Antigenic proteins of *Flavobacterium psychrophilum* recognized by ayu *Plecoglossus altivelis altivelis* antisera

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ABSTRACT: *Flavobacterium psychrophilum* is the causative agent of bacterial coldwater disease (BCWD) in ayu *Plecoglossus altivelis altivelis* and is responsible for substantial economic losses in ayu culture in Japan. To develop effective vaccines for the disease, we identified antigenic proteins of *F. psychrophilum* using immunoglobulin from ayu that had recovered from BCWD. The whole protein extracted from the bacterium was separated using 2-dimensional polyacrylamide gel electrophoresis and was transferred to a polyvinylidene fluoride membrane. Subsequently, antigenic proteins of the bacterium were detected using western blotting and ayu antisera against *F. psychrophilum*. Each protein spot showing antigenicity was subjected to tandem mass spectrometry (MS/MS) analysis using a MALDI-QIT-TOF mass spectrometer. Protein identification based on the MS/MS data was performed using the genome database for *F. psychrophilum* JIP02/86, and the subcellular localization for each identified protein was predicted with web-based tools (LipoP and PSORTb). In total, 62 antigenic proteins were identified: of these, 46 were putative cytoplasmic proteins (e.g. elongation factor Tu and heat shock protein 90). The remaining 21 proteins were identified as putative membrane-bound or secreted proteins and are potential vaccine candidates. These proteins include OmpA, Omp 121, M13 family metallopeptidase, and M48 family metalloprotease.

KEY WORDS: Bacterial coldwater disease · BCWD · 2D-PAGE · MS/MS analysis · Vaccine · Anti-ayu immunoglobin MAb

INTRODUCTION

Ayu *Plecoglossus altivelis altivelis*, a culinary delicacy and a popular game fish, is the most economically important fish species for Japanese freshwater fisheries. According to statistics of the Ministry of Agriculture, Forestry, and Fisheries, ayu fishery and farming output amounts to one-third of the total value of freshwater fishery and aquaculture production (www.e-stat.go.jp). Local fisheries stations produce hatchery stocks of ayu fry from 3 origins: amphidromous populations that spawn in freshwater and whose larvae migrate to the sea and then return as adults to freshwater habitats; landlocked populations from the landlocked waters of Lake Biwa, Japan; and local populations from different prefectures. These hatchery stocks are released into rivers for fishing, and they may have different characteristics in terms of catchability, length of fishing season, and resistance to bacterial cold water disease (BCWD; Miura et al. 2012).

*Flavobacterium psychrophilum* is the causative agent of BCWD and affects freshwater fish species worldwide. Four serotypes of *F. psychrophilum* based
on a thermostable antigen have been isolated in Japan: O-1, O-2, O-3, and O-4 were mainly found in strains isolated from coho salmon *Oncorhynchus kisutch*, ayu, rainbow trout *O. mykiss*, and amago *O. masou rhodurus*, respectively (Wakabayashi et al. 1994, Izumi & Wakabayashi 1999, Izumi et al. 2003). BCWD outbreaks were first reported in cultured ayu in Japan in 1987 (Wakabayashi et al. 1994). The disease has been reported in many freshwater systems in Japan and causes economic losses in cultured and wild ayu (Inouye 2000). Clinical signs include anemia and ulcerative skin lesions (Iida & Mizokami 1996). Sulfisozole sodium is an approved treatment for BCWD in Japan; however, frequent use of the antibiotic may result in antibiotic resistance and antibiotic residues. Several studies have reported the protective efficacy of an oil adjuvant-based vaccine using formalin-killed cells (Rahman et al. 2000, 2003, Nagai et al. 2003, Madetoja et al. 2006, Fredriksen et al. 2013). However, no suitable vaccine is available in ayu aquaculture in Japan.

LaFrentz et al. (2003) passively immunized rainbow trout with antiserum against *Flavobacterium psychrophilum* and reported that a specific antibody plays a critical role in protection. Similar results regarding the efficacy of passive immunization were also observed in ayu (G. Kato et al. unpubl. data). These results have stimulated research to better understand humoral immune responses to BCWD and to identify antigenic proteins. Proteins such as the 18 kDa OmpH-like surface protein (Massias et al. 2004, Dumetz et al. 2006) and ribosomal L10-like protein (Crump et al. 2007) were identified as antigens and shown to be effective when used in vaccines for rainbow trout. Proteomic analyses have been used to identify antigenic proteins of *F. psychrophilum* that are recognized by rainbow trout immunoglobulin (Ig; Sudheesh et al. 2007, Dumetz et al. 2008, LaFrentz et al. 2009, 2011, Gliniewicz et al. 2012). The antigenic proteins recognized by ayu Ig have not been studied.

Identification of a dominant antigen that can induce a protective host immune response is important for the development of an effective vaccine against BCWD. Ayu that recovered from BCWD were highly resistant to reinfection with *Flavobacterium psychrophilum* and had high antibody titers (Kintsuji et al. 2007). The aim of the present study was to use 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE), western blotting, and matrix-assisted laser desorption/ionization-quadrupole ion trap-time of flight (MALDI-QIT-TOF) mass spectrometry to identify antigenic proteins recognized by antisera from ayu that had recovered from BCWD.

### MATERIALS AND METHODS

#### Fish

Ayu from 2 stocks, GG42 and SE1, were used in this study. GG42 is a domesticated stock that has been maintained by intra-stock breeding for 42 generations at the Gunma Prefectural Fisheries Experimental Station. SE1 is an amphidromous stock that was derived from wild fish collected from upstream migrating populations in the Edogawa River. Fish were reared in 50k l tanks with flow-through conditions, and water temperature was maintained at 15°C.

#### Culture of *Flavobacterium psychrophilum*

*F. psychrophilum* strain GMA0330 isolated from wild diseased ayu from Gunma Prefecture in 2003 (Arai et al. 2007) was used for this study. Bacteria were cultured at 15°C for 24 h in modified Cytophaga broth (Wakabayashi & Egusa 1974). The culture was diluted in phosphate-buffered saline (PBS), and colony-forming units (cfu) were confirmed by noting growth after the bacterial suspension was spread on modified *Cytophaga* agar (Wakabayashi & Egusa 1974).

#### Challenge test and plasma preparation

Fish from the GG42 line (mean body weight of 15.9 g) were divided into 3 groups, and the fish in each of the groups were given an intraperitoneal injection with 1 of 3 doses of *Flavobacterium psychrophilum* (Table 1). Another experiment using SE1 fish (mean body weight of 17.9 g) was carried out in the same way. Fish were reared in 80 l tanks with flow-through conditions, and water temperature was maintained at 15°C. Mortality was recorded once a day for 20 d. Cultures from the kidney of dead fish and from survivors in all groups were grown on modified *Cytophaga* agar to confirm the presence or absence of *F. psychrophilum*. External clinical signs were also recorded.

Twenty days after fish were exposed to *Flavobacterium psychrophilum*, blood was collected from survivors by venipuncture with a heparinized syringe and then centrifuged at 5000 × *g* for 5 min (Table 1). Blood plasma was pooled and stored at 4°C until use. Control plasma from fish lines SE1 and GG42 could not be obtained for this experiment. Therefore, plas-
mas of uninfected fish from the next generations of these fish lines (SE2 and GG43) were used for the negative control.

Production of monoclonal antibody (MAb) against ayu serum Ig

MAb against ayu serum Ig was generated as previously described by Matsuyama et al. (2009). A female BALB/c mouse (4 wk old) was given an intraperitoneal injection with 0.2 mg of ayu Ig on Days 0, 21, and 42. On Day 45, spleen cells were harvested and fused with myeloma cell line Sp2/0 in polyethylene glycol. Hybridoma cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco) with penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), Brilclone hybridoma cloning medium (Archport), and HAT medium (Gibco) supplemented with 10% fetal bovine serum. Affinity of the MAb against the ayu Ig was confirmed by ELISA using 96-well plates coated with 5 µg ml⁻¹ of Ig. The MAb class and subclass were determined with a sandwich ELISA using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (GE Healthcare).

Microtiter agglutination test

The pooled plasma titer was determined using a microtiter agglutination test, following the method described by Lorenzen & Olesen (1997) with some modifications. Ten 2-fold serial dilutions (final dilution of 1:2048) of the plasma were made in 25 µl PBS in a conical bottom microtiter plate. Approximately 5.0 × 10⁵ bacterial cells that were suspended in 25 µl PBS were added to each well, and the plates were incubated overnight at 4°C. Bacterial cell agglutination was recorded by visualization against a dark background, and plasma titers were defined as the reciprocal value of the highest plasma dilution with macroscopically visible agglutination.

Indirect immunofluorescence assay

The pooled plasma was added to the bacterial suspension prepared as described above and incubated overnight at 4°C. The suspension was then centrifuged at 2000 × g (5 min), the cells were collected, washed 3 times, and re-suspended in PBS. A secondary antibody (anti-ayu Ig MAb) was added to the bacterial suspension, and incubated for 30 min at room temperature. A tertiary antibody (FITC-conjugated goat anti-mouse Ig [H+L], SouthernBiotech) was then added and incubated for 30 min at room temperature under subdued light. The bacterial cells were washed and re-suspended as above, and then stained with DAPI nucleic acid stain (Invitrogen) according to the manufacturer’s instructions. The bacterial suspension was spotted on a slideglass printed with a ring mark and examined with a BX50 fluorescence microscope (Olympus). Digital images were captured and analyzed with DP70-BSW software (Olympus).

2D-PAGE

Flavobacterium psychrophilum proteins were extracted from 20 mg cells using TRIzol Reagent (Life Technologies) following the manufacturer’s instructions and were dissolved in Destreak Rehydration Solution (GE Healthcare). The 2D Quant kit (GE Healthcare) was used to estimate the protein concentration. Protein (50 µg) from the rehydration solution was loaded onto an immobilized pH gradient gel (Immobiline DryStrip pH3-10 NL 7 cm; GE Healthcare). Isoelectric focusing electrophoresis (IEF) was performed with the CoolPhoreStar IPG-IEF (Anatec) at 500 V for 1.5 h, then ramped linearly from 500 to 3500 V for 1.5 h, and finally maintained at 3500 V for 3 h. After IEF, the strip gel was equilibrated twice for 20 min with 25 mM Tris-HCl (pH 6.8) containing 6 M Urea, 2% SDS, 30% glycerol, and 0.5% dithiothreitol (DTT). The equilibrated strip gel was then
subjected to SDS-PAGE with a 10 to 20% gradient polyacrylamide gel (BIO CRAFT), and the proteins were stained with Oriole Fluorescent Gel Stain (Bio-Rad Laboratories). A digital image of the gel was obtained using the FluoroPhoreStar 3000 (Anatech).

**Western blotting**

The *Flavobacterium psychrophilum* proteins separated by 2D-PAGE were transferred to a polyvinylidene fluoride (PVDF) membrane (ATTO) with EzBlot (ATTO) according to the manufacturer's instructions. After blocking with 3% skim milk (Wako), the membranes were incubated in a 1/50 dilution of the pooled plasma in the blocking solution for 1 h, followed by incubation with the MAb for 1 h. Antigen binding antibody complexes on the membranes were detected using goat anti-mouse IgG antibody conjugated with alkaline phosphatase (Bio-Rad Laboratories) and then visualized with an AP conjugate substrate kit (Bio-Rad Laboratories).

**Protein identification by mass spectrometry**

Protein spots in the 2D-PAGE gel were excised using a FluoroPhoreStar 3000 (Anatech) according to the manufacturer's instructions and then subjected to in-gel trypsin digestion, assisted by microwave (Juan et al. 2005, Sun et al. 2006). After the digestion, the tryptic digests were extracted from the gel piece with 50% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA) and with 75% ACN containing 0.1% TFA; each 15 min extraction was at room temperature. The mixture of these extracts was dried in a centrifugal concentrator (Concentrator plus 5305, Eppendorf) and then re-dissolved in 50% ACN containing 0.1% TFA. The sample was mixed with an equal volume of matrix reagent for MALDI mass spectrometry (dihydroxybenzoic acid, Shimadzu Corporation) and then subjected to the tandem mass spectrometry (MS/MS) analysis with MALDI-QIT-TOF mass spectrometry (Axima Resonance). Protein identification based on MS/MS data was performed by comparison with a database of all protein-coding sequences on the genome of *Flavobacterium psychrophilum* JIP02/86 (GenBank AM398681.1; MASCOT MS/MS Ions Search, Matrix Science). Signal peptides and subcellular localization of identified proteins were predicted with the aid of the LipoP 1.0 Server (www.cbs.dtu.dk/services/LipoP/) and PSORTb version 3.0.2 (www.psort.org/psortb/), respectively.

**RESULTS**

**Challenge test**

Cumulative mortality curves and survival rates for the challenged fish are shown in Fig. 1 and Table 1, respectively. Anemia and ulcerative skin lesions were noted in dead fish, and *Flavobacterium psychrophilum* was re-isolated from their kidneys. Survivors did not exhibit any clinical signs of BCWD, and the bacterium was not detected in their kidneys.

**Microtiter agglutination test and indirect immunofluorescence assay**

Except for SE1-H, pooled plasma titers were 16, whereas agglutination was not observed in the plasma obtained from uninfected fish (Table 1). Fluorescence was observed on the bacterial cells exposed to pooled plasma from fish that recovered from BCWD, but not on the bacterial cells exposed to pooled plasma from uninfected fish (Fig. 2).

**Identification of antigenic proteins recognized by ayu Ig**

Protein spots recognized by GG42 and/or SE1 plasma on the PVDF membrane, and corresponding
spots on the polyacrylamide gel, are shown in Fig. 3. In total, 97 protein spots were excised and subjected to protein identification by MS/MS analysis; 62 proteins were identified as known proteins of *Flavobacterium psychrophilum* JIP02/86. Of these, 41 were identified as putative cytoplasmic proteins (e.g. elongation factor Tu and heat shock protein [HSP] 90; Table 2), and 21 were identified as putative membrane-bound or secreted proteins (Table 3). The putative membrane-bound proteins included F0F1 ATP synthase subunit beta, OmpA family outer membrane protein, cell surface leucine-rich repeat-containing proteins (FP0167, FP0168, FP0169, and FP0177), hypothetical protein (FP0139), and Flavobacterium psychrophilum-specific protein antigen FspA. The putative secreted and membrane-bound proteins included some peptidases and proteases such as M16 family peptidase, aminopeptidase, Xaa-pro dipeptidyl-peptidase, M13 family metallopeptidase PepO, and M48 family metalloprotease YggG.

Some distinctive spots reacted only with GG42 or SE1 plasma. Five cytoplasmic proteins were identified from the spots recognized by GG42, whereas 4 cytoplasmic proteins and a secreted protease, M48 family metalloprotease YggG, were identified from the spots recognized by SE1 plasma. Further, *Flavobacterium psychrophilum* antigenic proteins were not recognized by pooled plasma obtained from uninfected fish from GG43 and SE2 by western blotting using the control plasmas (data not shown).

**DISCUSSION**

The outer membrane fraction of *Flavobacterium psychrophilum* induces protective immunity in rainbow trout and ayu (Rahman et al. 2002). Culture supernatant containing membrane vesicles induces immunity to *F. psychrophilum* in rainbow trout (Aoki et al. 2007). Thus, surface-exposed or secreted proteins are susceptible to antibody recognition and are therefore the most suitable vaccine candidates. In the present study, 21 putative extracellular localizing proteins were identified as antigenic proteins recognized by ayu Ig. Two types of outer membrane protein, OmpA family outer membrane protein P60 and Omp121 family outer membrane protein, were included in the identified proteins. In rainbow trout, OmpH-like surface protein induces a high antibody titer and a protective immune response against BCWD, and OmpA protein induces a humoral response that has bactericidal activity against *F. psychrophilum* (Dumetz et al. 2006, 2007). These reports suggest that Omp family proteins are good candidates for subunit or recombinant protein vaccines.
against BCWD. Furthermore, putative membrane-bound proteins that, in this study, reacted with ayu Ig (e.g. F0F1 ATP synthase subunit beta, cell surface leucine-rich repeat-containing proteins [FP0169 and FP0167], hypothetical protein [FP0139], and Flavosp inserted protein antigen FspA) have been shown to be antigenic proteins recognized by rainbow trout Ig (Crump et al. 2005, Dumetz et al. 2008, LaFrentz et al. 2011). Therefore, these proteins are immunodominant antigens of *F. psychrophilum* recognized by both rainbow trout and ayu, and are expected to be good vaccine candidates.

Five types of secreted or outer membrane-bound peptidases or proteases were also identified as antigenic proteins in this study. Early studies of *Flavobacterium psychrophilum* demonstrated that the bacterium is actively proteolytic, and proteases were suspected to be important virulence factors (Pacha & Porter 1968, Bertolini et al. 1994). Accordingly, the *F. psychrophilum* genome encodes 13 putative secreted proteases that are probably included in virulence and/or in the destruction of host tissues; these proteases include M13 family metallopeptidase PepO and probable M48 family membrane-bound proteins.
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*Table 2. Putative cytoplasmic proteins recognized by the plasma from ayu (GG42 and/or SE1 lines) that recovered from Flavobacterium psychrophilum infection. Overlaps in range numbers and letters indicate that several proteins were identified from the same protein spot. CYT: cytoplasmic. MASCOT, LipoP, and PSORTb are web-based tools used in the identification of proteins (see 'Materials and methods').*

Associated zinc metalloprotease (Du-
chaud et al. 2007). When a bacterial
toxin is the main cause of illness, toxoid
vaccines are used to induce an immune
response to the original toxins. Toxoids
of acetylcholinesterase and a lethal
toxic aspzcinc metalloendopeptidase
of Aeromonas hydrophila
and A. sal-
monicida have been constructed and
tested as toxoid vaccines against these
infections in fish (Pérez et al. 2002,
Schwenteit et al. 2013). Therefore, pre-
ventive strategies using antigenic pep-
tidases and proteases of
F. psychro-
philum
as toxoid vaccines might be
effective against BCWD in ayu.

In this study, 41 putative cytoplasmic
proteins, including HSP90 and elonga-
tion factor Tu, were identified as anti-
genic proteins. HSPs are highly im-
munogenic and can induce a strong
humoral and cellular immune response
(Zügel & Kaufman 1999). Elongation
factor Tu, from a variety of bacterial
species, is also immunogenic (Ying et
al. 2005, Boonjakuakul et al. 2007, Har-
ding et al. 2007). However, effective
vaccines against BCWD in rainbow
trout have not been developed from
HSP60, HSP70, and elongation factor

Land-locked stock and amphidrom-
ous stock show no significant differ-
ences in non-specific immunological
parameters such as respiratory burst,
phagocytic activities of blood leuko-
cytes, and serum-killing activity against
Flavobacterium psychrophilum
(Nagai et al. 2004). Thus, the mechanisms
underlying the resistance of the amphi-
dromous stock to BCWD are not fully
understood. In our study, the same level
of agglutination titer was observed in
GG42 and SE1 plasmas, but distinctive
spots that reacted with GG42 or SE1
were identified by western blotting.
These data suggest some differences in
the mechanisms of antigen recognition
in GG42 compared with SE1. Major his-
tocompatibility complex (MHC) class II
has an important role in the recognition of
antigenic proteins, because the MHC
class II molecule binds to exogenous-

Table 3. Putative membrane-bound or secreted proteins recognized by the plasma from ayu (GG42 and/or SE1 lines) that recovered from Flavobacterium psy-
chrophilum infection. Sg, signal peptide; Sp, signal peptide; SpI, signal peptide I; SpII, signal peptide II; CYT, cytoplasmic; CP, cytoplasmic; OM, outer membrane; PP, periplasmic; UK, unknown. MASCOT, LipoP, and PSORTb web-based tools used in the identification of proteins (see ‘Materials and methods’).
derived peptides and presents them to CD4+ T helper cells (Klein 1986). Furthermore, a correlation between resistance to bacterial and viral infections and MHC polymorphism has been reported in some fish species (Langefors et al. 2001, Lohm et al. 2002, Grimholt et al. 2003, Rakus et al. 2009). Therefore, investigation of ayu MHC class II genes may increase our understanding of the mechanisms underlying BCWD resistance in this species.

In this study, 62 antigenic proteins form *Flavobacterium psychrophilum* that included many newly reported antigens were identified by use of whole cell bacterial lysate and antisera from fish that had recovered from BCWD. Of these proteins, 21 are probably membrane-bound or secreted proteins that are very suitable vaccine candidates. This study provides a number of vaccine candidates and facilitates development of vaccines for the prevention of BCWD in cultured ayu.

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