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Use of *in vivo* induced technology to identify antigens expressed by *Photobacterium damselae* subsp. *piscicida* during infection of Senegalese sole (*Solea senegalensis*)

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ABSTRACT

Photobacterium damselae subsp. *piscicida* (*Phdp*), the causative agent of photobacteriosis, is an important pathogen in marine aquaculture that affects many different fish species worldwide, including *Solea senegalensis*, an important fish species for aquaculture in the south of Europe.

Bacteria express different repertoires of proteins in response to environmental conditions and when invading a host, sense *in vivo* environment and adapt by changing the expression of specific proteins. In the case of pathogens, identification of genes with up-regulated expression *in vivo* compared to *in vitro* conditions might give an insight into the genes relevant to the bacterial virulence.

In the present work, *in vivo* induced antigen technology (IVIAT) has been used to search for *Phdp* genes only expressed or up-regulated in infected *S. senegalensis*. An expression library from *Phdp* was assayed against pooled sera from convalescent *S. senegalensis* specimens and 18 clones were positive, indicating that proteins encoded are expressed by *Phdp* during *S. senegalensis* infection and are immunogenic for this fish species. In addition, five proteins were reactive against adsorbed sera, indicating their *in vivo* induced character. Inosine-5′-monophosphate dehydrogenase, serine hydroxy methyltransferase and alanyl-tRNA synthethase, involved in aminoacid and nucleotide metabolism, the protein with antioxidant activity alkyl hydroperoxide reductase and a non-ribosomal peptide synthetase responsible for the synthesis of the siderophore piscibactin have been identified as antigens induced in *Phdp* during *S. senegalensis* infection. Proteins induced during *in vivo* growth of *Phdp* represent promising targets for the development of novel antimicrobial or prophylactic agents in the treatment and prevention of photobacteriosis.

1. Introduction

Solea senegalensis is an important fish species in marine aquaculture in the south of Europe due to its high price, market demand and high growth potential of the industry [1,2]. However, infectious outbreaks affecting this species impose limits to the industry and include photobacteriosis as one of the most important diseases restricting *S. senegalensis* cultures.

Photobacterium damselae subsp. *piscicida* (*Phdp*), the causative agent of photobacteriosis, is an important pathogen in marine aquaculture that affects many different fish species worldwide, especially in Mediterranean countries and Japan [3–5]. Virulence factors of this pathogen include a polysaccharide capsular layer [6,7] and a metalloprotease A-B exotoxin that induces apoptosis in fish macrophages and neutrophils [8]. In addition, *Phdp* has been reported to be weakly or moderately adherent, capable of translocating over surfaces thanks to twitching motility, and invasive in some fish cell lines [9,10]. The adherence is mediated by a protein or glycoprotein receptor and an actin microfilament dependent mechanism seems to be involved [11]. However, information on the virulence factors contributing to the invasion of fish cells is still scarce. Furthermore, ability to scavenge iron from its host by using high-affinity iron binding siderophores has also been reported in *Phdp* [12].

The only commercial vaccine available against *Phdp* infections consists of an ECP-enriched bacterin used in Europe with variable

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results [5]. Investigation on the antigens eliciting protective immune response is being carried out and several antigens have been proposed as potential vaccine candidates. Thus, Heat Shock Protein 60 (Hsp60), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh), enolase and a 17 kDa lipoprotein have been reported as antigens conferring protection in cobia (*Rachycentron canadum*) [13,14] and another lipoprotein (PDP_0080) identified by reverse vaccinology has been found effective in *Dicentrarchus labrax* [15]. However, the protection has not been evaluated in *S. senegalensis*, and information on *Phdp* antigens eliciting protective response in this highly susceptible species is scarce.

It is well stablished that bacteria express different repertories of proteins under *in vitro* culture conditions compared to *in vivo* conditions due to missing *in vivo* signals, such as those coming from the interaction with host cells, extracellular environment, and restriction in nutrients such as free iron. *In vivo* induced antigen technology (IVIAT) is an immunological technique that uses antisera from an infected host to identify pathogen antigens that are expressed *in vivo* [16,17]. Thus, it allows for the detection of immunogenic epitopes in proteins not expressed by bacteria during *in vitro* growth.

In the present work, an expression library of *Phdp* Lg41/01 has been screened to identify clones immunoreactive against *S. senegalensis* sera convalescent from a *Phdp* infection in order to identify antigens expressed by the pathogen during *S. senegalensis* infection and determine their potential *in vivo* induced character.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Photobacterium damselae subsp. *piscicida* (*Phdp*) (strain Lg41/01) was isolated from diseased cultured *S. senegalensis* [18] and cultured in tryptic soy broth (Oxoid Ltd., UK) supplemented with 1.5% NaCl (TSBs) at 22 °C for 24 h. Genomic DNA from *Phdp* strain Lg41/01 was used to construct a protein expression library. *E. coli* BL21 (DE3), used as a host strain for recombinant protein expression, was cultured in Luria-Bertani (LB) (Difco, USA) broth and incubated at 37 °C. All strains were stored at −80 °C in media supplemented with 15% glycerol. When appropriate, kanamycin (kan) was added to LB medium (30 μg/mL). The pET30 plasmid expression system (Novagen, USA) was used to construct the *Phdp* Lg41/01 genomic expression library.

2.2. Construction of genomic expression library

An expression library was constructed with pET30c vector (Novagen), in which clones were inserted under the control of the T7 promoter. Genomic DNA from *Phdp* was isolated according to Martínez et al. (1998) [19] by salting out extraction and isopropanol precipitation, resuspended in MilliQ water and cut in 1000–3500 bp fragments by using HydroShear (GeneMachines, USA), at speed codes 8–10 for 30 cycles. DNA of the required size range (1000–3500 bp) was treated with Fast DNA End Repair Kit (Thermo Scientific, Spain). Repaired *Phdp* DNA was purified using GeneJET PCR Purification Kit (Thermo Scientific) in order to eliminate enzymes and salts from the previous repairing step. DNA fragment size was checked on agarose gels before ligation into pET30c plasmid (Novagen, USA). Briefly, pET30c vector was digested with *Eco*RV (Thermo Scientific), purified from agarose electrophoresis gel and dephosphorylated with calf intestinal phosphatase (Thermo Scientific) according to manufacturer's recommendations. The ligation mixture was used to transform chemically competent *E. coli* BL21 (DE3) cells by heat shock (42 °C, 30 s) and transformants were selected on LB plates supplemented with kanamycin (30 μg/mL) (Kan-LB) and incubated at 37 °C overnight. Colonies grown on LB plates were picked and used to inoculate microtiter wells containing LB broth supplemented with 15% glycerol. The quality of the library was assessed by PCR amplification using T7 promoter (5′-TAATACGACTCACTATAGGG-3′) and T7 terminator (5′- GCTAGTTATTGCTCAGCGG-3′) primers and electrophoresis on agarose gels of a random sample to determine the presence and size of inserts. Transformants were stored at −80 °C.

2.3. Fish challenge and convalescent sera collection

Senegalese sole (*S. senegalensis*) specimens (130 ± 15 g mean body weight, N = 40) were supplied by Planta de Cultivos Marinos (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain). Fish were transferred to the facilities at the University of Málaga (Málaga, Spain) and randomly stocked in two separated tanks (20 fish per tank) under optimal stocking density (7 kg m^{-2}) with recirculating aerated seawater at 20–22 °C (35 g L^{-1} salinity). Animals were fed daily (1% body mass) with pelleted food (Skretting, Spain) and acclimatized to laboratory conditions for 2 weeks before experiments. The day before, fish were checked for health status by random sampling of 6 fish specimens (3 per tank) and microbiological analysis of internal organs on tryptic soy agar (Oxoid Ltd., UK) supplemented with 1.5% NaCl (TSAs). In addition, absence of *Phdp* in internal organs was checked by PCR assays according to Osorio et al. (1999) [20]. *Phdp* Lg41/01 strain (LD50 = 2.2×10^4 CFU g^{-1}, determined by intraperitoneal injection in *S. senegalensis* specimens) was grown in TSBs at 22 °C for 24 h and suspended in sterile phosphate-buffered saline (PBS) to 10^5 CFU mL^{-1} (final concentration). Fish were anaesthetized with clove oil (100 ppm) and specimens intraperitoneally injected with the bacterial suspension (dose 10^4 CFU g^{-1}). Control fish were injected with the same volume of sterile phosphate buffered saline (PBS). Thirty days post-infection fish specimens were transferred to a new tank containing clove oil (200 ppm) to euthanize. Peripheral blood was sampled by caudal puncture of surviving specimens and sera obtained by allowing the blood to clot at room temperature. Collected sera were stored as single samples at −80 °C until assayed.

2.4. Determination of specific antibody presence by ELISA

Antibody levels in fish sera were measured using ELISA. *Phdp* (strain Lg41/01) cells were used as immobilized antigens. Briefly, ELISA flat-bottom 96–well plates (MaxiSorp immuno-plates, Nunc International, USA) were incubated at 4 °C overnight with the bacterial suspension (50 μl/well). Then, plates were washed three times with phosphate buffered saline (PBS) containing 0.25% (v/v) Tween 20 (PBS-T) and 200 μl/well of PBS-T supplemented with 3% (w/v) skim milk was added. Plates were incubated at 22 °C for 2 h and washed with PBS-T three times before adding serial dilutions (1:500; 1:1000; 1:2000) of fish serum samples and incubation at 22 °C for 2 h. Plates were washed again with PBS-T three times and 50 μl of anti-sole antibody developed in rabbit (Sigma Aldrich, Spain) against *S. senegalensis* IgM purified in our laboratory [21] was added to each well [dilution 1/1000 in PBS-T containing 0.25% (w/v) bovine serum albumin (BSA)]. After incubation at 22 °C for 2 h plates were washed again with PBS-T three times. Then, 50 μl/well of anti-rabbit IgG peroxidase conjugate (Sigma Aldrich) developed in goat was added (dilution 1/5000 in PBS-T + BSA) and incubated at 22 °C for 1 h. After a new wash step, 200 μl/well of *o*-phenylenediamine dihydrochloride (OPD, Sigma Aldrich) was added and plates incubated at 22 °C for 30 min in the dark. Chromogenic reaction was stopped by adding 50 μl/well of 3 M H_2SO_4 and plates were read spectrophotometrically at 490 nm using a