USE OF *IN VIVO*-INDUCED ANTIGEN TECHNOLOGY TO IDENTIFY BACTERIAL GENES EXPRESSED DURING *Solea senegalensis* INFECTION WITH *Photobacterium damselae* subsp. *Piscicida*

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Introduction

The marine fish pathogen *Photobacterium damselae* subsp. *piscicida* (*Phdp*) is responsible for important disease outbreaks affecting several fish species including flatfish *Solea senegalensis* (Kaup). *Phdp* is able to avoid host defences by invasion and intracellular survival in non-phagocytic cells, mainly epithelial cells. Virulence factors reported in *Phdp* include restricting complement-mediated activity, apoptosis of phagocytes caused by exotoxins secretion, iron acquisition mechanisms such as siderophores that enable the pathogen to obtain iron from transferrin and ability to bind haemin and antioxidant enzymatic activities capable to counteract superoxide radicals (Do Vale et al., 2005; Andreoni and Magnano, 2014). Commonly, genes expressed during pathogen infection are important for pathogenicity. *In vivo*-induced antigen technology (IVIAT) (Handfield et al., 2000) has been used to identify *in vivo*-induced genes using pooled sera from fish that have experienced photobacteriosis.

Materials and methods

Sera were obtained from surviving *S. senegalensis* specimens after sublethal infection with *Phdp* (Lg41/01) and subsequently pooled and adsorbed against *in vitro* grown *Phdp* Lg41/01 and *Escherichia coli* BL21 (DE3) cells and lysates according to Handfield et al. (2000). The efficiency of sera adsorption was evaluated based on the immunoreactivity after each adsorption step with whole and lysed *Phdp* cells grown *in vitro*. A genomic expression library of *Phdp* Lg41/01 was generated in *E. coli* BL21 (DE3) using pET-30 expression system (Novagen, San Diego, CA, USA). The expression library was probed with adsorbed and non-absorbed sera using immunoblot technique. Reactive clones of *in vivo*-induced and *in vitro* antigens were obtained, purified and their inserted DNA sequenced (Macrogen Europe, Amsterdam, The Netherlands). Nucleotide sequences were compared against the NCBI protein database using BLASTx.

Results

A progressive reduction in sera immunoreactivity against *in vitro* grown *Phdp* cells was detected after the adsorption rounds, especially after the first adsorption step. Thus, following adsorption steps substantially removed antibodies against *in vitro* expressed antigens and resulted in relative enrichment in antibodies recognizing *in vivo* expressed antigens. The library from *Phdp* Lg14/01 constructed in *E. coli* BL21 (DE3) consisted of approximately 6500 recombinants.

A total of 117 clones were selected for their reactivity with pooled adsorbed and non-adsorbed sera from convalescent *S. senegalensis* specimens after a first round of screening. In a second screening, 14 out of 117 candidate clones showed positive reaction, among which two clones were clearly positive and two gave weak reaction against adsorbed sera. Predicted proteins codified by inserted sequences have intracellular and membrane cell location and are involved in virulence, synthesis of intermediary products, energy metabolism and gene replication. Inosine-5'-monophosphate dehydrogenase (IMPDH) and alkyl hydroperoxide reductase (AhpC) have been identified as *in vivo* induced antigens expressed during *S. senegalensis* infection with *Phdp*. Iron/manganese superoxide dismutase (Fe/Mn-SOD) and alanyl-tRNA synthetase (AlaRS) proteins have also been identified, though with weak signal.

Discussion and conclusion

Identification of immunogenic bacterial proteins during *Phdp* infection is essential for understanding bacterial pathogenesis and development of effective vaccines. AhpC peroxidase activity has a protective role by reducing hydrogen peroxide, peroxynitrite and organic hydroperoxides. Immunization with AhpC conferred protection against *Helicobacter pylori* infection (O'Riordan et al., 2012). IMPDH catalyzes the conversion of products essential in *de novo* synthesis of guanine nucleotides. Adequate levels of purine nucleotides are critical for cell proliferation, nucleic acid replication, cell signaling and as a biochemical energy source. This gene is an important therapeutic target against bacterial diseases (Shu and Nair,

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2008). In conclusion, different genes expressed during *Phdp* infection in *S. senegalensis* have been identified. Among them, IMPDH and AhpC have been identified as *in vivo* induced antigens expressed during *S. senegalensis* infection with *Phdp*. Thus, they are likely to play a role in the virulence of *Phdp*. The antigenic character of these proteins makes them potential targets for the development of new vaccines.

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