Evaluation of *Streptococcus iniae* killed bacterin and live attenuated vaccines in hybrid striped bass through injection and bath immersion

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ABSTRACT: *Streptococcus iniae* poses a serious threat to finfish aquaculture operations worldwide. Stringent regulatory standards limit the use of antibiotics to treat *S. iniae* infections; improved vaccination strategies are thus of great interest. We investigated the potential for efficient, non-injectable batch vaccination via the use of live attenuated vaccines. Three attenuated *S. iniae* strains with genetic mutations eliminating the production of virulence factors—capsular polysaccharide (Δ*cpsD*), M-like protein (Δ*simA*), and phosphoglucomutase (Δ*pgmA*)—were evaluated in parallel with an adjuvanted, formalin-killed, whole-cell *S. iniae* bacterin. Juvenile hybrid striped bass (HSB; *Morone chrysops* × *M. saxatilis*) were vaccinated through intraperitoneal (i.p.) injection or bath immersion and held for 800 degree-days prior to challenge with a lethal dose of the virulent wild-type (WT) *S. iniae* parent strain. The Δ*cpsD*, Δ*pgmA*, and bacterin vaccines provided the highest level of vaccination safety (0% mortality), whereas the Δ*simA* mutant, although it caused 12 to 16% vaccination-related mortality, was the only vaccine candidate to provide 100% protection in both i.p. and immersion delivery models. Our studies demonstrate the efficacy of live attenuated vaccines for prevention of *S. iniae* infection, and identify immersion delivery of live vaccines as an attractive option for use in commercial aquaculture settings.

KEY WORDS: *Streptococcus iniae* · Live attenuated vaccine · Hybrid striped bass · M-like protein · Capsular polysaccharide · Phosphoglucomutase

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INTRODUCTION

Streptococcus iniae was first isolated from an Amazon freshwater dolphin *Inia geoffrensis* in the 1970s (Pier & Madin 1976) and since then has been shown to cause infection in a wide variety of fresh and saltwater finfish. *S. iniae* outbreaks predominantly occur in intensive aquaculture operations and affect many commercially important fish species, including tilapia, salmon, trout, yellowtail, barramundi, flounder, and hybrid striped bass (HSB) (Agnew & Barnes 2007). Common clinical signs of *S. iniae* infection include loss of orientation, lethargy, ulcers, and exophthalmia (Eldar et al. 1995). Mortality resulting from *S. iniae*

infection is often attributed to meningoencephalitis (Eldar et al. 1995), and is responsible for annual losses exceeding USD 100 million in aquaculture (Shoemaker et al. 2001). Despite the prevalence of *S. iniae* as an aquatic pathogen, therapeutic options for treating or preventing infection are limited.

While vaccines have been a viable therapeutic strategy to prevent infections caused by many bacterial fish pathogens (Sommerset et al. 2005), attempts to develop *Streptococcus iniae* vaccines have been met with limited success in part due to a general lack of efficacy over extended time periods (Agnew & Barnes 2007) and the re-emergence of serotypic variants (Bachrach et al. 2001). The predominant vaccination approach for *S. iniae* utilizes whole-cell, killed bacterins (Eldar et al. 1997, Klesius et al. 2000). There are currently 2 whole-cell killed commercial *S. iniae* vaccines, Norvax® Strep *Si* and AquaVac™ Garvetil™ (Intervet/Schering-Plough Animal Health), but neither is approved for use in the USA.

Transposon and targeted mutagenesis techniques have facilitated the development of live attenuated vaccines as an alternative vaccination strategy for *Streptococcus iniae* (Buchanan et al. 2005, Locke et al. 2008). The attractiveness of live attenuated vaccines relative to bacterin vaccines lies in their ability to stimulate a robust humoral and cell-mediated immune response, which can translate into long-term adaptive protection against subsequent exposure to a virulent wild-type (WT) strain (Marsden et al. 1996, 1998, Zlotkin et al. 2003). A key step towards targeted live attenuated vaccine development involves understanding *S. iniae* virulence factors. To date, only a handful of these determinants have been characterized for *S. iniae* in the context of fish virulence: phosphoglucomutase (Buchanan et al. 2005), capsular polysaccharide (Locke et al. 2007a, Lowe et al. 2007, Shutou et al. 2007), streptolysin S (Fuller et al. 2002, Locke et al. 2007b), and M-like protein (Baiano et al. 2008, Locke et al. 2008).

Mutation of any *Streptococcus iniae* virulence gene resulting in significant *in vivo* attenuation warrants consideration for preliminary testing of the mutant as a vaccine candidate. Previously, through intraperitoneal (i.p.) vaccination of HSB, we found protection levels of 100% against WT *S. iniae* (after 1400 degree-days) with an M-like protein mutant strain (Δ*simA*) (Locke et al. 2008) and 90 to 100% protection (after 2000 degree-days) with a phosphoglucomutase-deficient transposon mutant (Δ*pgmA*) (Buchanan et al. 2005). Our allelic replacement of the *cpsD* gene in the *S. iniae* capsular polysaccharide biosynthesis operon resulted in a highly attenuated mutant strain (Δ*cpsD*) (Locke et al. 2007a), and preliminary testing showed efficacy of this mutant at higher doses in a similar HSB i.p. vaccination

model (data not shown). However, vaccination by immersion has a number of advantages over injection-based approaches, including reduced handling and stress to fish, lower cost, avoidance of damage to fillets, and the potential to more effectively stimulate the teleost immune response by delivery through a route utilized by aquatic pathogens.

We explored the protective capacity of a formalin-killed *Streptococcus iniae* bacterin (WT K288) in parallel with 3 isogenic live attenuated mutant *S. iniae* strains (Δ*cpsD*, Δ*simA*, and Δ*pgmA*), which are deficient in capsular polysaccharide, M-like protein, and phosphoglucomutase, respectively. Each vaccine was evaluated in juvenile HSB in parallel through i.p. injection and bath immersion. Survival following a lethal challenge with WT *S. iniae* was used to assess vaccine efficacy after an 800 degree-day holding period. These studies are the first to evaluate immersion delivery of live attenuated *S. iniae* vaccines and to directly compare efficacy of multiple live *S. iniae* vaccine candidates to a killed bacterin.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and DNA techniques. *Streptococcus iniae* WT strain K288 was isolated from the brain of a diseased HSB at the Kent SeaTech aquaculture facility in Mecca, CA (Buchanan et al. 2005). The Δ*simA* (Locke et al. 2008) and Δ*cpsD* (Locke et al. 2007a) allelic replacement mutants and the Δ*pgmA* transposon mutant (Buchanan et al. 2005) were all generated in the strain K288 background (Table 1). Mutant and WT *S. iniae* strains were cultured at 30°C in Todd-Hewitt broth (THB; Hardy Diagnostics) or on Todd-Hewitt agar plates (THA). Enumeration of colony-forming units (CFU) was performed by serially diluting bacteria in phosphate-buffered saline (PBS; pH 7.4) and plating on THA. A Colony Fast-Screen Kit (EPICENTRE Biotechnologies) was used to isolate genomic DNA for PCR identification of *S. iniae* colonies isolated from challenged fish. Vaccine strains were identified through amplification of regions spanning the allelic exchange or transposon insertion sites as previously described (Buchanan et al. 2005, Locke et al. 2007a, 2008).

Preparation of formalin-killed bacterin. WT K288 *Streptococcus iniae* was grown to early stationary phase (optical density at 600 nm [OD_{600}] ≈ 0.80; Spectronic 20D spectrophotometer). CFU were enumerated through plating serial dilutions of the 0.80 culture prior to formalin inactivation via room temperature (21 ± 1°C) incubation for 72 h with 0.07% formaldehyde (v/v). A 100 µl aliquot of the culture was plated on THA and incubated for 48 h at 30°C to ensure total killing.

Table 1. *Streptococcus iniae* vaccine strains and doses. CFU = colony-forming units, i.p. = intraperitoneal, WT = wild-type

Strain	Description	I.p. dose (CFU)	Immersion dose (CFU ml^{-1})	Reference
K288	Bacterin prepared from WT strain	3×10^8	4×10^7	Buchanan et al. (2005)
ΔsimA	M-like protein (SiM) allelic replacement mutant	3×10^6	3×10^7	Locke et al. (2008)
ΔcpsD	Capsular polysaccharide allelic replacement mutant	3×10^7	3×10^7	Locke et al. (2007a)
ΔpgmA	Phosphoglucomutase transposon insertion mutant	3×10^7	3×10^7	Buchanan et al. (2005)

Bacteria were centrifuged ($3250 \times g$ for 15 min) and resuspended in supernatant to an OD_{600} of 5.0. EMULSIGEN® oil-in-water adjuvant (MVP Laboratories) was added to a final concentration of 20% (v/v) with the bacterial solution. The bacterin was stored at 4°C and used within 1 wk of preparation.

Preparation of live vaccines. Glycerol aliquots of each *Streptococcus iniae* strain were used to start static 3 ml overnight cultures (~16 h) which were then diluted 1:10 in fresh THB and grown to mid-log phase (OD_{600} = 0.40; Spectronic 20D spectrophotometer). Cultures were centrifuged ($3500 \times g$ for 7 min), washed once in PBS, re-centrifuged, then resuspended in an equal volume of PBS. Bacterial suspensions were diluted in PBS to achieve desired injection doses in a 100 μl injection volume (Table 1).

HSB. Juvenile HSB (*Morone chrysops* × *M. saxatilis*; Keo Fish Farms, Keo, AR) were held in recirculating 1000 l tanks at 20 ± 2°C prior to use in vaccine challenges. All fish were fed daily with 3.5 mm high-protein (40%) sinking pellet feed (Nelson's Silver Cup Trout Feed) and were maintained on a 12 h light:12 h dark cycle.

I.p. vaccination. Each *Streptococcus iniae* strain was serially diluted in PBS and plated on THA to confirm the dose injected. The formalin-killed bacterin was also delivered in a 100 μl volume (~3×10^8 CFU fish^{-1}). Mock vaccinate groups included fish injected with PBS only and a group injected with adjuvant plus THB (100 μl volumes). Groups of 25 juvenile HSB (~15 g) were anesthetized with MS-222 (25 mg l^{-1}; Western Chemical), weighed, and fin-clipped (right or left caudal, pectoral, or pelvic) for identification by treatment group. Bacteria were delivered i.p. using a 1.0 ml syringe and 27-gauge needle. Initially, vaccinate groups were held separately in 100 l tanks at 26 ± 1°C with flow-through water and aeration for 2 wk. Fish were fed sparingly and monitored daily for survival. Brain cultures were taken from mortalities via brain swab following preparation as previously described (Phelps et al. 2009) to identify the possibility of infection from the vaccine strain. Swabs were streaked on THA and screened by PCR to positively identify vaccine strains. After the 2 wk holding period, vaccinated fish were transferred into 1000 l recirculating tanks

and cohabitated. Fish were held for an additional 3 wk (~800 degree-days total) at 20 ± 1°C and fed to satiation daily. Two days prior to WT challenge, 3 fish were randomly sampled from each group for analysis of *S. iniae*-specific serum immunoglobulin M (IgM) levels; however, high assay background variation prevented conclusive analysis of immunoglobulin response trends.

Immersion vaccination. A 1.0 l culture of each *Streptococcus iniae* strain (mid-log phase, cultured as described for live i.p. vaccines) was added to 9.0 l of dechlorinated system water in an aerated 5.0 gallon (19 l) plastic bucket (~3.0×10^7 CFU ml^{-1} final concentration) (Table 1). The K288 bacterin was diluted 1:80 in system water (~4×10^7 CFU ml^{-1}) for HSB immersion vaccinations. A 10% THB solution served as a mock immersion vaccination control. Fish (n = 25 treatment group^{-1}) were anesthetized, fin-clipped, and weighed prior to immersion in their respective solutions for a period of 90 min. All immersion vaccinations were performed in 10.0 l volumes. Immersion vaccinated fish were maintained and fin-clipped as described for the i.p. vaccinate groups.

WT *Streptococcus iniae* challenge. After 800 degree-days, vaccinates were removed from the holding tanks and sorted into 100 l flow-through challenge tanks. Over 2 d, tanks were heated up from 20°C to the challenge temperature of 26°C. Fish were anesthetized and challenged i.p. with 1×10^6 CFU (in 100 μl) of WT K288 (preparation as described for live vaccines), then fed lightly and monitored for survival for 2 wk post challenge. Bacteria recovered from all mortalities were confirmed to be WT *S. iniae* through culture and PCR analysis. Vaccine trials were conducted in a facility certified by the Association and Accreditation of Laboratory Animal Care (AAALAC International) following protocols approved by the Institutional Animal Care and Use Committee (IACUC).

Statistical analysis. Survival data were analyzed using a log-rank test included in the GraphPad Prism software suite (GraphPad Software). A p-value < 0.05 was considered statistically significant. Relative percent survival (RPS) was calculated by the following equation: {1 − [(vaccinate mortality)/(control mortality)]} × 100 (Amend 1981).

RESULTS

Survival post-vaccination

Both i.p. and bath-immersion vaccination of HSB using the 3 attenuated *Streptococcus iniae* mutants and the killed bacterin were generally well tolerated by the fish (Table 2). As expected, no post-vaccination mortalities occurred in any of the mock vaccination control groups or in the killed bacterin group. Consistent with previous HSB i.p. virulence studies for the Δ*pgmA* (Buchanan et al. 2005) and Δ*cpsD* (Locke et al. 2007a) mutants, i.p. vaccination with 10^7 CFU of each of these strains generated no mortalities. These 2 mutants were also avirulent when delivered through bath immersion. The Δ*simA* mutant was the only vaccine candidate to generate vaccination-related mortalities in both groups (16 % with i.p., 12 % with immersion). *S. iniae* recovered from the brains of dead fish from the Δ*simA* mutant group were positively identified as the mutant strain through PCR analysis.

Survival post-WT challenge

Fish were held for 5 wk post-vaccination (800 degree-days), a sufficient holding period to ensure that any protection against WT
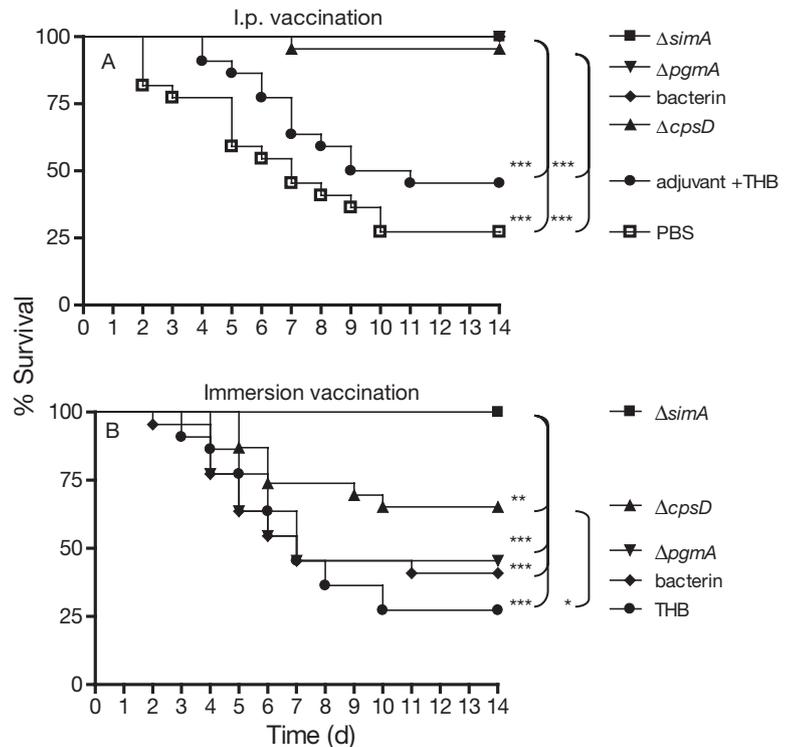

Fig. 1. Survival of hybrid striped bass (HSB; *Morone chrysops* × *M. saxatilis*) vaccinated against *Streptococcus iniae*. HSB vaccinated through (A) intraperitoneal (i.p.) injection or (B) bath immersion were challenged with a lethal dose of wild-type (WT) *S. iniae* after an 800 degree-day holding period. Kaplan-Meier plots show survival over a 14 d monitoring period post-challenge. Significant differences between survival data sets: *p < 0.05, **p < 0.01, ***p < 0.001. PBS = phosphate-buffered saline, THB = Todd-Hewitt broth. See Table 1 for description of *S. iniae* mutants Δ*simA*, Δ*cpsD*, and Δ*pgmA*

Table 2. Summary of *Streptococcus iniae* vaccine trial data. i.p. = intraperitoneal, na = not applicable, PBS = phosphate-buffered saline, THB = Todd-Hewitt broth, WT = wild-type See Table 1 for description of *S. iniae* mutants Δ*simA*, Δ*cpsD*, and Δ*pgmA*

Vaccination group	Vaccination survival (%)	WT challenge survival (%)	Relative % survival
I.p. injection			
Δ*simA*	84	100	100
Δ*cpsD*	100	96	95
Δ*pgmA*	100	100	100
PBS control	100	27	na
K288 bacterin	100	100	100
Adjuvant + THB control	100	46	26
Bath immersion			
Δ*simA*	88	100	100
Δ*cpsD*	100	64	51
Δ*pgmA*	100	46	26
K288 bacterin	100	41	19
THB control	100	27	na

Streptococcus iniae challenge (Fig. 1) was a result of adaptive immunity rather than prolonged upregulation of the innate immune system. Following i.p. challenge of all vaccinated fish with 1×10^6 CFU of WT *S. iniae*, 27 % survival was seen in the THB and PBS mock vaccination groups (Table 2). The THB plus adjuvant i.p. control group had slightly increased survival (46 %) compared to the corresponding PBS control group, perhaps through non-specific immune stimulation, though the 2 survival curves were not significantly different (p = 0.13). No mortalities were observed in fish that had been vaccinated i.p. with Δ*simA*, Δ*pgmA*, or the killed bacterin. One fish died (96 % survival) in the Δ*cpsD* i.p. vaccinate group following WT challenge. With the exception of the Δ*simA* vaccinate group (100 % survival), where no mortalities were seen, immersion vaccination groups suffered lower post-challenge survival levels than they did following injection vaccination: 64 % for Δ*cpsD*, 46 % for Δ*pgmA*, and 41 % for bacterin.

Overall vaccine efficacy

The adaptive immuno-protection conferred by each vaccine was assessed through calculation of RPS. For i.p. vaccination, the Δ*pgmA* and killed bacterin i.p. vaccine groups had perfect protection profiles (100% RPS) during the trial (Table 2). However, for immersion vaccination, the Δ*pgmA* and bacterin vaccines were the worst overall immersion candidates, with RPS values of 26 and 19%, respectively; the groups were not significantly different from the survival of the THB immersion control group (p = 0.51 and p = 0.66, respectively). The Δ*cpsD* mutant had 95% RPS through i.p. delivery, a value not significantly different from the Δ*pgmA* and bacterin groups (p = 0.37). The Δ*cpsD* immersion-vaccinated group had an RPS value of 51% and was significantly more protective (p = 0.01) than the THB immersion control group. The Δ*simA* mutant was the only vaccine to achieve 100% RPS in all surviving fish through both delivery methods.

DISCUSSION

As knowledge of *Streptococcus iniae* virulence increases and a growing number of virulence factor-deficient mutants are available, the vaccine potential of the most attenuated of these mutants can be assessed. We evaluated 3 such *S. iniae* mutants lacking capsular polysaccharide (Δ*cpsD*), M-like protein (Δ*simA*), and phosphoglucomutase (Δ*pgmA*). The efficacy of each live mutant was compared through i.p. injection and bath immersion in parallel with an isogenic, adjuvanted, formalin-killed bacterin. Both live and killed vaccine candidates generated 95 to 100% RPS values when delivered by injection. However, through immersion delivery, only the Δ*simA* live mutant provided a high level of protection (100% RPS), demonstrating for the first time the superior efficacy of live *S. iniae* vaccines in this commercially desirable delivery method. The present study also highlights some of the challenges faced with live vaccines, such as vaccination safety and the balance between attenuation, efficacy, and the conservation of immunodominant epitopes.

Despite its high protective capacity in both delivery models, the Δ*simA* mutant was the only 1 of the 3 live strains tested that lacked complete attenuation at the delivered dose. The 16% mortality observed after i.p. vaccination in the present study is greater than the 8% mortality observed at a similar dose in previous work with this mutant (Locke et al. 2008), potentially attributable to the higher temperature at vaccination employed here compared to previous work (26 vs. 24°C), although these differences may not be biologically significant. Increase in temperature correlates

positively with *Streptococcus iniae* virulence (Kitao 1993), and the use of outbred fish may also account for some of the variability in HSB susceptibility to *S. iniae* infection. Regardless, in order to minimize pre-vaccination mortalities, the Δ*simA* mutant will require further mutagenesis-based attenuation or optimization of vaccination conditions, for example by lowering the immunization dose or using cooler water temperature.

Past research has indicated that the Δ*simA* mutant has a reduced ability to invade and adhere to epithelial cells (Locke et al. 2008). Because the Δ*simA* mutant retained sufficient abilities for host interaction to function in immersion vaccination, it suggests that the role of SiM in adherence and invasion of epithelial cells (Locke et al. 2008) may be shared or redundant with other *Streptococcus iniae* surface factors. The Δ*simA* mutant was able to provide complete immune protection against WT challenge and we speculate that the SiM protein itself is not an essential antigenic epitope involved in the adaptive immune response to *S. iniae*.

Properties of an ideal live vaccine include sufficient attenuation, low cytotoxicity, complete immune clearance, conservation of key antigenic epitopes, and stimulation of long-term adaptive immune protection. Studies of the Δ*pgmA* mutant, through the present study and earlier work using an extended 2000 degree-day trial (Buchanan et al. 2005), show that so far this mutant appears to meet all of these key criteria when delivered through injection. Despite a perfect i.p. safety and efficacy profile, the Δ*pgmA* mutant was a poor immersion vaccine candidate. Mutagenesis of *pgm* appears to have significantly reduced the ability of the bacteria to utilize natural water-based transmission routes, or perhaps weakening of the cell wall (Buchanan et al. 2005) has lowered the ability of this mutant to handle osmotic stress and therefore has diminished viability in water-based immersion delivery.

The adjuvanted, formalin-killed bacterin used in the present study was prepared according to a commercial protocol used at an aquaculture facility that vaccinates fish using autogenous *Streptococcus iniae* isolates and thus serves as a relevant comparator vaccine. The bacterin, when injected at a log-greater CFU than the live attenuated vaccines, provided complete protection. Efficacy of the whole-cell bacterin may also be in part due to the inclusion of supernatant from the liquid culture in the injected bacterin. Previous work with whole-cell killed bacterins has demonstrated the importance of small extracellular products in the adaptive immune response (Klesius et al. 2000); live vaccines offer the advantage that they inherently produce these small immunogenic compounds *in vivo* and in their native form. Despite high levels of protection when delivered i.p., the killed bacterin performed

poorly in the immersion trial and did not provide statistically significant protection against WT *S. iniae*. Development of localized immunity at the site of i.p. injection vaccination (followed by i.p. challenge) may have contributed slightly to the efficacy of injected vaccination over immersion vaccination. Nevertheless, the present results indicate a failure to induce significant protection by immersion.

While injection vaccination is precise and reproducible, it is also labor- and cost-intensive and is thus restricted in application to only higher-value aquaculture species such as salmonids and HSB. Immersion vaccination is more economical and more closely mimics natural infection routes utilized by aquatic pathogens, resulting in a potentially more robust immune response. I.p. vaccination with all candidate *Streptococcus iniae* vaccines provided >95% RPS; however, immersion administration of these same vaccines failed to provide high levels of adaptive immune protection, with the exception of the *S. iniae* Δ*simA* mutant. Other studies have shown similar trends with superior protection of killed bacterins for closely related streptococcal species when delivered through injection versus immersion (Iida et al. 1982, Evans et al. 2004). The ability to effectively deliver a vaccine through immersion may be a key feature of live attenuated vaccines that cannot be replicated with killed alternatives and may make them more attractive to commercial users.

Successful passive vaccination experiments suggest that the immune response to *Streptococcus iniae* infection is largely antibody-mediated (Shelby et al. 2002, Evans et al. 2006). Kinetic studies of the specific IgM response of fish to vaccination with live *S. iniae* strains could be an interesting comparison to the response generated with killed bacterins. Another avenue of investigation with live vaccines should focus on assessing their cross-protective capabilities against *S. iniae* serotypes. Emergence of serologically distinct strains in operations where fish have been vaccinated is a problem with *S. iniae* vaccination efforts (Bachrach et al. 2001). Studies have shown that vaccination with a bacterin composed of multiple serotypes provides a superior protective advantage (Klesius et al. 2000), but just how much cross-protection is generated with live vaccines is unknown.

Live bacterial vaccines have demonstrated sufficient efficacy and safety for use in finfish aquaculture. Three live attenuated vaccines have already received approval by the United States Department of Agriculture (USDA) for use in US aquaculture: AQUAVAC-ESC® (Intervet/Schering-Plough Animal Health) for prevention of enteric septicemia in catfish caused by *Edwardsiella ictaluri* (Klesius & Shoemaker 1999), AQUAVAC-COL™ (Intervet/Schering-Plough Animal Health) for prevention of columnaris in catfish caused by *Flavobacterium columnare* (Shoemaker et al. 2007), and Renogen™ (Novartis Animal Health) for prevention of infections in salmonids caused by *Renibacterium salmoninarum* and *Piscirickettsia salmonis* using an *Anthrobacter davidanieli* strain (Salonius et al. 2005). These successes demonstrate the potential of live attenuated vaccines for aquaculture application. Two issues would need to be considered prior to application of a live attenuated *Streptococcus iniae* vaccine. First, the vaccine strain would need to be incapable of reversion back to WT; this is true of the allelic exchange mutants used in the present study where the virulence gene has been deleted. The other main criterion would be the absence of any antibiotic resistance genes; these are found in the allelic exchange mutants used in the present study, but they could easily be removed via subsequent targeted deletion mutagenesis.

In sum, we have evaluated a panel of 3 attenuated live *Streptococcus iniae* vaccine candidates and an adjuvanted WT bacterin using injection and immersion delivery. While high levels of protection were achieved with all vaccines delivered through i.p. injection, the Δ*simA* mutant was the only vaccine to provide complete protection when delivered via immersion delivery. This finding highlights a potential advantage of invasive live attenuated vaccines over killed-bacterin alternatives in an immersion application. Future large-scale studies should investigate the long-term efficacy and multi-strain cross-protective capacity of these promising live vaccine candidates. Oral delivery of the live attenuated vaccines should also be tested with these vaccine strains, as this commercially desirable method of vaccination, like immersion delivery, utilizes a known route of infection in *S. iniae* (Perera et al. 1997, Bromage & Owens 2002) and may be well suited for use with live attenuated mutants.

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