Veterinary Microbiology 162 (2013) 151–159

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

The conserved surface M-protein SiMA of *Streptococcus iniae* is not effective as a cross-protective vaccine against differing capsular serotypes in farmed fish

Fabian Aviles, Meiman May Zhang, Janlin Chan, Jerome Delamare-Deboutteville, Timothy J. Green, Cecile Dang, Andrew C. Barnes *

The University of Queensland, School of Biological Sciences and Centre for Marine Science, Brisbane, QLD 4072, Australia

ARTICLE INFO

Article history:
Received 20 June 2012
Received in revised form 22 August 2012
Accepted 22 August 2012

Keywords:
Streptococcus iniae
SiMA
Vaccine
Barramundi
Antibody response
Macrophage
Mgx

ABSTRACT

Streptococcus iniae causes invasive infections in fresh and saltwater fish and occasional zoonoses. Vaccination against *S. iniae* is complicated by serotypic variation determined by capsular polysaccharide. A potential target for serologically cross-protective vaccines is the M-like protein SiMA, an essential virulence factor in *S. iniae* that is highly conserved amongst virulent strains. The present study determined how SiMA is regulated and investigated potential as a cross-protective vaccine for fish. Electrophoretic mobility shift suggested that SiMA is regulated by the multigene regulator Mgx via a binding site in the −35 region of the *simA* promoter. Moreover, expression of *simA* and *mgx* was highly correlated, with the highest level of *simA* and *mgx* expression during exponential growth under iron limitation (20-fold increase in relative expression compared to growth in Todd-Hewitt broth). Based on these results, a vaccination and challenge experiment was conducted in barramundi (*Lates calcarifer*) to determine whether SiMA is protective against *S. iniae* infection and cross-protective against a different capsular serotype. The challenge resulted in 60% mortality in control fish. Formalin-killed bacterins prepared from the challenge strain resulted in 100% protection, whereas bacterins prepared from a serotypically heterologous strain resulted in significantly reduced protection, even when culture conditions were manipulated to optimise SiMA expression. Moreover, recombinant SiMA protein was not protective against the challenge strain in spite of eliciting specific antibody response in vaccinated fish. Specific antibody did not increase oxidative activity or phagocytosis by barramundi macrophages. Indeed incubating *S. iniae* with antisera significantly reduced phagocytosis. Lack of specific-antibody mediated opsonisation in spite of 100% protection against challenge with the homologous vaccine suggests that other immune parameters result in protection of challenged fish.

1. Introduction

Streptococcus iniae is a pathogen of wild and farmed marine and freshwater fish in all temperate aquatic regions, causing substantial losses to aquaculture (Agnew and Barnes, 2007; Shoemaker et al., 2001) and is occasionally zoonotic in a susceptible demographic (Lau et al., 2006; Weinstein et al., 1997). Consequently, it is important to control *S. iniae* infections in farmed fish, but vaccination has met with occasional vaccine failure (Bachrach et al., 2001; Nawawi et al., 2008) through the emergence of novel serotypes (Bachrach et al., 2001). Antigenic serotype in *S. iniae* is determined by capsular polysaccharide (CPS) (Barnes et al., 2003b; Eyngor et al.,

* Corresponding author at: Aquatic Animal Health Laboratory, The University of Queensland, School of Biological Sciences, Brisbane, QLD 4072, Australia. Tel.: +61 7 3346 9416; fax: +61 7 3365 4755.
E-mail address: a.barnes@uq.edu.au (A.C. Barnes).

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http://dx.doi.org/10.1016/j.vetmic.2012.08.018

2008) and Streptococci are renowned for developing new capsular serotypes under high selective pressure (Weinberger et al., 2009; Zhang et al., 2009). Although polyvalent vaccines have been considered for control of *S. iniae* (Klesius et al., 2000), identification of more highly conserved protective surface antigens will be required for cost-effective cross-protective vaccines in fish (Baiano and Barnes, 2009).

In Group A Streptococcus (GAS), M protein, encoded by *emm* gene, has been extensively studied as a target of protective immunity (Smeesters et al., 2010), but hypervariability of its N-terminal domain has generated more than 100 known serovars amongst which lack of cross-protection is commonplace (Kwinn and Nizet, 2007). Unlike *emm* genes in GAS, the *emm*-like genes, *simA* and *simB* of *S. iniae* are highly conserved with only the *simA* variant found in all virulent strains (Baiano et al., 2008). Moreover, SiMA is a major virulence factor, contributing to macrophage resistance and adherence to fish epithelial cells (Baiano et al., 2008; Locke et al., 2008), with knockout mutants being attenuated (Locke et al., 2008). High conservation, surface location and key role in virulence make SiMA a potential target for serologically cross-protective vaccines against *S. iniae* in fish (Baiano and Barnes, 2009; Baiano et al., 2008). However, it is pertinent to consider why cross-protective immunity is not elicited in vaccinated fish with existing bacterins. One reason may be that SiMA is not expressed during normal culture and appears to be expressed only during growth in fish serum (Barnes et al., 2003a), therefore is not presented to the immune system in broth-grown bacterins used for vaccinating fish (Klesius et al., 2000; Sommerset et al., 2005). An understanding of the regulatory mechanisms behind SiMA expression is therefore required.

In GAS, expression of the genes encoding M-like proteins (such as *emm*), and other virulence factors in response to environment is controlled by the multigene regulator Mga, and their transcription responds to elevated carbon dioxide levels and changes such as iron limitation, osmolarity and temperature (McIver et al., 1995b). Recognition of these environmental cues by Mga results in co-ordinate regulation of multiple virulence-associated genes to ensure their expression at specific stages of an infection. A homologue of Mga, Mgx has been identified in *S. iniae* (Baiano et al., 2008), thus we investigated regulation of SiMA by Mgx in *S. iniae* in response to environmental cues. Then, having established optimal conditions for SiMA expression, we evaluated bacterins prepared from cultures grown under these conditions and recombinant SiMA for protective/cross-protective efficacy in a vaccination and challenge in fish.

2. Materials and methods

2.1. Bacterial strains and routine culture

S. iniae strain QMA0076, originally isolated from diseased barramundi in Queensland, Australia, has been previously characterised and the complete *simA* sequence published (Baiano et al., 2008). Strain QMA0248 was isolated from diseased barramundi in NSW in 2010 and was found to be virulent in barramundi in a pilot challenge and is serotypically different to QMA0076. The *simA* gene sequence of QMA0248 is 100% identical to *simA* of QMA0076. *S. iniae* strains were maintained as frozen stocks in Todd-Hewitt Broth (THB) (Oxoid, Thebarton, Australia), containing 20% glycerol at −80 °C. *S. iniae* were routinely recovered from stock on Columbia agar base containing 5% defibrinated sheep's blood (Oxoid) and used without further subculture on solid media.

2.2. Regulation of simA by Mgx

The *mgx* gene was amplified and 6 x His tagged by PCR with a proofreading polymerase (PrimeStar, Takara, Shiga, Japan) and the primers New fused MGX F and 6 His MGXR (Supplementary Table 1). The product was ligated by directional cloning into the pBAD/TOPO ThioFusion Expression vector (Invitrogen) and the construct was transformed into chemically competent *Escherichia coli* which were screened on Lysogeny Broth (LB) agar plates containing 100 mg/L ampicillin (Baiano et al., 2008). 6His-tagged Mgx was expressed in *E. coli* BL21 Star grown using standard methods and Mgx fusion protein was purified with ProBond (Invitrogen).

A 261 bp fragment of the *simA* promoter region (P_{sim}) incorporating the −35 region was amplified by PCR using primers Prom R and Prom F (Supplementary Table 1).

Binding of recombinant Mgx protein to the −35 region of the *simA* promoter (P_{sim}) was assessed by electrophoretic mobility shift assay (EMSA) (Lightshift, Pierce, Rockford, IL). Briefly, 3′ biotin-labelled P_{sim} was incubated with purified Mgx protein and analysed by native polyacrylamide gel (5%) electrophoresis. DNA–protein complexes were blotted onto a positively charged nylon membrane (Pierce) using a semi-dry blotting apparatus (Hoeffer TE70, GE Healthcare). Biotin-labelled DNA–protein complexes were visualised via chemiluminescent-labelled streptavidin with an ImageQuant ECL400 (GE Healthcare). Epstein Barr Nuclear Antigen 1 (EBNA) protein and a 60 bp DNA duplex containing the EBNA binding signal supplied with the kit were used as positive control.

2.3. Regulation of simA expression in culture

Starter cultures were prepared in three different liquid culture media: Tryptone Soya Broth (TSB), Todd-Hewitt Broth and Vegetable Peptone Broth (Oxoid) and used at 1% and 2% (v/v) to inoculate 500 μL of each broth in 48 well tissue culture plates (Krystal, Greiner Bio-One). Growth (OD_{600}) was recorded every 60 min for 24 h at 28 °C using a Fluostar Optima luminometer (BMG Labtech, Melbourne, Australia). Each assay was replicated at least twice.

Iron limitation was induced by the addition of 2,2-dipyridyl (DP) (Sigma Chemicals, Castle Hill, Australia) (Allard et al., 2006) to the THB at 10, 100 and 1 mM. Effect of salinity was investigated by adding NaCl to THB at a range of concentrations from 0 to 0.56 M (equivalent to seawater i.e. 35‰).