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Streptococcus iniae Phosphoglucomutase Is a Virulence Factor and a Target for Vaccine Development

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Streptococcus iniae **represents a major health and economic problem in fish species worldwide. Random Tn*917* mutagenesis and high-throughput screening in a hybrid striped bass (HSB) model of meningoencephalitis identified attenuated *S. iniae* mutants. The Tn*917* insertion in one mutant disrupted an *S. iniae* homologue of a phosphoglucomutase (*pgm*) gene. Electron microscopy revealed a decrease in capsule thickness and cell wall rigidity, with ΔPGM mutant cells reaching sizes ~3-fold larger than those of the wild type (WT). The ΔPGM mutant was cleared more rapidly in HSB blood and was more sensitive to killing by cationic antimicrobial peptides including moronecidin from HSB. In vivo, the ΔPGM mutant was severely attenuated in HSB, as intraperitoneal challenge with 1,000 times the WT lethal dose produced only 2.5% mortality. Reintroduction of an intact copy of the *S. iniae pgm* gene on a plasmid vector restored antimicrobial peptide resistance and virulence to the ΔPGM mutant. In analysis of the aborted infectious process, we found that ΔPGM mutant organisms initially disseminated to the blood, brain, and spleen but were eliminated by 24 h without end organ damage. Ninety to 100% of fish injected with the ΔPGM mutant and later challenged with a lethal dose of WT *S. iniae* survived. We conclude that the *pgm* gene is required for virulence in *S. iniae*, playing a role in normal cell wall morphology, surface capsule expression, and resistance to innate immune clearance mechanisms. An *S. iniae* ΔPGM mutant is able to stimulate a protective immune response and may have value as a live attenuated vaccine for aquaculture.**

With increased development of intensive operations, disease has become a significant hurdle to the profitable culture of fish and shellfish. Streptococcal infections in fish, in particular those produced by the pathogen *Streptococcus iniae*, have increased markedly with intensification of aquaculture practices (37). *S. iniae* causes a fatal meningoencephalitis and is associated with large-scale mortality in a wide variety of marine and freshwater cultured fish species, as well as in wild species (5, 39, 46). More than 30 species of fish have documented susceptibility to *S. iniae* disease, including trout (10), yellowtail (19), tilapia (39), barramundi (6), and hybrid striped bass (HSB) (11). *S. iniae* is distributed globally and is estimated to cause yearly economic losses of hundreds of millions of dollars. Occasionally, *S. iniae* can cause serious zoonotic infections in humans who injure themselves while handling infected fish (20, 42).

Relatively little is known of the genetics of *S. iniae* or of the pathogenic mechanisms underlying its virulence. Here, we describe a severely attenuated mutant of *S. iniae*, identified through random transposon mutagenesis and direct screening for virulence in HSB. Analysis of the transposon insertion site revealed a disruption of an open reading frame (ORF) with similarity to bacterial phosphoglucomutase (*pgm*) genes. The enzyme phosphoglucomutase (PGM) interconverts glucose-6-phosphate and glucose-1-phosphate, and it has recently been discovered to play an important role in polysaccharide capsule production and virulence in a variety of gram-positive and gram-negative bacterial pathogens. In the present study, we characterize phenotypic changes associated with loss of PGM in *S. iniae*, including alterations in cell wall morphology, capsule production, and susceptibility to fish innate immune defenses. Loss of PGM is associated with markedly reduced virulence in *S. iniae*, and preliminary data are provided to suggest that avirulent, PGM-deficient *S. iniae* may serve as an effective live attenuated vaccine to protect aquacultured fish against fatal meningoencephalitis.

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MATERIALS AND METHODS

Bacteria strains, culture, transformation, and DNA techniques. Wild-type (WT) *S. iniae* strain K288 was isolated from the brain of a diseased HSB at Kent SeaTech. K288 was identified unambiguously as *S. iniae* through biochemical testing and by analysis of ribosomal 16S sequence (data not shown) and proven to be virulent in a HSB model by intraperitoneal (IP) injection of 4×10^5 CFU, a lethal dose in all fish tested. *S. iniae* was propagated in Todd-Hewitt broth (THB) or on Todd-Hewitt agar (THA) at 30°C unless otherwise indicated, with antibiotic selection of 2 μg/ml chloramphenicol (Cm), 5 μg/ml erythromycin (Em), and 500 μg/ml kanamycin when required. *Escherichia coli* was grown in Luria-Bertani (LB) medium at 37°C using 500 μg/ml of Em, 15 μg/ml of Cm, or 100 μg/ml of ampicillin for selection unless otherwise indicated. *S. iniae* was rendered competent for electroporetic transformation by growth in THB plus 0.6% glycine, following procedures for group B *Streptococcus* (12). Transformants were identified by recovery for 2 h at 30°C in SOC medium (Invitrogen Corp., Carlsbad, CA) for *E. coli* and THB plus 0.25 M sucrose for *S. iniae*, followed by plating on appropriate antibiotic selective media. For use in fish challenges, overnight cultures of *S. iniae* were diluted 1:20 and grown to mid-log phase (optical density at 600 nm [OD_{600}] = 0.4) corresponding to $\sim 4 \times 10^8$ CFU/ml. Plasmid DNA was isolated from *E. coli* with QiaPrep kits (QIAGEN, Valencia, CA). For *S. iniae*, a 15-min incubation at 37°C in 100 U of mutanolysin (Sigma, St. Louis, MO) preceded plasmid isolation with the QiaPrep kit.

Genomic DNA was isolated with the UltraClean DNA Isolation Kit (MoBio, Carlsbad, CA).

Transposon mutagenesis. Transposon mutagenesis of strain K288 followed procedures described for *Streptococcus mutans* using the temperature-sensitive plasmid pTV_1OK bearing transposon Tn*917* (13), with slight modifications. Individual colonies of K288 transformed with pTV_1OK were inoculated into THB plus kanamycin and grown to $OD_{600} = 0.9$ at the permissive temperature (30°C) for plasmid replication. Cultures were diluted 1:100 in THB plus Em and grown at a nonpermissive temperature (37°C) to an OD_{600} value of 0.9, then plated on THA plus Em for isolation of candidate insertion mutants. Libraries of *S. iniae* mutants bearing random transposon insertions into the genome were verified by Southern blot analysis (data not shown).

In vivo screening of transposon mutants for loss of virulence. HSB (*Morone chryosops* × *Morone saxitilis*) aged <1 year and with an average weight of approximately 30 g each were used for an in vivo challenge model of *S. iniae* infection. HSB were challenged IP with 100 μl of WT or mutant *S. iniae* bacteria resuspended in phosphate-buffered saline (PBS) at known inocula and injected using a 27-gauge needle. Fish were held with aeration and flowthrough water at 24 to 27°C for 7 days after challenge and were monitored twice daily for mortalities. Brain biopsy cultures were taken from selected dead fish to confirm infection with the challenge *S. iniae* strain, based on the appropriate antibiotic sensitivity profile. Screening the transposon library in this manner revealed numerous attenuated mutants, including mutant TnM2 with a transposon disruption of a putative *pgm* gene.

Identification of the *pgm* gene. The Tn*917* insertion site in the attenuated mutant TnM2 was identified by direct sequencing from genomic DNA using the modified primer (fimer) 5′-GAAACATTGGTTTAGTGGGAATTTGTAC-3′ and 0.1 μl of Thermofidelase (Fidelity Systems) in a 20-μl cycle sequencing reaction mixture (Big Dye v3.0; Applied Biosystems, Inc.). Chromosome walking using a single-primer PCR technique was used to discover the entire sequence of *pgm* and flanking regions (17, 18). Sequence files were analyzed with Chromas (Technelysium, Tewantin, Australia), aligned with BioEdit (Ibis Therapeutics, Carlsbad, CA), and annotated with Artemis, version 5.0 (Sanger Institute, Cambridge, United Kingdom). The amino acid sequence of PGM was compared to sequences in the GenBank databases with the BlastP program (2).

Complementation and heterologous expression of *S. iniae pgm*. The entire *pgm* gene plus the upstream putative promoter region (1865 bp) was amplified from WT *S. iniae* K288 genomic DNA using forward primer 5′-GAACTAGCTAGT TACTTTTGTAACTG-3′ and reverse primer 5′-CTAATTCACAAAAGTGTT GATTTCAG-3′ in a standard PCR using Platinum Supermix (Invitrogen, Carlsbad, CA) and 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s), and elongation (72°C for 1.5 min). The resulting product was T-A cloned into pCR2.1Topo (Invitrogen, Carlsbad, CA). The *pgm* gene was cut from the pCR2.1Topo intermediate with BamHI and XbaI and cloned into the corresponding sites in the *E. coli*-streptococcus shuttle expression vector pDC123 (7) bearing Cm^r to create pSiPGM. This recombinant vector was used to transform WT *S. iniae* K288, *S. iniae* transposon mutant TnM2, and an *E. coli* K-12 ΔPGM mutant (1); similar transformations were performed with pDC123 to serve as a vector-only control. Transformants were identified by Cm^r and confirmed by restriction enzyme and PCR analysis of plasmid preparations from transformed cells.

Phosphoglucomutase activity assays. Cells extracts were prepared from bacteria in mid-log phase ($OD_{600} = 0.4$), collected by centrifugation, and washed twice in chilled PBS. For *E. coli*, cells were resuspended in 50 mM triethanolamine buffer with 5 mM $MgCl_2$ (pH 7.2), and suspensions were frozen at −80°C. Cell lysates were prepared using sonication (three bursts of 10 s each). For *S. iniae*, cell pellets were frozen. Cells lysates were prepared using the Cellytic B plus reagent (Sigma Aldrich, St. Louis, MO) following the manufacturer's instructions. All cell lysates were centrifuged (30 min at 10,000 × *g*), and the supernatants (crude cell extracts) were stored at −80°C until use. The specific activity of PGM in cell extracts was measured as the conversion of α-glucose-1-phosphate to glucose-6-phosphate in a reaction coupled to reduction of glucose-6-phosphate by glucose-6-phosphate dehydrogenase and quantitated spectrophotometrically at 340 nm by monitoring the formation of NADPH from $NADP^+$ at 30°C. The PGM assay solution contained final concentrations of 5 mM $MgCl_2$, 0.4 mM $NADP^+$, 2 U/ml glucose 6-phosphate dehydrogenase, and 50 μM α-glucose-1,6-bisphosphate. After the cell extract was added, the reaction was initiated with addition of α-glucose-1-phosphate to a concentration of 1.4 mM. Parallel phosphomannomutase assays were performed with the above reagents plus the addition of 2 U/ml each of phosphomannoisomerase and phosphoglucoisomerase and activated with a final concentration of 1.4 mM α-mannose-1-phosphate. All chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Fish virulence studies. Groups of 40 HSB (each, ~30 g) were challenged IP with 2×10^5 to 2×10^8 CFU of WT or mutant *S. iniae* bacteria from log-phase ($OD_{600} = 0.4$) cultures resuspended in 100 μl of PBS. Fish were held with aeration and flowthrough water at 24 to 27°C for 14 days after challenge and were monitored twice daily for mortalities. To compare the virulence of the complemented mutant TnM2[pSiPGM] to the WT and TnM2 strains, groups of 28 fish for each treatment were injected with 4×10^5 CFU and monitored for 7 days. Three fish were selected at random from each group at 24 h postchallenge and sacrificed, and the number of CFU of *S. iniae* per milliliter of blood was enumerated by plating dilutions on THA.

Assays for buoyancy, surface charge, and hydrophobicity. To measure buoyancy, sequential overlay gradients of 1 ml (each) of 70%, 60%, and 50% Percoll were prepared in 5-ml glass test tubes. One milliliter of overnight bacterial culture was placed on top of the Percoll layers, the tubes were centrifuged in a swinging bucket centrifuge for 8 min at 500 × *g*, and the migration of the bacteria to various Percoll interphases was recorded. To measure surface charge, bacteria were grown to stationary phase, harvested by centrifugation, and washed twice in morpholinepropanesulfonic acid (MOPS) buffer (20 mM; pH = 7.0). Cells were resuspended in MOPS to $OD_{600} = 6.0$, and cytochrome *c* (Sigma, St. Louis, MO) was added to a final concentration of 0.5 mg/ml. After 15-min incubation at 23°C, samples were centrifuged (13,000 × *g* for 5 min), and the amount of cytochrome *c* remaining in the supernatant was quantitated spectophotometrically at 530 nm. To measure hydrophobicity, stationary-phase cultures were washed twice in PBS and resuspended in PBS at $OD_{600} = 1.0$. A total of 300 μl of *n*-hexadecane was layered on top of the cell suspension, and the tubes were vortexed for 60 s. Samples were incubated for 30 min at 23°C to allow for phase separation. The aqueous phase was removed, and the OD_{600} value was recorded to determine the quantity of remaining bacteria.

Electron microscopy. *S. iniae* were grown to late log phase ($OD_{600} = 0.7$) and collected by centrifugation. Pellets were resuspended in 0.07 M cacodylate buffer and transferred to 2.0-ml microfuge tubes. Samples were washed twice (0.07 M cacodylate buffer), resuspended in 0.2 M cacodylate plus 3.6% glutaraldehyde, incubated on ice for 1 h, and then washed three additional times. Polycationic ferritin (Sigma) was added to a final concentration of 1.0 mg/ml, and the samples were incubated for 30 min, washed twice, and stored at 4°C. Prior to electron microscopy, bacteria were washed three times in 0.1 M sodium phosphate buffer (pH 7.3), postfixed for 1 h in 0.1 M phosphate-buffered 2% osmium tetroxide, and rinsed three times in distilled water (dH_2O). Dehydration was performed using ethanol at 30%, 50%, 70%, 95%, and 100% concentrations. Bacteria were immersed in two rinses of propylene oxide and incubated for 2 h in a mixture of 50% propylene oxide and 50% epoxy resin. Mollenhaure's formulation of Epon-Araldite was used to embed bacteria for thin sectioning. Sixty-nanometer sections were placed on 300-mesh copper support grids and viewed using a Zeiss EM 10 electron microscope at an acceleration voltage of 80 kV with ×16,000 and ×50,000 magnifications. Negatives were enlarged and printed to final magnifications of ×35,000 and ×109,000.

Blood survival assay. Mid-log-phase *S. iniae* suspensions of ~300 CFU in 100 μl of PBS were added to 300 μl of fresh heparinized HSB blood in 2-ml siliconized plastic tubes and incubated for 1 h at 30°C on an orbital shaker. After 1 h, 100-μl aliquots were taken from each sample in duplicate and plated on THA for enumeration of surviving bacteria. Each experiment was repeated three times, each time using the blood of a different HSB.

Antimicrobial peptide sensitivity. Early-log-phase cultures of *S. iniae* ($OD_{600} = 0.2$) were diluted in fresh THB to ~2×10^5 CFU/ml. A total of 180 μl of this bacterial suspension was added to replicate wells of a 96-well plate. Dilutions of the antimicrobial peptides (AMPs) moronecidin (2 μM) (from HSB) and CRAMP (16 μM) (from mouse) were prepared in dH_2O and added to the wells in 20 μl of distilled H_2O; dH_2O alone was used as a negative control. To measure antimicrobial killing kinetics, 20-μl aliquots of each well were serially diluted in PBS and plated at specified time points after addition of the antimicrobial peptide for CFU determination. Each experiment was performed in triplicate.

Characterization of the *S. iniae* infection process. HSB (*Morone chryosops* × *Morone saxitilis*) aged <1 year and with an average weight of ~20 g each were used to characterize the attenuation infection process of mutant TnM2. Groups of 50 HSB were challenged by IP injection of 2.5×10^5 CFU/100 μl of PBS of log-phase WT or TnM2 *S. iniae*. Fifty control fish were injected with 100 μl of PBS (each). Fish were held with aeration and flowthrough water at 24 to 27°C after challenge. Three fish were sacrificed at various time points during the first 124 h of infection, and bacterial load in the brain, blood, and spleen was calculated by weighing tissue samples and homogenizing tissue samples in PBS (Tissue Tearor; Biospec Products, Bartlesville, OK). Serial dilutions of each tissue homogenate in PBS were plated in duplicate on blood agar for enumeration of CFU. All TnM2 bacteria recovered were confirmed to have maintained the Em^r

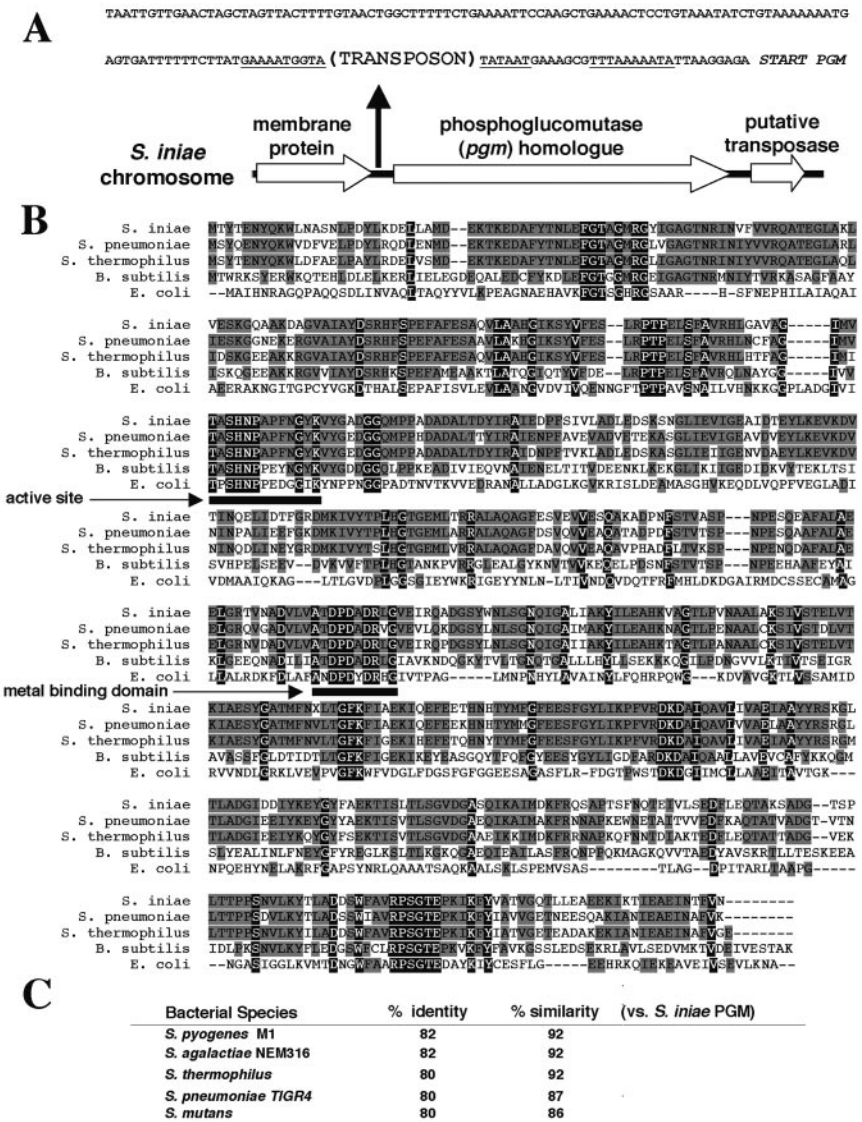

FIG. 1. Sequence features of *Streptococcus iniae* PGM. (A) Chromosomal location of *S. iniae* PGM and site of Tn*917* insertion in promoter motifs upstream of translational start site. Underlined areas indicate conserved promoter motifs. (B) Alignment of *S. iniae* PGM with known bacterial PGMs and indication of putative conserved functional domains. (C) Degree of homology of *S. iniae* PGM to other streptococcal PGMs.

phenotype. Brain and spleen tissues from each of the three fish sampled at 96 h were pooled and placed in 10% buffered formalin until processing. Tissue pools were trimmed into cassettes and embedded in paraffin, and sections were cut for routine histology. Serial sections were stained with either hematoxylin and eosin (H&E) or Giemsa stain; all sections were examined in a blinded fashion and scored as the number of positive traits seen in the sections of the tissue examined. H&E-stained sections were used to score the respective spleen and brain changes, while Giemsa stain-treated sections were used to subjectively quantify the degree of bacteria associated with splenic ellipsoids and the meninges, respectively. The following criteria were used to assess the splenic response to infection: congestion, capsular hypertrophy, peritoneal inflammation, and ellipsoidal degeneration. Criteria for brain changes include meningeal inflammation and ventricle (optic lobe) inflammation.

Vaccine trial. To test the ability of TnM2 to function as a live attenuated vaccine, groups of 40 fish (each, ~30 g) were injected IP with 4×10^5, 4×10^6, 4×10^7, or 4×10^8 CFU of TnM2 in PBS. Forty controls were injected with PBS alone. Fish were held for 2,000 degree days (~4 months); no mortalities were observed. Fish were then challenged by injection with the previously determined lethal dose (4×10^5 CFU) of K288, held in mixed groups for 21 days at 24 to 27°C, and monitored for mortality. Brain biopsy cultures were taken from all dead fish and cultured to confirm *S. iniae* meningoencephalitis as the cause of death.

Nucleotide sequence accession number. The nucleotide sequence for *pgmA* identified in this study has been deposited in GenBank under accession number AY846302.

RESULTS

Sequence characteristics of *S. iniae* PGM. The attenuated mutant TnM2 was identified in a screen of a Tn*917* chromosomal insertion library of WT *S. iniae* strain K288 for loss of virulence in HSB. By direct sequencing and single-primer chromosome walking, the site of the sole Tn*917* insertion in TnM2 and surrounding DNA sequence was determined (Fig. 1A). Tn*917* was found to have inserted in a predicted promoter

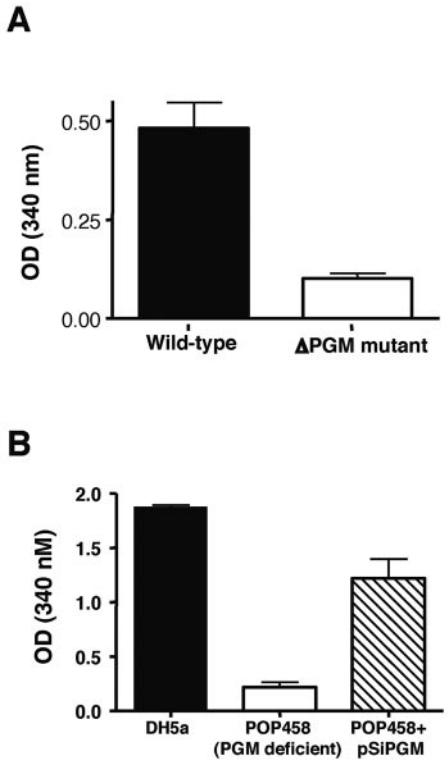

FIG. 2. *Streptococcus iniae* PGM is a functional phosphoglucomutase enzyme. (A) Loss of PGM activity in *S. iniae* ΔPGM mutant. (B) Heterologous expression of *S. iniae* PGM restores phosphoglucomutase activity to *E. coli* PGM mutants.

region 32 bp upstream of the start ATG codon of the ORF, sharing strong sequence homology to genes encoding PGM enzymes. Herein, this ORF is designated *pgmA* BlastP analysis in GenBank of the deduced amino acid sequence of the candidate *S. iniae* PGM revealed strong sequence homology and identity with known α-PGM enzymes from other gram-positive bacteria species (Fig. 1B and C), as well as several known α-PGMs from gram-negative and vertebrate species (not shown). In particular, the functional regions characteristic of α-PGM proteins were conserved in the *S. iniae* homologue, including a metal binding domain and active site residues (8).

PGM activity in *S. iniae* is linked to *pgmA*. Biochemical assays were performed to confirm that the *pgmA* ORF encoded a functional PGM enzyme. Measured PGM activity decreased more than fivefold in mutant TnM2 compared to the WT *S. iniae* parent strain (Fig. 2A). Complementation of TnM2 with plasmid pSiPGM restored PGM activity to approximately WT levels. In parallel assays, we were unable to detect phosphomannomutase activity in *S. iniae* cell extracts and thus conclude that PGM enzyme lacks the dual activity described in a subset of known PGM enzymes. A mutant strain of *E. coli* (Pop458) with a disrupted native *pgm* gene had greatly reduced PGM activity compared to that of WT *E. coli*. Heterologous expression of pSiPGM in *E. coli* Pop458 significantly increased PGM activity ($P < 0.0001$) (Fig. 2B). Together, these studies demonstrate that *pgmA* is both necessary and sufficient for PGM enzymatic activity.

TABLE 1. Attenuation of *Streptococcus iniae* ΔPGM mutant (TnM2) in a hybrid striped bass infection model[a]

Bacterium	Dose (CFU)	No. of fish	% Mortality
WT *S. iniae*	4×10^5	40	100
ΔPGM mutant	4×10^5	40	0
	4×10^6	40	0
	4×10^7	40	0
	4×10^8	40	2.5

[a] Fish (≈30 g each) were injected intraperitoneally with the specified bacterial dose and held at 26°C for 14 days; mortalities were identified and removed daily.

***S. iniae* PGM expression is linked to virulence.** The HSB challenge model was used to evaluate the virulence potential of *S. iniae* mutant TnM2 with reduced PGM activity. Whereas 100% of HSB injected with 4×10^5 WT *S. iniae* died of meningoencephalitis, mortality was absent in fish challenged with TnM2 at the same dose; the rate of mortality was only 2.5% in fish challenged with the mutant at a 1,000-fold higher inoculum ($P < 0.0001$) (Table 1). In a separate experiment, survival curves were plotted for HSB after intraperitoneal challenge with either 4×10^5 CFU of WT *S. iniae*, mutant TnM2, or TnM2 complemented with pSiPGM. While no mortality was seen in fish infected with TnM2, reintroduction of *pgmA* to the

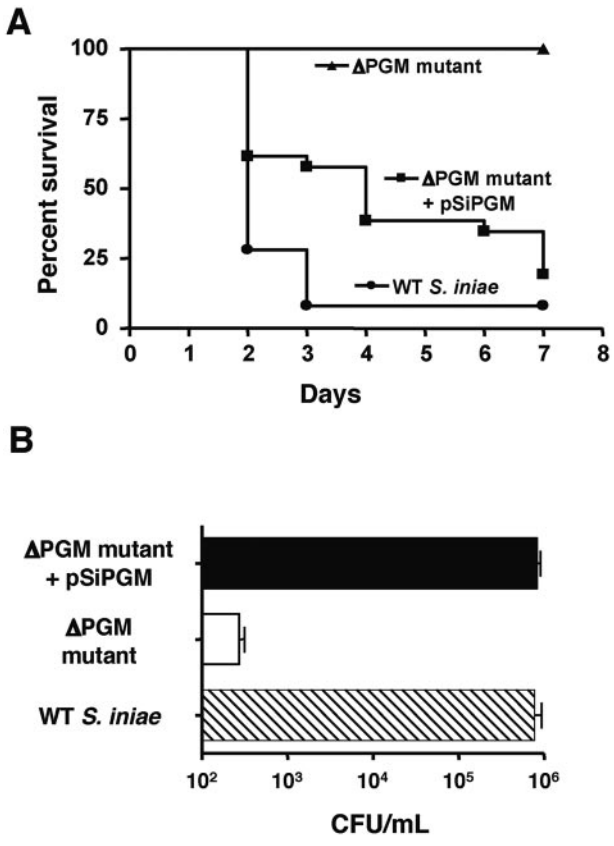

FIG. 3. Phosphoglucomutase is a virulence factor in the pathogenesis of *Streptococcus iniae* meningoencephalitis. (A) Kaplan-Meier survival plot of HSB challenged with 4×10^5 CFU of wild-type *S. iniae*, the ΔPGM mutant, or the complemented mutant. (B) Bacterial counts in blood of fish 24 h after infection.

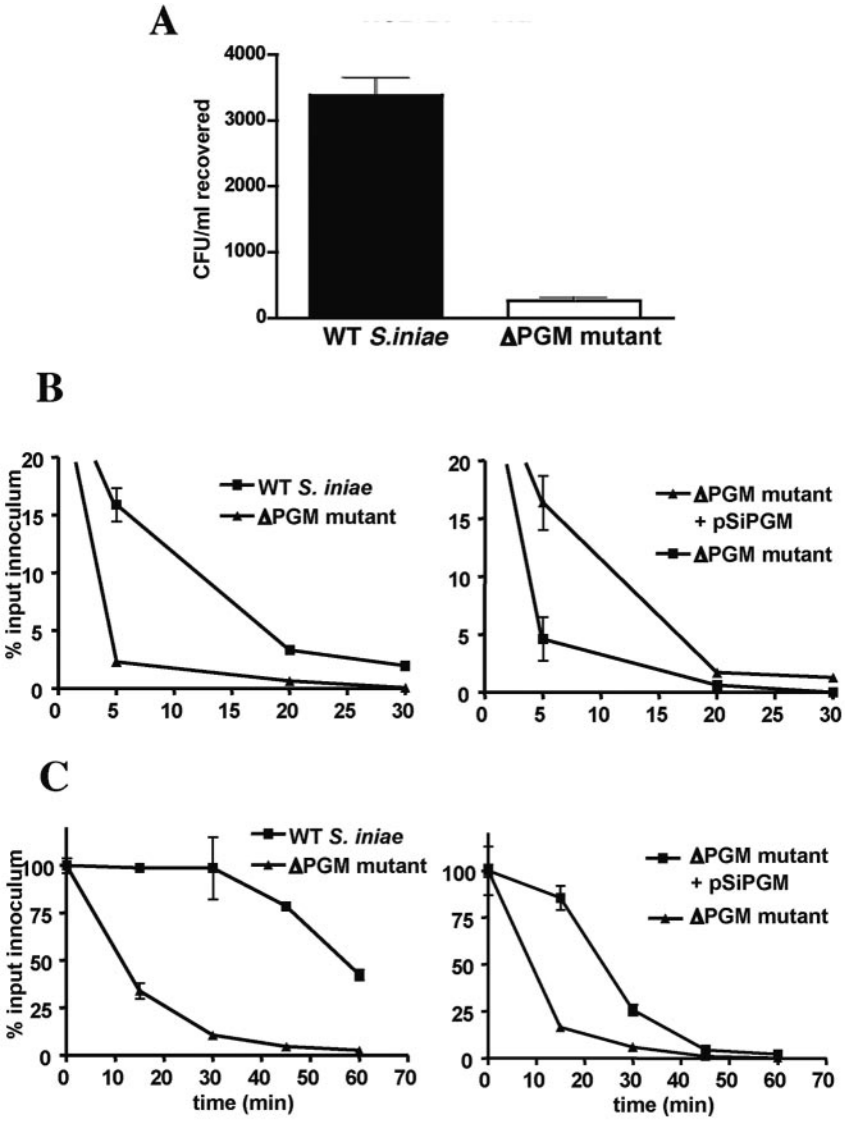

FIG. 4. Phosphoglucomutase contributes to *Streptococcus iniae* bloodstream survival and resistance to AMP killing. (A) CFU recovered (mean ± standard error) of wild-type *Streptococcus iniae* and the isogenic ΔPGM mutant after incubation in HSB blood. Kinetics of killing of wild-type *S. iniae*, the ΔPGM mutant, or the complemented mutant by the HSB AMP moronecidin (B) and the murine AMP mCRAMP (C).

mutant on a plasmid vector resulted in mortality comparable that observed with WT *S. iniae* (Fig. 3A). Measurement of bacterial load in the blood of infected fish at 24 h appeared to predict mortality. Bacterial levels in the blood of HSB infected with WT *S. iniae* or TnM2[pSiPGM] were 1,000-fold higher than those observed with TnM2 (Fig. 3B). The severe attenuation of mutant TnM2 in animal challenges identifies *pgmA* as being associated with one or more virulence phenotypes of *S. iniae*.

The *S. iniae* ΔPGM mutant is more sensitive to innate immune clearance mechanisms. To measure the relative susceptibility of WT *S. iniae* and the ΔPGM mutant TnM2 to phagocytic clearance, a survival assay was performed with fresh blood isolated from HSB. Whereas the WT strain proliferated markedly (~4-fold) in HSB blood, mutant TnM2 decreased in number (Fig. 4A). Cationic AMPs are recognized to be important components of innate defense and phagocyte killing in higher organisms (45). HSB produce the AMP moronecidin (also known as piscidin) possessing broad-spectrum antimicrobial activity (21, 40). The ΔPGM mutant TnM2 was found to be significantly more sensitive than the WT *S. iniae* strain to moronecidin; similar results were seen with the murine cationic AMP CRAMP. In vitro killing kinetics for each AMP showed that the rate of killing of the ΔPGM mutant was accelerated significantly compared to the WT *S. iniae* strain, whereas complementation of the mutant with pSiPGM produced a commensurate delay in the time course of AMP killing (Fig. 4B).

***S. iniae* PGM mutants have an altered cell phenotype.** Assays were employed to identify general cell characteristics associated with loss of PGM activity in *S. iniae*. Logarithmic-phase growth of WT *S. iniae* and the ΔPGM mutant in THB were equivalent (Fig. 5). Migration through a Percoll gradient was increased in the ΔPGM mutant TnM2 compared to the

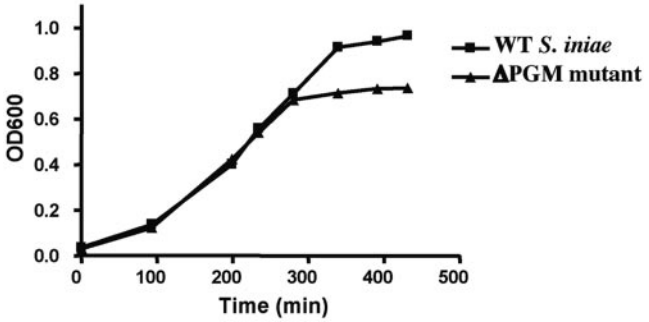

FIG. 5. *Streptococcus iniae* and the ΔPGM mutant have identical growth through the logarithmic phase in Todd-Hewitt broth as measured by optical density at 600 nm. The ΔPGM mutant does not achieve equivalent cell density at stationary phase.

WT *S. iniae* strain, indicative of a decrease in buoyancy (data not shown). More ΔPGM mutant cells were found to partition from the aqueous phase into *n*-hexadecane than did WT *S. iniae* cells, consistent with an overall increase in cell hydrophobicity (data not shown). The ΔPGM mutant also bound less cytochrome *c* than did the WT *S. iniae* strain (data not shown), indicating that the mutant possessed a net decrease in surface negative charge. Our transmission electron microscopic analysis of WT and ΔPGM mutant *S. iniae* labeled with polycationic ferritin revealed that the mutant exhibited decreased amounts of surface-associated exopolysaccharide capsule (4) and that the average cell volume of ΔPGM mutant bacteria was three to five times larger than that of the WT strain (Fig. 6).

Analysis of the aborted infectious process of ΔPGM mutant *S. iniae*. To further characterize the basis for the severely attenuated virulence of PGM-deficient *S. iniae*, we followed the course of infection in HSB challenged with WT and ΔPGM mutant *S. iniae*. At 4 h postinfection, WT bacteria were found at 10^5 CFU/ml in blood, $>10^4$ CFU/g in brain, and $\sim 10^6$ CFU/g in spleen (Fig. 7A). Bacterial counts in WT *S. iniae*-infected fish increased steadily over time, surpassing 10^7 CFU/g in all tissues between 24 and 96 h of infection; widespread mortality was observed. Histological examination of brain revealed severe granulomatous meningeal inflammation with associated bacterial foci, and histology of the spleen showed large numbers of bacteria within degenerate splenic ellipsoids (Fig. 7B; Table 2). HSB challenged intraperitoneally with the *S. iniae* ΔPGM mutant similarly exhibited dissemination of the bacteria to blood, brain, and spleen such that 4-h counts of these tissues were only modestly less than those observed with WT infection (Fig. 7A). In stark contrast to the WT strain, the mutant was rapidly cleared from both blood and brain to undetectable levels within 24 h and slowly cleared from the spleen to undetectable levels by 5 days of infection (Fig. 7A). No mortalities were observed in the ΔPGM mutant-infected group of HSB. Histological examination of brain and spleen from fish infected with the ΔPGM mutant did not reveal evidence of the inflammatory damage produced in WT-infected HSB (Fig. 7B; Table 2).

Efficacy of live attenuated vaccination with ΔPGM mutant against *S. iniae* infection. We hypothesized that the aborted infectious process of the attenuated ΔPGM mutant would elicit an immune response capable of protecting HSB against subsequent WT *S. iniae* infection. A vaccination trial was performed by infecting HSB with various concentrations of the *S. iniae* ΔPGM mutant TnM2 (or PBS as a negative control), allowing them to spontaneously clear the infection, and then housing them for a period of 2,000 degree days (~4 months). At this point, all animals were challenged with a typically lethal dose (4×10^5 CFU) of WT *S. iniae* strain K288. Only a 3% survival rate was observed with control fish that were mock immunized with PBS, while all groups of fish that were previously challenged with various doses of the attenuated ΔPGM mutant experienced survival rates of at least 90% (Fig. 8). At the highest concentration of mutant used for live attenuated vaccination (2×10^8 CFU), 100% of fish survived subsequent challenge with a lethal dose of the WT *S. iniae* strain. This preliminary trial indicates that the ΔPGM mutant TnM2 elicits an effective immune response and may have value as a live attenuated vaccine to protect HSB against *S. iniae* infection.

DISCUSSION

Our studies have identified a mutant of *S. iniae* with severely attenuated virulence in a model of fish meningoencephalitis.

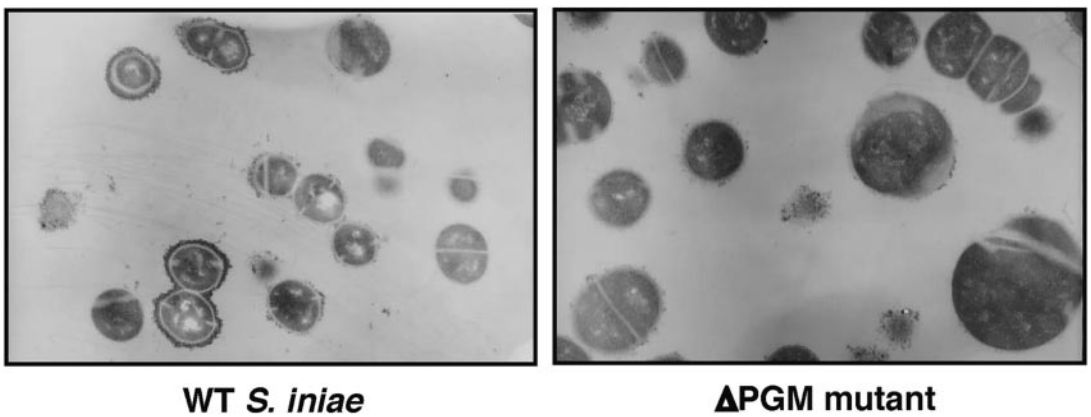

FIG. 6. Phenotypic differences observed between *Streptococcus iniae* and the isogenic ΔPGM mutant include increased cell size and decreased level of encapsulation, as appreciated by transmission electron microscopy.

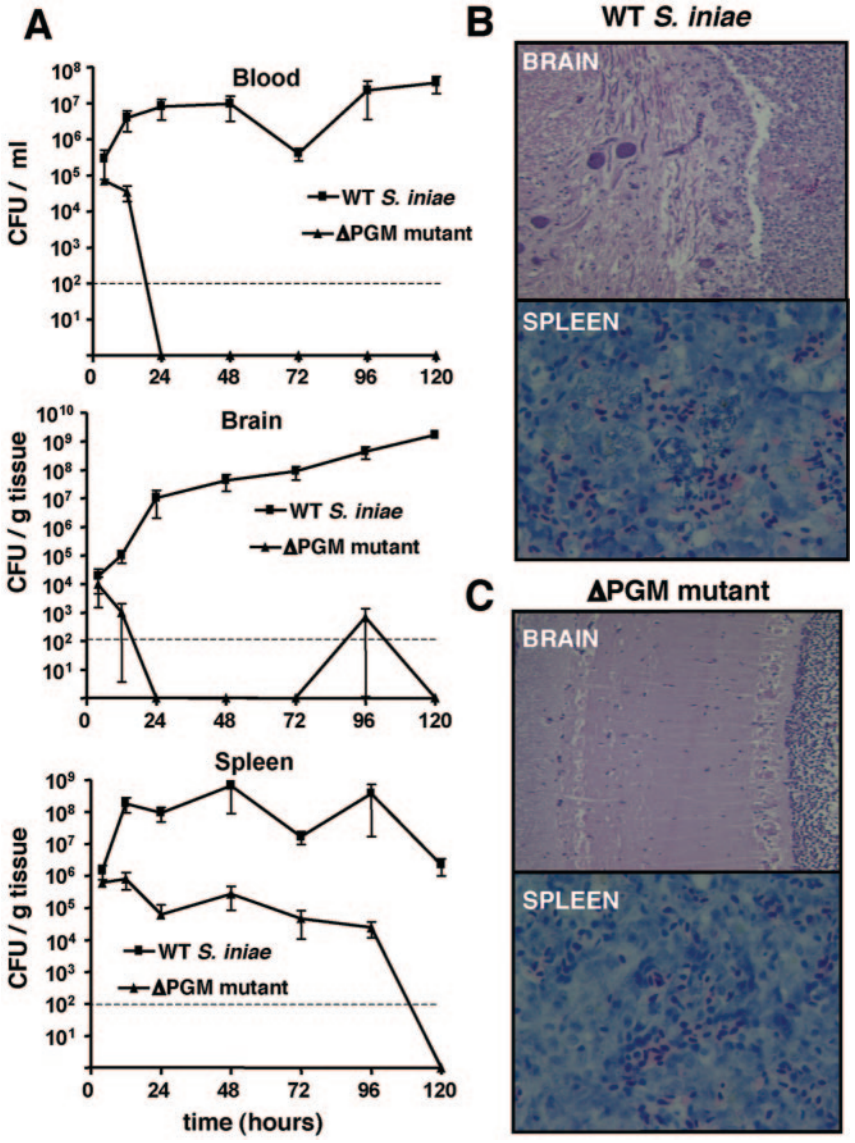

FIG. 7. Characterization of the aborted infectious process produced by the *Streptococcus iniae* phosphoglucomutase mutant (ΔPGM). (A) Bacterial counts in blood, spleen, and brain of HSB at different time points after intraperitoneal challenge. (B and C) Representative histological sections of brain (48 h; H&E stain, ×20 magnification) and splenic (120 h; Giemsa stain, ×40 magnification) tissues. With wild-type *S. iniae* infection, severe granulomatous meningeal inflammation and bacterial infiltration are seen in brain tissue (B, top), and large numbers of bacteria are associated with degenerate splenic ellipsoids (B, bottom). (C) In contrast, fish exposed to the ΔPGM mutant lacked significant histopathologic abnormalities in the meninges (top) or splenic (bottom) tissues.

The transposon mutation maps to the promoter of an ORF encoding the *S. iniae* homologue of α-PGM, sharing >80% amino acid identity to PGMs of other streptococcal species and all conserved regions for enzymatic activity. Single-gene complemenation analysis confirms that both PGM enzymatic activity and virulence attenuation are associated with the *pgmA* gene. The phenotype of the *S. iniae* ΔPGM mutant is characterized by increased cell size, decreased buoyancy, decreased encapsulation, decreased surface negative charge, more rapid clearance in blood, increased susceptibility to cationic AMPs, and a profound decrease in disease potential in vivo. To our knowledge, *pgmA* represents the first molecular virulence determinant identified for *S. iniae* pathogenicity in fish.

In bacteria, PGM catalyzes reactions at the branch point that determines whether incoming sugars will be catabolized for energy or, alternatively, utilized in the synthesis of more complex structures such as polysaccharides, usually through sugar nucleotide intermediates. In many species of bacteria, disruption of PGM activity can reduce the availability of sugar building blocks needed for key biosynthetic pathways, consequently reducing the virulence potential of the ΔPGM mutant. For example, the production of exopolysaccharide capsule in *Streptococcus pneumoniae* and *Streptococcus thermophilus* requires the presence of a functional PGM enzyme (15, 25). Cell wall polysaccharide changes associated with PGM deletion in gram-negative bacteria include altered lipopolysaccharide in *Borde-*

TABLE 2. Histological survey of brain and spleen of hybrid striped bass infected with wild-type *Streptococcus iniae* and the ΔPGM mutant

Test and tissue studied	No. of samples infected/no. tested (h)				
	PBS	WT *S. iniae* (48)	ΔPGM (48)	WT *S. iniae* (96)	ΔPGM (96)
Giemsa stain					
Spleen: bacteria associated with ellipsoids	0/2	3/3	0/3	3/3	0/3
Brain: bacteria associated with meninges	0/2	3/3	0/3	3/3	0/3
Brain histology					
Meningeal inflammation	0/2	2/3	0/3	3/3	0/3
Ventricle (optic lobe) inflammation	0/2	0/3	0/3	2/2	0/3
Relative severity of histological lesions (0–4)					
Spleen	0	4	1	3	1
Brain	0	3	0	4	0

tella bronchiseptica (44), truncated lipo-oligosaccharide in *Neisseria meningitidis* (32), and shorter O-polysaccharide side chains in *Stenotrophomonas maltophilia* (30). Our ultrastructural examination suggests decreased but not absent expression of the recently described (4), as-yet-biochemically-uncharacterized *S. iniae* surface capsule in the ΔPGM mutant.

Streptococcal surface capsules are generally complex anionic polysaccharides that contribute importantly to virulence by virtue of their antiphagocytic properties (26). Streptococcal capsular polysaccharides are also frequently a principal target of protective immunity in natural infection and can represent effective vaccine antigens particularly when conjugated to protein carriers (23). Decreased buoyancy, decreased surface negative charge, and increased hydrophobicity as measured in our *S. iniae* ΔPGM mutant are changes consistent with diminished expression of a polyanionic surface capsule. Direct correlations between buoyancy, degree of encapsulation, and animal virulence have been demonstrated for group B *Streptococcus* (14), group A *Streptococcus* (43), and *Streptococcus equi* (3). Of particular interest, ΔPGM mutants of *S. pneumoniae* lacking surface capsule are avirulent in mice (16). In *S. iniae*, transposon disruption of the putative capsule operon was recently shown to be correlated with decreased buoyancy and decreased virulence in zebra fish (31). We hypothesize that diminished capsule expression is an important aspect of the increased susceptibility to phagocytic clearance and virulence attenuation of the *S. iniae* ΔPGM mutant.

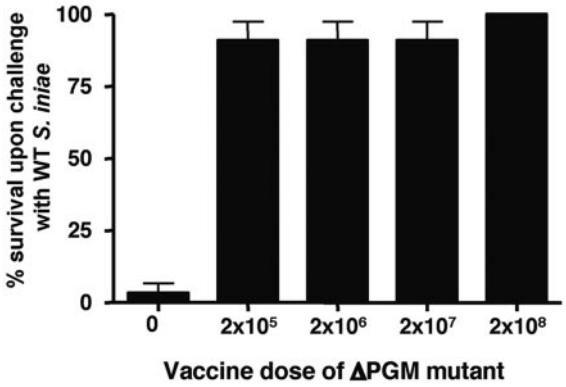

FIG. 8. Live attenuated vaccination with the *Streptococcus iniae* ΔPGM mutant protects HSB against a lethal wild-type infectious challenge. Groups of 30 HSB (each, ~30 g) were injected IP with stated doses of ΔPGM mutant vaccine or PBS control. After 2,000 degree days, HSB from each treatment were challenged IP with a lethal dose of 2×10^5 CFU of wild-type *S. iniae* and survival monitored for 21 days.

Electron microscopy also revealed a marked increase in the cell size of the ΔPGM mutant in comparison to WT *S. iniae*. Precedent exists in studies of bacterial ΔPGM mutants for alterations in cell morphology in response to loss of enzyme activity. The bacteriophage C21, which is unable to infect WT *E. coli* strains (38), was found to efficiently bind and infect *E. coli* ΔPGM mutants grown in glucose media (1). This finding was theorized to reflect defects in normal cell wall polysaccharides due to the lack of production of sugar nucleotides (such as UDP-glucose) dependent upon functional PGM (1). A subsequent structural study of *E. coli* ΔPGM mutants measured their length to be only ~70% that of the corresponding WT strains (27). With *Lactococcus lactis*, ΔPGM mutants grown on maltose (favoring catabolic processes) were observed by phase-contrast microscopy to be significantly larger than WT cells (24). Each of these phenotypes suggests significant alterations in cell wall architecture and, in the case of the gram-positive *L. lactis*, an increased cell size similar to that observed with our *S. iniae* ΔPGM mutant was documented.

AMPs are an evolutionarily conserved component of the innate defense system of higher organisms against microbial infection (45), as recently confirmed by targeted gene knockout in mammals (34). AMPs have been identified in numerous fish species (35) and represent a key component of the killing mechanism of their phagocytic cells (33). The 22-amino-acid cationic AMP moronecidin is produced in the gills, spleen, head kidney, intestine, skin, blood cells, and mast cells of HSB (21, 40). We found that loss of PGM activity in *S. iniae* results in markedly increased susceptibility to moronecidin antimicrobial action. We speculate that the increased cell size of the *S. iniae* ΔPGM mutant may reflect a decrease in cell wall rigidity that could render the bacterium more susceptible to lysis by the hypothesized pore-forming mechanism of this and other AMPs. In addition, the decreased net negative charge of the ΔPGM cell surface (as confirmed by diminished cytochrome *c* binding) may decrease the electrostatic attraction of the cationic AMP for its cell wall target of action, a well-described factor in determining the relative susceptibility of bacteria to AMP killing (36). Interestingly, a study of the role of PGM in *B. brochiseptica* determined that decreased animal virulence

could be correlated with increased cationic AMP susceptibility, as corroborated experimentally with cecropin B (44).

S. iniae infection represents a serious threat to the economic viability of intensive aquaculture of several finfish species. Antibiotic treatment protocols and traditional bacterin-type vaccines to control *S. iniae* infection each possess significant practical limitations. Our preliminary vaccine trial indicates that ΔPGM mutant TnM2 indeed elicits an effective immune response and may have value as a live attenuated vaccine to protect HSB against *S. iniae* infection. This degree of attenuated infection and antigenic load was sufficient to induce an effective immune response, as live vaccinated fish demonstrated >90% survival upon exposure to a lethal dose of WT *S. iniae* 4 months (2,000 degree days) later. Furthermore, in our examination of the aborted infectious process of the ΔPGM mutant, we observed the mutant to disseminate to multiple organs within 4 h but to be rapidly cleared from blood and brain while persisting at diminished levels in splenic tissue for up to 5 days after infection. The use of live attenuated vaccines in aquaculture could offer benefits, including elicitation of mucosal immunity, longer presentation time of unaltered antigens, and stimulation of both cell-mediated and humoral immunity (28, 29, 41). A number of recent studies of fish have found live attenuated vaccines against other bacterial pathogens to be effective (9, 22, 28), and the development of a successful live attenuated vaccine to treat the globally important fish pathogen *S. iniae* would be greatly welcomed.

In summary, we have identified the PGM gene of *S. iniae* and linked its activity to a variety of structural and functional phenotypes that translate to dramatically decreased virulence in a model of fish meningoencephalitis. Future research avenues include unraveling the biochemistry of the *S. iniae* polysaccharide capsule to ascertain the role that PGM plays in its expression, identifying the PGM-dependent cell surface components of *S. iniae* that contribute to AMP resistance, and exploring the utility of ΔPGM mutants as live attenuated vaccines in larger scale trials for applications in aquaculture.

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