taken and pooled from four to six HSB fingerlings. Fresh blood was collected via caval vein blood draw. A 75 μl sample of blood was added to 2 ml siliconized plastic centrifuge tubes containing approximately 5×10^7 c.f.u. of a mid-exponential-phase culture in a 25 μl volume and incubated at 30 °C for 1 h on an orbital shaker (225 r.p.m.). Reactions were performed in quadruplicate for each strain tested. A 50 μl volume from each reaction was spread on THA, and a control plate was prepared at the initiation of the assay as a reference for starting c.f.u. Each experiment was repeated three times.

**Resistance to oxidative killing.** The Δpdi mutant and WT S. iniae were grown to mid-exponential phase and diluted in PBS such that adding 100 μl of culture to a 96-well plate delivered 2×10^3 c.f.u. per well. A 100 μl volume of dilute H_2O_2 was then added to each well, bringing the final concentration to 0.03, 0.06 or 0.09 %. After 1 h incubation at 30 °C, the reaction was quenched by the addition of 10 μl of a 1000 U catalase ml^-1 solution in PBS. c.f.u. 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.

**AMP susceptibility assays.** Mid-exponential-phase cultures of S. iniae were diluted in fresh THB, and 1×10^7 c.f.u. in a total volume of 180 μl was added to replicate wells of a 96-well plate. Dilutions of AMPS were prepared in deionized H_2O (D_2O), and 20 μl was added to experimental wells for a final concentration of 1.5 μM moroecin (Lauth et al., 2002) or 60 μM polymyxin B (Sigma). D_2O alone was used as a negative control. For the measurement of antimicrobial killing kinetics, 25 μl aliquots were taken from each well at various time points, serially diluted in PBS and plated on THA for surviving c.f.u. determination. Each experiment was performed in triplicate.

**Lysozyme sensitivity assays.** For the determination of the sensitivity of S. iniae to lysozyme, cultures were washed once in PBS, and resuspended in PBS to OD_600 0.4. Cultures were then diluted 1:1000 in DMEM (Gibco) plus 10 % FBS (Gibco) plus 20 % THB, and 180 μl diluted bacteria (3.6×10^4 c.f.u.) was added to a 96-well plate. A 20 μl volume of chicken egg white lysozyme (Sigma) was added to a final concentration of 80 μg ml^-1 to each well. Plates were incubated at 30 °C and serial dilutions were plated on THA after 30 min to enumerate surviving bacteria. To test sensitivity of growing cultures to lysozyme, the above procedure was followed, except that bacteria were allowed to grow for 1 h (sufficient time to reach exponential-phase growth) prior to adding lysozyme, incubating for 30 min, and plating to enumerate surviving bacteria.

To test the sensitivity of a growing culture of Δpdi S. iniae to lysozyme, cultures were grown in DMEM/10 % FBS/20 % THB to mid-exponential phase (OD_600 0.4), and diluted 1:20 in the same medium in glass test tubes and incubated at 30 °C. Growth was monitored by measuring the OD_600 and lysozyme was added to a final concentration of 80 μg ml^-1 during exponential-phase growth (around OD_600 0.1). All growth curves were conducted in triplicate.

**Invasion and adherence assays.** Invasion and adherence assays were carried out in 96-well collagenized plates (Nunc). One day prior to each assay, WBE27 cells were seeded in the wells at a density of ~1×10^5 cells per well in 100 μl DMEM 10 % FBS medium and were allowed to grow to confluence overnight. Cell culture medium was removed, and 100 μl mid-exponential-phase bacterial cultures diluted 1:40 in DMEM 2 % FBS medium was added to confluent cells at an m.o.i. of 5 (bacteria to cells). The plate was then centrifuged at 350 g for 15 min to allow contact between bacteria and the cell monolayer. At 30 min post-centrifugation, the medium was removed, and the wells were washed four times with 200 μl DMEM 2 % FBS for the removal of any non-adoherent bacteria. The cells were then lysed by trituration with 100 μl 0.01 % Triton X-100, and 25 μl was removed, serially diluted and plated on THA for the enumeration of adherent c.f.u. Invasion assays were carried out in a similar manner, except that at 1 h post centrifugation, cell medium was removed, wells were washed once, and then were incubated with 150 μl fresh DMEM containing 300 μg gentamicin ml^-1 (Invitrogen) and 30 μg penicillin ml^-1 (Invitrogen) for an additional hour. All strains of S. iniae were tested for susceptibility to these antibiotic concentrations at 1 h. Cells were then washed three more times and lysed by trituration. Serial dilutions of lysed cell supernatant were plated on THA for the enumeration of surviving intracellular bacteria. 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-exponential phase and diluted 1:10 in THB in a 96-well plate. Eight replicates for each strain were monitored for growth over 12 h by the use of an EL808 plate reader and Gen5 software (BioTek Instruments). The incubation temperature was held constant at 30 °C, and the plates were shaken before each reading (every 30 min). Autolysis assays were performed as previously described (Kristian et al., 2005); an EL808 plate reader and Gen5 software were used to monitor decreases in OD.

Statistical analyses. Data resulting from *in vitro* assays were assessed by 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 ± SE) are from single representative assays unless otherwise stated. Fish challenges were repeated with similar results. Statistical values obtained from mortality curves of *in vivo* challenges were generated from chi-squared tests.

RESULTS

Identification of *S. iniae* virulence gene *pdi*

Using an HSB infection model, we identified the attenuated transposon mutant TnM7 from a Tn917 chromosomal insertion library of WT *S. iniae* strain K288, which had been created previously (Buchanan et al., 2005). TnM7 is one of approximately 40 attenuated *S. iniae* transposon mutants that were identified through initial virulence screening in HSB. To identify the gene or operon that had been disrupted by transposon insertion in the TnM7 chromosome, we used single-primer PCR (Karlyshev et al., 2000) to amplify genomic regions around the transposon insertion site and sequence the amplified DNA fragments.

The transposon insertion mapped immediately upstream of an ORF encoding a potential protein with high sequence similarity to bacterial polysaccharide deacetylases (by BLASTX analysis). We termed this putative gene and its predicted product *pdi* and Pdi, respectively. The full sequence and chromosomal context of *pdi* were identified using BLASTN and a genomic library of contiguous sequence data generated from the pyrosequencing of the parent *S. iniae* strain K288 genome (Locke et al., 2008).

Sequence analysis of Pdi

Primary analysis of *S. iniae* Pdi showed that the protein possesses a probable signal peptide (SignalP probability = 0.875; cleavage probability = 0.716), suggesting that it is membrane-bound or, less likely, secreted. A motif search and protein family analysis using Interpro and Pfam revealed that Pdi, and its orthologues, share a deacetylase enzymic motif near the C terminus (Pfam Id Polysacc_deac_1 PF01522). This motif is also shared by the protein PgdA (Fig. 1b), which is highly conserved in many bacteria and has been well characterized for its role in virulence in *S. pneumoniae* (Vollmer & Tomasz, 2000) and *Listeria monocytogenes* (Boneca et al., 2007). Using PgdA sequence data from *S. pneumoniae* and *Streptococcus pyogenes*, a search of our *S. iniae* genomic library and the Baylor *S. iniae* genome project revealed a PgdA orthologue in *S. iniae* that is distinct from Pdi (Fig. 1), confirming that Pdi and PgdA are paralogous proteins. In fact, FigFam analysis revealed that Pdi and PgdA belong to two distinct protein families (FIG012537 and FIG015749, respectively), and that most streptococcal species whose genomes have been sequenced possess both proteins (Table 2).

Pdi homologues were found in other microbial genomes, and their sequences were aligned to the Pdi sequence (Fig. 1a) to infer a phylogenetic tree (Fig. 2). Pdi is conserved in the genomes of *S. iniae* phylogenetic neighbours (Table 2, Fig. 2). The conservation of its parologue, PgdA, in all streptococci (Fig. 2) suggests that the proteins are not functionally redundant. Paralogues are often generated from gene duplication events that create variants of proteins with different substrate specificities or allosteric regulatory properties (Hooper & Berg, 2003). Although there is extensive variability within and between the Pdi and PgdA family members (Figs 1 and 2), these homologues share highly conserved residues (Fig. 1).

Targeted mutagenesis of the *S. iniae* *pdi* gene

Upon successful generation of an isogenic Δpdi mutant by precise in-frame allelic replacement of *pdi* with *cat* in the WT *S. iniae* strain (Supplementary Fig. S1), we used the HSB i.p. challenge model to further evaluate the virulence of the newly created Δpdi knockout. Significant attenuation was observed: no mortality occurred in fish challenged with the Δpdi knockout at a dose that killed 70% of fish that received WT (*P* = 0.0011), and only 30% mortality occurred in fish challenged with the Δpdi knockout at a dose that killed 90% of WT-injected fish (*P* = 0.0031) (Fig. 3).

Effects of *pdi* deletion on *S. iniae* chain length and buoyancy

WT *S. iniae* bacteria occur in short chains and are β-haemolytic on sheep blood agar (Fig. 4a and Supplementary Fig. S2b). The Δpdi allelic exchange mutant lost its ability to maintain association in pairs or short chains, and cocci were seen predominantly as single cells (Fig. 4b). Expression of the *pdi* gene in the constitutive high-expression plasmid pDestErm was used to complement the Δpdi knockout mutant and restored chain length (Fig. 4c).

Cultures of the Δpdi mutant grown in liquid media exhibited greater buoyancy than the WT *S. iniae* parent strain (Supplementary Fig. S2a); the WT phenotype was partially restored in the plasmid-complemented strain. No differences in β-haemolytic activity of the strains were observed on sheep blood agar plates (Supplementary Fig. S2b). A similar growth profile was observed between the WT and the mutant through exponential phase (*P* = 0.6);
thus, no apparent gross defect in viability or fitness is conferred from loss of the \( pdi \) gene (Supplementary Fig. S2c). Phenotypes of impaired chain formation and differences in culture buoyancy of \( D_{pdi} \) imply that the activity of Pdi involves alteration of molecules on the cell surface of \( S.\) \( iniae \).

**Effect of \( pdi \) mutation on \( S.\) \( iniae \) susceptibility to lysozyme killing**

We found that the \( S.\) \( iniae \) \( \Delta pdi \) mutant was significantly \((P<0.0005)\) more sensitive to lysozyme than WT (Fig. 5a). In a manner similar to that reported for deacetylase

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**Fig. 1.** Alignment of \( S.\) \( iniae \) Pdi and closely related proteins. These representations depict orthologues of Pdi found in other species as well as paralogous peptidoglycan deacetylase proteins (PgdA). (a) Amino acid alignment of conserved regions between polysaccharide deacetylases and peptidoglycan deacetylases that are found in multiple species. A full list of abbreviations is provided in Table 1. (b) Alignment of the predicted \( S.\) \( iniae \) Pdi and PgdA amino acid sequences shows low overall similarity but highly conserved patches, suggesting that the two proteins are paralogous and that Pdi has novel functional features in addition to the deacetylase motif shared with PgdA.
mutants in other bacterial species, we observed markedly reduced sensitivity in cells in exponential-phase growth (Boneca et al., 2007; Psylinakis et al., 2005; Vollmer & Tomasz, 2000) (Fig. 5a, b).

No difference in autolytic potential between S. iniae WT and the Δpdi mutant

We recognized the possibility that the virulence attenuation observed in HSB could be due to a greater propensity for autolysis in the Δpdi mutant versus WT S. iniae; however, our experiments showed that the rates of autolysis for the two strains in a standardized assay did not differ significantly ($P = 0.95$) (Supplementary Fig. S3).

S. iniae pdi promotes survival in blood

To investigate additional possible mechanisms for the in vivo attenuation of the Δpdi mutant, we assessed the ability of the Δpdi mutant to survive in fresh fish blood. The mutant was significantly less able to survive in whole blood than WT ($P < 0.002$), while the complemented strain significantly restored whole blood survival to levels similar to those of the WT ($P < 0.01$) (Fig. 6).

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

Reactive oxygen species and AMPs are mechanisms used by phagocytes and other immune cell types to mediate clearance of bacterial invaders in whole blood (Gallo & Nizet, 2003; Locke et al., 2007a; Soehnlein et al., 2008). To further analyse 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 found no significant difference ($P = 0.8$) in the susceptibility to hydrogen peroxide or AMP killing between the two strains (Supplementary Fig. S4a–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 oxidative killing mechanisms or AMPs.

S. iniae Δpdi mutant shows an impaired ability to adhere to and invade epithelial cells

Compared with the WT parent strain, we found the Δpdi mutant to be significantly reduced, by approximately twofold, in its ability to adhere to ($P < 0.02$) and invade ($P < 0.007$) fish epithelial cells (white bass epithelial cell line WBE27) (Fig. 7).

DISCUSSION

Enzymes that deacetylate polysaccharide molecules on the surface of bacterial cells have only recently been appreciated as virulence factors for various pathogenic species. *Staphylococcus epidermidis* uses the surface-attached polysaccharide deacetylase enzyme IcaB to deacetylase poly-$N$-acetylglucosamine (GlcNAc) residues of the polysaccharide...
**Fig. 2.** Phylogenetic analysis of Pdi homologues. A maximum-likelihood tree produced by MUSCLE alignment followed by PhyML analysis (http://www.phylogeny.fr) with 100 bootstrap tests. These bootstrap values are shown as branch labels. The tree shows two major clusters, one of PgdA orthologues and another of Pdi orthologues. *S. iniae* Pdi and PgdA are underlined.
PgdA has been reported to promote survival of L. monocytogenes (Vollmer & Tomasz, 2000, 2002). Additionally, peptidoglycan cell wall and provide resistance to the activity of N-acetylglucosamine (GlcNAc) deacetylases (Blair et al., 2005). These results indicate a possible role for deacetylation as a general mechanism for evasion of host innate immune responses, primarily through modification of GlcNAc residues.

Through our studies of our S. iniae Tn917 insertion mutant library (Buchanan et al., 2005), we discovered mutant TnM7 with attenuated virulence in an HSB model of fish meningoencephalitis. Due to the unavailability of a fully assembled S. iniae genome sequence, we used a genomic library that we created by pyrosequencing S. iniae strain K288, and were able to map the transposon insertion of TnM7 to a location upstream of a putative polysaccharide deacetylase gene (pdi) in the S. iniae chromosome. Through phylogenetic and protein family analyses, we have concluded that S. iniae pdi is a paralogue of the highly conserved Gram-positive pgdA gene, but that Pdi and PgdA are likely not redundant. Although the two predicted proteins contain the same motif (Pfam PF01522) and share conserved amino acid residues, they belong to different protein families (Fig012537 and Fig015749, respectively). Moreover, while S. pneumoniae genomes only have PgdA orthologues, the S. iniae genome contains the two paralogues, pdi and pgdA. The conservation of Pdi in the genomes of bacteria closely related to S. iniae suggests that this protein is ancestral in streptococci rather than horizontally acquired, and the conservation of its parologue, PgdA, in all streptococci suggests that the proteins are not functionally redundant. Paralogues are often generated from gene duplication events that create variants of proteins with different substrate specificities or allosteric regulatory properties (Hooper & Berg, 2003). Taken together, our in silico analyses of the similarity and variability among Pdi and PgdA homologues imply that: (1) while Pdi and PgdA are two distinct paralogues, they share a molecular motif that is most likely responsible for their deacetylase enzymic activity; (2) while pdi is a gene likely to be responsible for the production of a deacetylase with some sequence similarity to PgdA, it is in fact a novel gene that has yet to be characterized. Thus, any identified roles of the S. iniae pdi gene in virulence could be divergent and distinct from those attributed to pgdA. Based on these results, pdi and pgdA may both have the capacity to play unique roles in the virulence of other streptococcal pathogens that contain both genes, such as S. pyogenes and Streptococcus agalactiae.

Observations of morphological differences between the isogenic Δpdi mutant and WT strain provide strong circumstantial evidence that pdi works on the cell surface intercellular adhesin (PIA) molecule. The deacetylation of PIA promotes virulence mechanisms of Staphylococcus epidermidis such as biofilm formation, surface colonization and resistance to human AMPs (Vuong et al., 2004). In S. pneumoniae and Listeria monocytogenes, mutagenesis studies have revealed that cell surface peptidoglycan deacetylases (pgdAs) play a role in virulence (Boneca et al., 2007; Vollmer & Tomasz, 2002). The PgdA enzymes are metal-dependent deacetylases and members of the family 4 carbohydrate esterases (Blair et al., 2005). In S. pneumoniae, PgdA has been shown to de-N-acetylate GlcNAc residues of the peptidoglycan cell wall and provide resistance to the activity of lysozyme (Vollmer & Tomasz, 2000, 2002). Additionally, PgdA has been reported to promote survival of L. monocytogenes in macrophages and also to confer resistance to host lysozyme (Boneca et al., 2007). These results indicate a possible role for deacetylation as a general mechanism for evasion of host innate immune responses, primarily through modification of GlcNAc residues.

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Observations of morphological differences between the isogenic Δpdi mutant and WT strain provide strong circumstantial evidence that pdi works on the cell surface deacetylation of the PIA molecule.
of \textit{S. iniae}. The \textit{Δpdi} mutant has an impaired ability to form chains characteristic of the WT \textit{S. iniae} strain, and its cells are predominantly found as single cocci. We believe that this phenotype contributes to the observed increased buoyancy in liquid media, since longer chains are more likely to settle out of solution. Because complementation of the mutant restores both of these phenotypes, we hypothesize that the increased buoyancy of \textit{Δpdi} liquid cultures and the inability to form chains are due to the loss of Pdi function at the \textit{S. iniae} cell surface. A peptidoglycan deacetylase in \textit{Lactococcus lactis} that affects chain length morphology and resistance to lysozyme killing also protects against cell autolysis (Meyrand \textit{et al.}, 2007). However, we were unable to detect any differences in autolysis.

The attenuated virulence of the \textit{Δpdi} mutant observed in the HSB infection model is reflected in the decreased ability of the mutant to survive in whole blood. Gene deletion strains of \textit{S. iniae}, \textit{S. pyogenes} or \textit{S. agalactiae} with diminished ability to survive in fresh blood show reduced animal virulence (Buchanan \textit{et al.}, 2005; Datta \textit{et al.}, 2005; Liu \textit{et al.}, 2004; Locke \textit{et al.}, 2007a). These results suggest a fitness advantage conferred by the \textit{S. iniae pdi} gene against immune clearance by cells or soluble components present in the fish blood circulation. One possible explanation is that the Pdi-dependent ability of \textit{S. iniae} to form longer chains reduces the efficiency of phagocytic uptake and clearance. Another possible explanation for this decreased survival is that deacetylation of \textit{S. iniae} cell surface molecules introduces significant changes in molecular patterns that are otherwise easily recognized by the host immune system, thus allowing WT \textit{S. iniae} to survive and persist \textit{in vivo} (Arancibia \textit{et al.}, 2007; Nigro \textit{et al.}, 2008). In examining specific potential effectors of phagocytic killing, we have found no difference in the kinetics of the killing of WT and \textit{Δpdi} by hydrogen peroxide (a component of the oxidative burst) and the cationic HSB AMP moronecidin.

Resistance to cell wall hydrolysis by lysozyme has been reported as a key protective role of the peptidoglycan deacetylases \textit{pgdA} in bacterial species such as \textit{Bacillus cereus} (Psilakis \textit{et al.}, 2005), \textit{S. pneumoniae} (Vollmer & Tomasz, 2000) and \textit{L. monocytogenes} (Boneca \textit{et al.}, 2007). Notably, \textit{pgdA} mutants are sensitive to lysozyme killing primarily in stationary phase and are resistant when cells are actively growing. Similarly, \textit{S. iniae Δpdi} mutants express an increased sensitivity to the enzymic activity of lysozyme when not growing, but \textit{Δpdi} mutant cells in growth phase are apparently no more resistant to lysozyme killing. Yet, while the \textit{S. iniae Δpdi} mutant is significantly...
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 (white bars) was observed to have a significantly reduced ability to adhere to *(P<0.02)* and invade ***(P<0.007)*** WBE27 cells compared with WT (black bars). Values are mean ± SEM.

The impaired ability of the Δpdi mutant to adhere to and invade host cells may indicate a novel role for *pdi* in *S. iniae* virulence. To date, no association between a polysaccharide deacetylase of a pathogenic bacterial species and the ability of an organism to bind to or enter host cells has been described. Adherence to 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, including *S. iniae* (Eyngor et al., 2007; Locke et al., 2007b; Rajam et al., 2008; Williamson et al., 2008). It has recently been shown that transcytosis across host cell barriers may be a key virulence mechanism of *S. iniae*, for which adherence to and invasion of host cells would play an integral role (Eyngor et al., 2007). Fish mortality associated with *S. iniae* infection is most often attributed to fatal meningoencephalitis (Agnew & Barnes, 2007; Locke et al., 2007b), in which the bacterial agent must cross the blood–brain barrier to cause the fatal pathology of this disease. Although our HSB model of virulence in fish was conducted by i.p. injection, the impaired ability of the Δpdi mutant to adhere to and invade host cells may render the bacteria less able to initiate the proper contact needed for dissemination and entry into a variety of tissue microenvironments, including the central nervous system (Zlotkin et al., 2003). In addition, the reduction of this virulence mechanism may leave the bacteria more susceptible to unfavourable host conditions and cause the bacteria to forfeit the ability to mechanically hide from host defences (Eyngor et al., 2007), thus contributing largely to the attenuation witnessed in *ex vivo* and *in vivo* experiments. Apparently, removal of deacetylase activity in the Δpdi mutant interferes with certain steps in the pathogenic process by decreasing attachment to and invasion of epithelial cells, while simultaneously increasing sensitivity to host lysozyme and clearance by host phagocytes. In our fish infection challenge, these phenomena may play a critical role in determining the outcome of infection.

In summary, as a product of high-throughput genome-wide approaches, we discovered a putative cell surface polysaccharide deacetylase gene (*pdi*) that we suggest plays a novel role in *S. iniae* fish virulence. The predicted amino acid sequence of the *pdi* gene product contains active residues similar 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 role in resistance to lysozyme killing. We propose that the sensitivity of the Δpdi mutant to lysozyme, and its shortened chain length, impaired ability to survive in whole blood and impaired ability to adhere to and invade host cells, indicate mechanisms of pathogenesis associated with this gene and define Pdi as a novel and significant virulence factor for *S. iniae* infection. Because of its predicted membrane-localization signal peptide, Pdi 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 post-transcriptional regulation of other virulence molecules, which may...
themselves confer an ability to adhere to and invade host cells. This virulence factor is of interest as a recombinant vaccine target or for the development of attenuated Δpdi strains for vaccination programmes in the aquaculture industry.

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