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

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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 Δ*pdi* 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 Δ*pdi*. 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.

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 (Lau *et al.*, 2003). To date, at least 25 human cases of invasive streptococcal infection attributed to *S. iniae* have been confirmed in the USA, Canada, China and Taiwan, many of which were in immunocompromised patients (Agnew & Barnes, 2007; Sun *et al.*, 2007; Weinstein *et al.*, 1997); since there is currently no prospective epidemiological surveillance for human *S. iniae* infections, the true number may be much higher (Facklam *et al.*, 2005; Lau *et al.*, 2006).

In spite of the economic and human health risks that *S. iniae* presents, a fully assembled genome sequence for this emerging pathogen is not yet available, and very little is known about its disease mechanisms. To better understand the molecular genetic basis of virulence in this versatile pathogen, we created a library of randomly generated transposon mutants that we screened for virulence

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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 Δ*pdi* mutant, are available with the online version of this paper.

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), streptolysin S (Fuller *et al.*, 2002; Locke *et al.*, 2007a) and capsular polysaccharide (Barnes *et al.*, 2003; Locke *et al.*, 2007a; Lowe *et al.*, 2007; Miller & Neely, 2005). In addition, recent whole-genome pyrosequencing of *S. iniae* strain K288 has uncovered an *mga*-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 deacetylase gene, *pdi* (polysaccharide deacetylase of *S. iniae*), implicated in *S. iniae* virulence. We provide an analysis of the conserved amino acid residues of Pdi homologues in representative Gram-positive bacteria. 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 erythromycin 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 on 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 (OD$_{600}$ 0.40), unless otherwise stated. The bacteria were rendered electrocompetent 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 % CO$_2$. 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 pTV1OK 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 pTV1OK were inoculated in THB plus kanamycin (500 μg ml^{-1}) and grown to OD$_{600}$ 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 OD$_{600}$ 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′-AATCTGTACCACTAATAACTC-3′. The external forward primer 5′-AATGTACAAAATAACAGCGA-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: FJ664396).

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; McNeil *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.*, 1996; 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 (Dereeper *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.hgsc.bcm.tmc.edu/bcm/blast/microbialblast.cgi?organism =Siniae) was utilized for comparative analyses.

Allelic exchange mutagenesis of the *pdi* locus. To confirm that the attenuation of TnM7 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 Δ*pdi* 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 amplicon 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 Mach 1 *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 MC1061 *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 anti-biotic selection of Cm^r and Erm^s 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 Δ*pdi* mutant.

***In vivo* fish challenges.** Groups of 10 HSB fingerlings (~15.8±3.7 g) were challenged through i.p. 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 1×10^5 or 1×10^4 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 caudal vein blood draw. A 75 μl sample of blood was added to 2 ml siliconized plastic centrifuge tubes containing approximately 5×10^2 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 Δ*pdi* 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 2×10^5 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 1×10^5 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 (dH_2O), 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_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 Δ*pdi* *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-adherent 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 Tn*917* 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 paralogue, 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 Δ*pdi* 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 Δ*pdi* knockout. Significant attenuation was observed: no mortality occurred in fish challenged with the Δ*pdi* 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 Δ*pdi* 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 Δ*pdi* 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 Δ*pdi* knockout mutant and restored chain length (Fig. 4c).

Cultures of the Δ*pdi* 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);

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(a) SEq_zoo_PoDe   NLIVaPDlFEsqLKaMKEaGYyFLSpeEayrVLsan-----------------ElPaeKV
    SPy_M1_PoDe    NLIVnPDlFDqqLqkMKDeGYyFLSpeEvyraLsnn-----------------ElPakKV
    SIn_UCSD_PdI   NLIVaPDvFEshIKqLSeAGYyFLSpeEayrVLsan-----------------ElPsdKV
    SAg_2603V_R_PoD NLIVaPDiFEshIKrLKkeGYyFLapnEayraLnen-----------------alPekKV
    LLa_la_Il1403_P NnIn-ivtFEneLKaLKEkGYttVSgsDaekILttk-----------------EkPsdKM
    SAu_aureus_N315 NysVsqsqFEsqIKwLKshdakFLTlkEflyykkg-----------------kfP-kRs
    ECo_F11_PoDe   stttsvraFnnqMawLRDrGYatLSmaqlegyVknn-----------------inlpaRa
    BAn_Ames_PoDe  -LyVpPEqfFEkhMqyLKDnGYtmLTferwndInrvn----------------Kp
    SPn_R6_PgdA    QIILyPspvvenLeeIalpvsaFfdviqssyLLekdaal----------yqsyfDkkhqKV
    SPy_M1_PgdA    snsLlsDgFEvksgnLifdkkltIplttlfdVInpdflansdraaydnyrtykEdhpkKL
    LLa_Il1403_PgdA kapyeksdkEalaKqVnadkqilIerqkeaeLIanvqn-------------qyankKL
    LMo_PgdA       eItnselilpvkIpgapEpnkvkVklaDiassVnkrylp-----ssvkvpevpkaktnKr
    consensus      nliv-pd-fe--lk-lke-gy-fls--e---vl---------------e-p--kv
                   241......250.......260.......270.......280.......290.......

    SEq_zoo_PoDe   VWLTFDDs-mvdfYnvAFPILKKYdIKATNnVITGfteag----naanLTldqMKEMKqa
    SPy_M1_PoDe    VWLTFDDs-midfYnvAYPILKKYdaKATNnVITGltemg----saanLTlkqMKEMKqv
    SIn_UCSD_PdI   VWLTFDDs-midfYrvAYPILKKYqakATNnVITGftqnn----svanLTidqMKEMKkh
    SAg_2603V_R_PoD IWITFDDG-nadfYtkAYPILKKYkVKATNnIITGfVqeg----resnLnvqqMlEMKqn
    LLa_la_Il1403_P VWLTFDDG-svtmYteiFPLLKKYNMhATNFIITGyVnkg----qggiLTweqIKEMKas
    SAu_aureus_N315 VWInFDDm-detiYenAYPILKKyIpATgFIITGhVgeenf-hnldmISkkeLKEMykt
    ECo_F11_PoDe   VvITFDDG-lksvsryAYPVLKqYgMKATaFIVTsrIkrhpqkwnpksLqfmsVsELnei
    BAn_Ames_PoDe  IFITmDDG--rknnmnAlhILqKlkddtfQpaaTefLtan-eidkpnrLStddIKqMmds
    SPn_R6_PgdA    VaLTFDDGpnpattpqvletLaKYdIKATfFVLgknVsgn----------edlVKrIKse
    SPy_M1_PgdA    VaLTFDDGpdptttpqvldILaKYqaKGTfFMIgskVvnn----------enltKrVsda
    LLa_Il1403_PgdA IaLTFDDGpnpnttpqllkIfsdaqVpATfFaLgkqaqac----------pqiIKEeadr
    LMo_PgdA       IaLTFDDGpsasvtpgvldtLKRhnVKATfFVLgssViqn----------pglVKrelee
    consensus      vwltfDDg-----y--afpilkky-ikatnfvitg-v-----------lt---mkemk--
                   301......310.......320.......330.......340.......350.......

    SEq_zoo_PoDe   -GMs-FQdHTvnHPdLSq--------aSaasQtaEMkdSKeyLDReLhqdTIaIAYPaGr
    SPy_M1_PoDe    -GMs-FQdHTvnHPdLeq--------aSpdvQttEMkdSKdyLDKqLnqnTIaIAYPsGr
    SIn_UCSD_PdI   -GMs-FQdHTvnHPdLSq--------qnevtQnfEMqdSmefLDKeLsqkTIaIAcPaGr
    SAg_2603V_R_PoD -GMs-FQgHTvtHPnLSl--------lTpelQtqEMtlSKqfLDqkLsqdTLaIAYPsGr
    LLa_la_Il1403_P -GLvdFQSHTvsHPdLgk--------lTleaQrtELeqSKadLDKnLeqkTdiICYPaCg
    SAu_aureus_N315 -GLweFeTHThdlhnLSknnksklmkaSeatiikDLnkSekyLtKnfkksqktIAYPYGl
    ECo_F11_PoDe   rdVfdFQSHThflhrIdgyrrpillsrSehnilfDfarSRraLaq-fnphvLyLsYPFGg
    BAn_Ames_PoDe  -GIfsiQSHTanHtmMah----------snnydeELrgSKekIEaltgkkvIaLAYPVGs
    SPn_R6_PgdA    --ghvvgnHSwsHPiLSq--------lSldeakkqItdTedvLtKvLgssSklMrpPYGa
    SPy_M1_PgdA    --gheianHTwdHPnLTn--------lSvseiqhqVnmTnqaIEKacgkkpryLrpPYGa
    LLa_Il1403_PgdA --gnevaSHTwdHkdLvt--------lSpdqQkqEIesanqlInKitgknvtlfrpPYGs
    LMo_PgdA       --ghgvgnSHSwdHPqLTk--------qStqevynqIlkTqkaVfdqtgyfpttMrpPYGa
    consensus      -gm--fqsHt--hp-ls----------s---q--em--sk--ldk-l---ti-iayPyG-
                   361......370.......380.......390.......400.......410.......
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(b) SIn_PgdA   1   MKKLNTIIFAIISVIFIIFWGITVNNWQINnqvkaiiKsekKhterYLsIkSvksLenGd
    SIn_Pdi    1   ----------------------------mrhnnrrKrqqKerkvFLfLlSfliValGf

    SIn_PgdA   61  ktFHYfspLanSdhfyQDNLPMSVYQKATNdkedVfiePiIeetPLKNVKTVTIHEVRYK
    SIn_Pdi    31  fvFHYkdsLqfSktttQD-----------ktsqVstnPkVknn---------------

    SIn_PgdA   121 HGLFQVnKaSdQvvsRyhvneAfehVRLsqLfkEKEETVNQKLKDLSHGkwNhehlDAmT
    SIn_Pdi    63  -----IkKdTnQtknKttwtkAeqpVKIpiLmy-------------HAihNmapeEAaS

    SIn_PgdA   181 ekkhIltDqfFDiasnQLllddkisLplsDlFdIVnpdlLeGDVKVAYDDYQKKKEEERQR
    SIn_Pdi    104 anlIVapDvfFEshikQLseqgyyfLspeEaYrVLskneLp-----------------

    SIn_PgdA   241 dkKIVaLTFDDgpnpvttPqvlsILdKYhAKGTffMLgskisgN------eDlVKkvKea
    SIn_Pdi    144 sdKVVwLTFDDsmidfyr-vayplILkKYqAKATnnVItgftqnNSVANLTiDqMKeMKkh

    SIn_PgdA   295 GheignHSwdHPnLTklspdqvsFqIknTnmaIEKvtgkapIffrpPyGatnanvqsIAa
    SIn_Pdi    203 GmsfqdHTvnHPdLSqqnevtqnFeMqdSmefLDKelsqktIaiacPaGrysdktleIAs

    SIn_PgdA   355 ltQvlwtVdTrDwenhntEAILaniKaqLrPgGIILMHDVHQTTVNALPTVLDYLkSqDy
    SIn_Pdi    263 rlNyklgVtTnEglasaaDGLLslnRvrIlPn---------------------tTaEi

    SIn_PgdA   415 kfvTVSQVLGHS
    SIn_Pdi    300 lmaTITQ-----
```
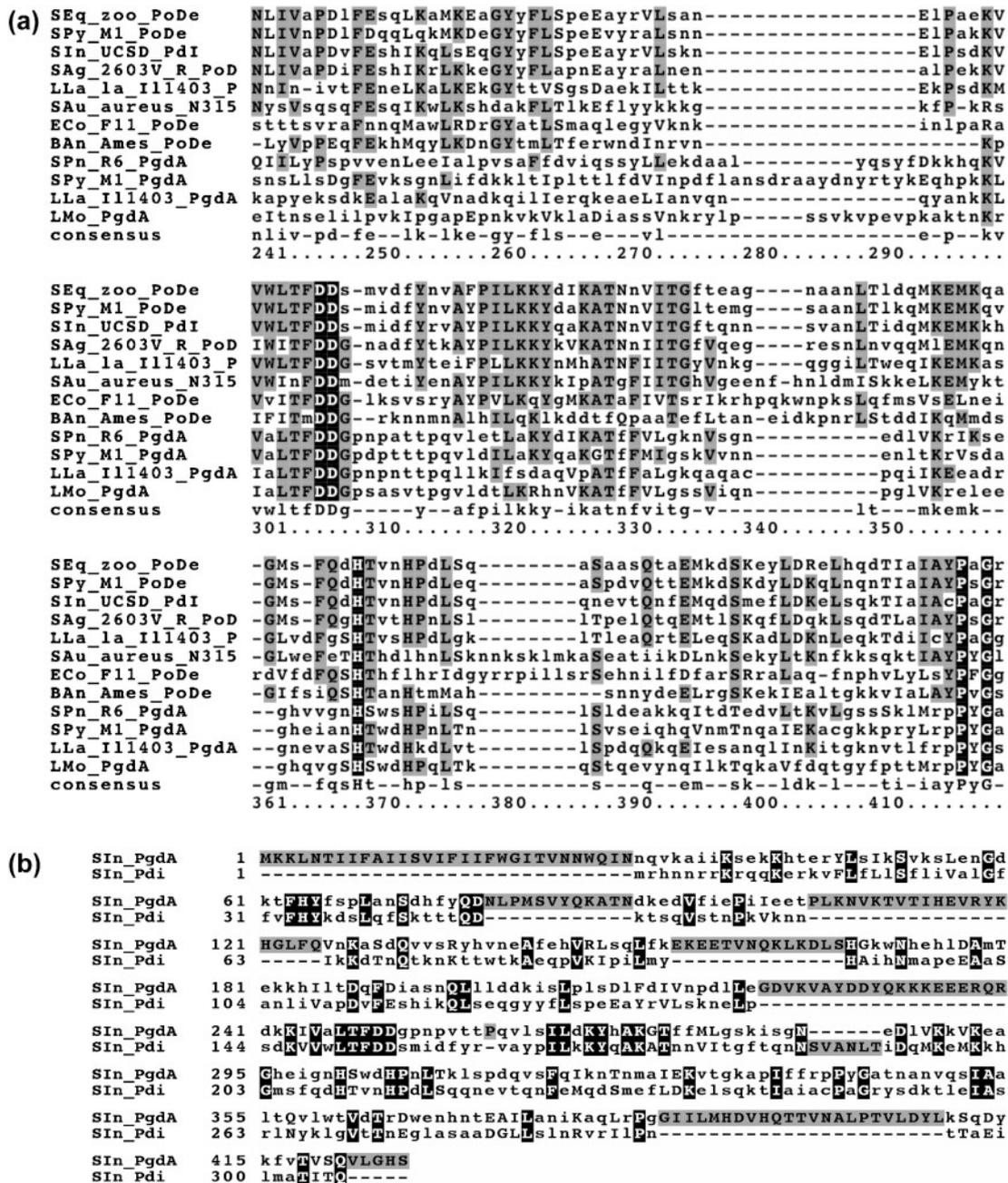
Fig. 1. Alignment of *S. iniae* Pdi and closely related proteins. These representations depict orthologues of Pdi found in other species as well as paralogous peptidoglycan deacetylase proteins (PgdA). (a) Amino acid alignment of conserved regions between polysaccharide deacetylases and peptidoglycan 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.

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 Δ*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* Δ*pdi* mutant was significantly ($P=0.0005$) more sensitive to lysozyme than WT (Fig. 5a). In a manner similar to that reported for deacetylase

Table 1. Abbreviations used in the alignments and phylogenetic analysis

Abbreviation	Definition
ECo	*Escherichia coli*
SAu	*Staphylococcus aureus*
SEp	*Staphylococcus epidermidis*
SIn	*Streptococcus iniae*
SPy	*Streptococcus pyogenes*
SPn	*Streptococcus pneumoniae*
SAg	*Streptococcus agalactiae*
SEq	*Streptococcus equi*
STh	*Streptococcus thermophilus*
SMu	*Streptococcus mutans*
SSa	*Streptococcus sanguinis*
SSu	*Streptococcus suis*
SUb	*Streptococcus uberis*
SMi	*Streptococcus mitis*
SGo	*Streptococcus gordonii*
BAn	*Bacillus anthracis*
BCe	*Bacillus cereus*
LLa_la	*Lactococcus lactis* subsp. *lactis*
LMo	*Listeria monocytogenes*
PoDe	Polysaccharide deacetylase
PgdA	Peptidoglycan deacetylase
IcaB	PIA biosynthesis deacetylase
Pdi	Polysaccharide deacetylase of *S. iniae*

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).

Table 2. Distribution of *pgdA* and *pdi* homologues in streptococcal genomes

The list was compiled through subsystem-based genome analysis (Overbeek *et al.*, 2005) in the NMPDR database (McNeil *et al.*, 2007). +, Present; −, absent.

Genome	*pod/pdi*	*pgdA*
S. pyogenes	+	+
S. equi	+	+
S. agalactiae	+	+
S. suis	+	+
S. thermophilus	+	+
S. uberis	+	+
S. iniae	+	+
S. gordonii	+	+
S. sanguinis	+	+
S. pneumoniae	−	+
S. mitis	−	+

No difference in autolytic potential between *S. iniae* WT and the Δ*pdi* mutant

We recognized the possibility that the virulence attenuation observed in HSB could be due to a greater propensity for autolysis in the Δ*pdi* 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 Δ*pdi* mutant, we assessed the ability of the Δ*pdi* 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 Δ*pdi* mutant was less able to survive in whole fish blood, we compared the resistance of WT and the Δ*pdi* 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 Δ*pdi* mutant to whole blood killing is not derived from enhanced sensitivity to the innate immune system oxidative killing mechanisms or AMPs.

S. iniae Δ*pdi* mutant shows an impaired ability to adhere to and invade epithelial cells

Compared with the WT parent strain, we found the Δ*pdi* 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 deacetylate poly-*N*-acetylglucosamine (GlcNAc) residues of the polysaccharide
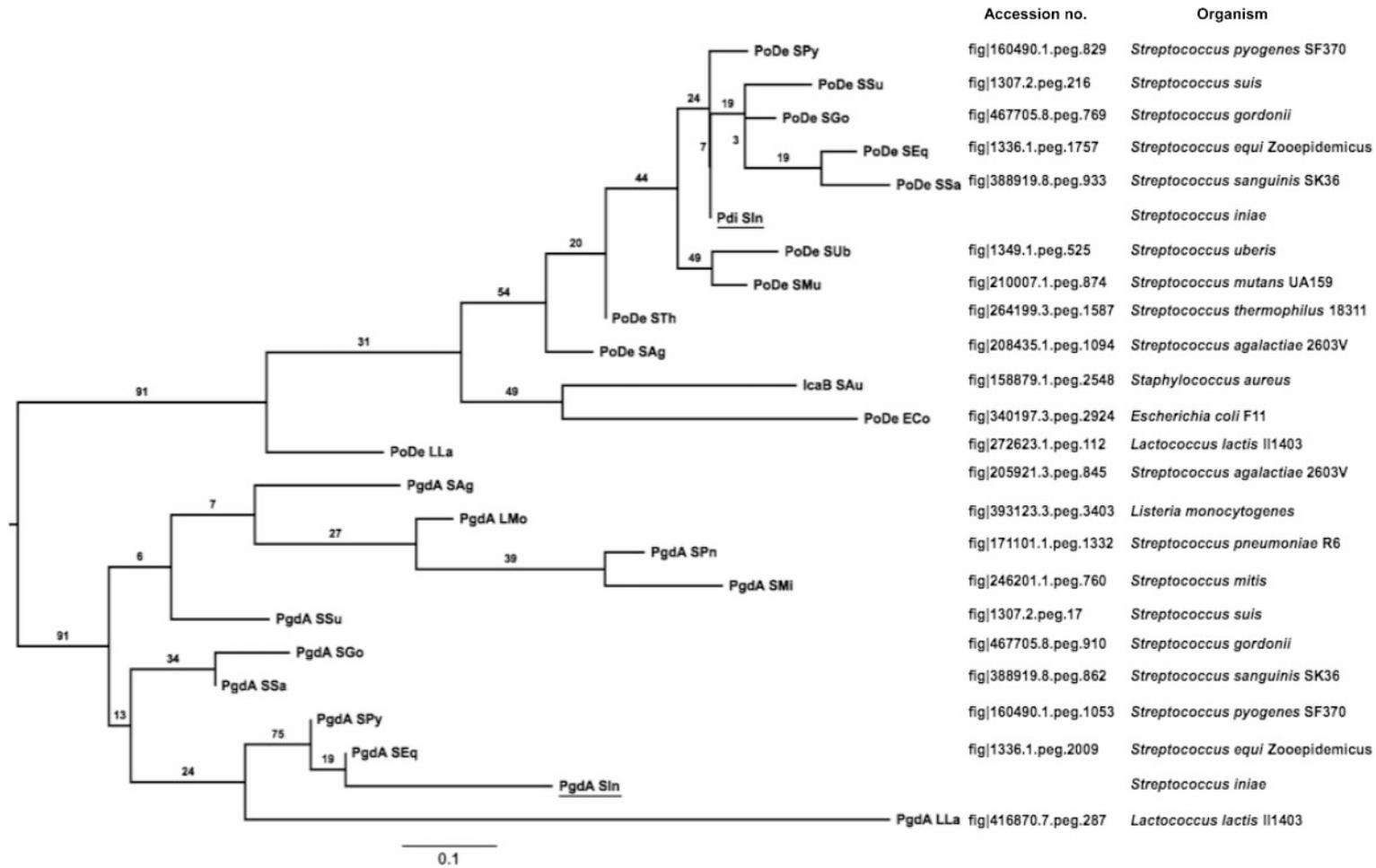
	Accession no.	Organism
PoDe SPy	fig|160490.1.peg.829	*Streptococcus pyogenes* SF370
PoDe SSu	fig|1307.2.peg.216	*Streptococcus suis*
PoDe SGo	fig|467705.8.peg.769	*Streptococcus gordonii*
PoDe SEq	fig|1336.1.peg.1757	*Streptococcus equi* Zooepidemicus
PoDe SSa	fig|388919.8.peg.933	*Streptococcus sanguinis* SK36
Pdi SIn		*Streptococcus iniae*
PoDe SUb	fig|1349.1.peg.525	*Streptococcus uberis*
PoDe SMu	fig|210007.1.peg.874	*Streptococcus mutans* UA159
PoDe STh	fig|264199.3.peg.1587	*Streptococcus thermophilus* 18311
PoDe SAg	fig|208435.1.peg.1094	*Streptococcus agalactiae* 2603V
IcaB SAu	fig|158879.1.peg.2548	*Staphylococcus aureus*
PoDe ECo	fig|340197.3.peg.2924	*Escherichia coli* F11
PoDe LLa	fig|272623.1.peg.112	*Lactococcus lactis* Il1403
PgdA SAg	fig|205921.3.peg.845	*Streptococcus agalactiae* 2603V
PgdA LMo	fig|393123.3.peg.3403	*Listeria monocytogenes*
PgdA SPn	fig|171101.1.peg.1332	*Streptococcus pneumoniae* R6
PgdA SMi	fig|246201.1.peg.760	*Streptococcus mitis*
PgdA SSu	fig|1307.2.peg.17	*Streptococcus suis*
PgdA SGo	fig|467705.8.peg.910	*Streptococcus gordonii*
PgdA SSa	fig|388919.8.peg.862	*Streptococcus sanguinis* SK36
PgdA SPy	fig|160490.1.peg.1053	*Streptococcus pyogenes* SF370
PgdA SEq	fig|1336.1.peg.2009	*Streptococcus equi* Zooepidemicus
PgdA SIn		*Streptococcus iniae*
PgdA LLa	fig|416870.7.peg.287	*Lactococcus lactis* Il1403

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.
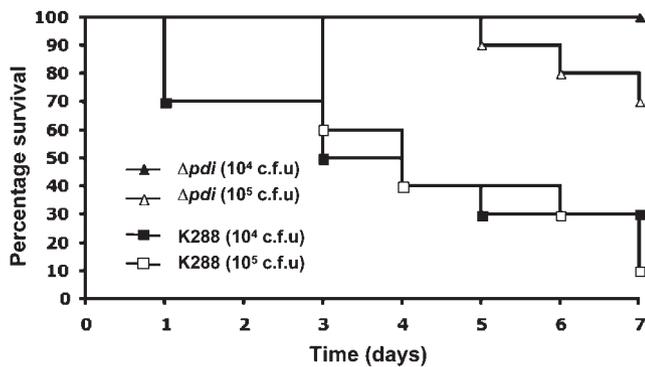
intercellular adhesin (PIA) molecule. The deacetylation of PIA promotes virulence mechanisms of *Staphylococcus epidermidis* such as biofilm formation, surface colonization and resistance to human AMPs (Vuong *et al.*, 2004). In *S. pneumoniae* and *Listeria monocytogenes*, mutagenesis studies have revealed that cell surface peptidoglycan deacetylaces (*pgdA*s) 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.

Through our studies of our *S. iniae* Tn*917* 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 paralogue, 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 Δ*pdi* mutant and WT strain provide strong circumstantial evidence that *pdi* works on the cell surface
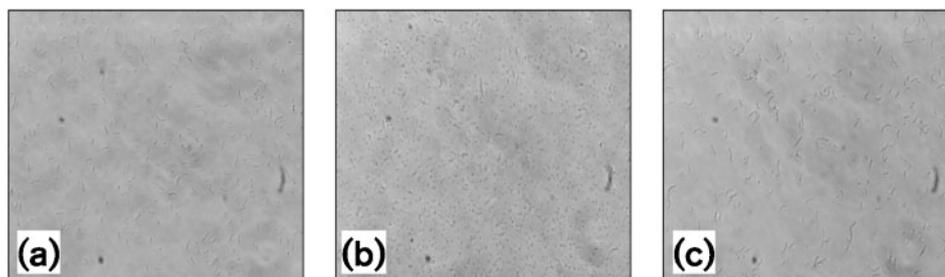

Fig. 4. The Δ*pdi* mutant lacks the ability to remain in chains. Photomicrographs taken of early stationary phase cultures (OD_{600} 0.8) indicate that the *S. iniae* Δ*pdi* mutant is impaired in its ability to remain in chains. In (a), WT, most bacteria appear in short chains, while in (b), mutant, most bacteria exist as single cocci. In (c), the mutant is complemented to restore the WT phenotype.
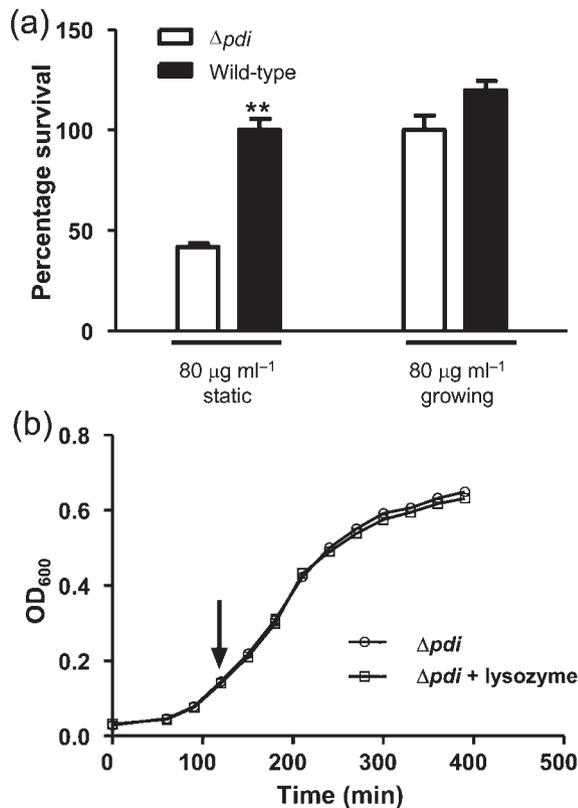
Fig. 5. The *S. iniae* Δ*pdi* mutant is more susceptible to lysozyme than WT. (a) Observed differences in the kinetic killing of WT (black bars) and the Δ*pdi* mutant (white bars) by lysozyme show that the mutant is significantly more susceptible to lysozyme killing when static cells are exposed (**P=0.0005), but significant differences were not detected when cells were dividing (P=0.0803). Each experiment was performed with exposure to 80 μg lysozyme ml^{-1} for 30 min. Values plotted are mean ± SEM. (b) Addition of 80 μg lysozyme ml^{-1} to an exponential-phase culture of the Δ*pdi* mutant does not result in killing. The arrow indicates the time point when lysozyme was added to the culture. Values plotted are mean ± SEM.

Fig. 6. The *S. iniae* Δ*pdi* mutant survives significantly less well than WT in whole fish blood. The WT and Δ*pdi* mutant were incubated with pooled blood from HSB fingerlings (average n=5). The Δ*pdi* mutant showed a significantly reduced ability to survive in whole fish blood compared with WT *(P<0.002), with survival being restored to levels similar to those of the WT in the complemented strain, which were significantly greater than those in the Δ*pdi* mutant **(P<0.01). Values are mean ± SEM.

of *S. iniae*. The Δ*pdi* mutant has an impaired ability to form chains characteristic of the WT *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 Δ*pdi* liquid cultures and the inability to form chains are due to the loss of Pdi function at the *S. iniae* cell surface. A peptidoglycan deacetylase in *Lactococcus lactis* that affects chain length morphology and resistance to lysozyme killing also protects against cell autolysis (Meyrand *et al.*, 2007). However, we were unable to detect any differences in autolysis.

The attenuated virulence of the Δ*pdi* 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 *S. iniae*, *S. pyogenes* or *S. agalactiae* with diminished ability to survive in fresh blood show reduced animal virulence (Buchanan *et al.*, 2005; Datta *et al.*, 2005; Liu *et al.*, 2004; Locke *et al.*, 2007a). These results suggest a fitness advantage conferred by the *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 *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 *S. iniae* cell surface molecules introduces significant changes in molecular patterns that are otherwise easily recognized by the host immune system, thus allowing WT *S. iniae* to survive and persist *in vivo* (Arancibia *et al.*, 2007; Nigro *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 Δ*pdi* 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 *pgdA* in bacterial species such as *Bacillus cereus* (Psylinakis *et al.*, 2005), *S. pneumoniae* (Vollmer & Tomasz, 2000) and *L. monocytogenes* (Boneca *et al.*, 2007). Notably, *pgdA* mutants are sensitive to lysozyme killing primarily in stationary phase and are resistant when cells are actively growing. Similarly, *S. iniae* Δ*pdi* mutants express an increased sensitivity to the enzymic activity of lysozyme when not growing, but Δ*pdi* mutant cells in growth phase are apparently no more resistant to lysozyme killing. Yet, while the *S. iniae* Δ*pdi* mutant is significantly
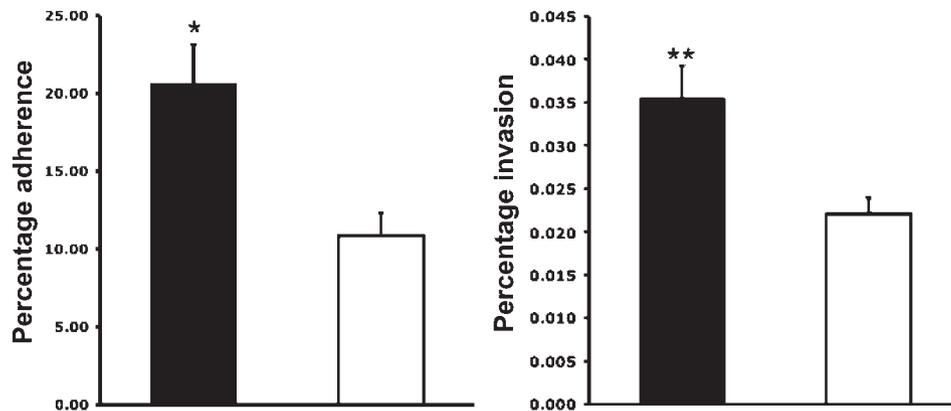
Fig. 7. Adherence to and invasion of white bass epithelial cell line WBE27 by *S. iniae* WT and Δ*pdi* mutant. Adherence and invasion of innate immunity barriers, such as epithelial cells and the blood–brain barrier are thought to be keys for virulence in many streptococcal species. The *S. iniae* Δ*pdi* 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.

(threefold) more susceptible to lysozyme than WT, the differences are not nearly as great as the three-log difference observed between WT and deacetylase mutants of other pathogenic species (Boneca *et al.*, 2007). This is likely the result of *S. iniae* possessing a functional PgdA; therefore, deacetylase activity in the Δ*pdi* mutant is only reduced, not abolished. We hypothesize that while the decreased resistance to lysozyme of the isogenic Δ*pdi* mutant is important, this does not fully account for the attenuated virulence. It is important to note that we used lysozyme from a non-host species (chicken) in these assays, and it is possible that different results would be observed in similar experiments performed using fish lysozyme.

The impaired ability of the Δ*pdi* 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 menigoencephalitis (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 Δ*pdi* 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 deacetylation activity in the Δ*pdi* 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 Δ*pdi* 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 Δ*pdi* strains for vaccination programmes in the aquaculture industry.

ACKNOWLEDGEMENTS

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