2010

Cloning, Expression, and Immunogenicity of *Flavobacterium columnare* Heat Shock Protein DnaJ

Oscar Olivares-Fuster
Auburn University

Jeffery S. Terhune
Auburn University

Craig A. Shoemaker
United States Department of Agriculture, craig.shoemaker@ars.usda.gov

Covadonga R. Arias
Auburn University, ariascr@auburn.edu

Journal of Aquatic Animal Health 22:78–86, 2010

DOI: 10.1577/H09-029.1

[Article]

Cloning, Expression, and Immunogenicity of *Flavobacterium columnare* Heat Shock Protein DnaJ

Oscar Olivares-Fuster and Jeffery S. Terhune

Department of Fisheries and Allied Aquacultures, Auburn University, 217 Swingle Hall, Auburn, Alabama 36849, USA

Craig A. Shoemaker

U.S. Department of Agriculture, Agricultural Research Service, Aquatic Animal Health Research Unit, Auburn, Alabama 36832, USA

Covadonga R. Arias*

Department of Fisheries and Allied Aquacultures, Auburn University, 217 Swingle Hall, Auburn, Alabama 36849, USA

Abstract.—The *Flavobacterium columnare* heat shock protein (HSP) gene *dnaJ** was isolated, cloned, expressed, and used as an antigen in a recombinant vaccine strategy for channel catfish *Ictalurus punctatus*. The *F. columnare dnaJ** sequence was obtained from genomovars I and II and showed intraspecies variability. Recombinant protein was expressed and purified from *Escherichia coli* cultures and injected intraperitoneally (12 μg of purified DnaJ/fish) into fingerling channel catfish. In addition, induced (expressing the recombinant DnaJ) and uninduced (no recombinant protein being produced) *E. coli* cultures were also used to immunize fish. At 28 d postimmunization, antibody response was evaluated and the fish were challenged with *F. columnare*. A specific immune response against DnaJ was observed in fish immunized with DnaJ or *E. coli* cultures expressing DnaJ. No protection against the disease, however, was observed in *F. columnare*-challenged fish that had been immunized with DnaJ. Some level of protection was observed in fish immunized with uninduced and induced *E. coli* lysates. Although HSPs have been shown to be immunodominant and good candidates for subunit vaccines in other animals, DnaJ failed to protect against columnaris disease in channel catfish.

Flavobacterium columnare, the etiologic agent of columnaris disease, is a Gram-negative bacterium that can infect most freshwater fish species. The disease generally begins as an external infection on the fins, body surface, or gills. Fin lesions can become necrotic, while skin loses pigmentation and is covered with yellowish mucoid material. Lesions can develop exclusively on the gills, which usually results in subacute disease and mortality. In some cases, columnaris becomes systemic, with little or no gross pathological changes occurring (Hawke and Thune 1992). *Flavobacterium columnare* infects aquacultured (Wise et al. 2002), ornamental (Decostere et al. 1999), and wild species (Olivares-Fuster et al. 2007) and is distributed worldwide (Shotts and Starliper 1999). Currently considered the second most important disease affecting the aquaculture of channel catfish *Ictalurus punctatus* in the USA, economic losses associated with columnaris disease are in excess of millions of dollars annually (USDA 2003a, 2003b).

Differences in virulence are known to exist among *F. columnare* isolates, resulting in variable mortality of fish (Pacha and Ordal 1970; Shoemaker et al. 2008). Susceptibility to columnaris disease may also be affected by environmental factors, such as water temperature, salinity, high fish density, and poor nutrition (Wakabayashi 1993; Altinok and Grizzle 2001). Minimizing fish stress helps in preventing columnaris outbreaks, but the ubiquitous presence of this pathogen in aquatic environments makes eradication of the disease in aquaculture systems difficult. Current treatment practices by the industry include the use of topical chemotherapeutants (e.g., potassium permanganate) or administering medicated feed, such as Terramycin (oxytetracycline) or Aquaflor (florfenicol), which was recently approved by the U.S. Food and Drug Administration. However, therapeutic use of antibiotics is expensive and usually is not very effective since sick fish do not eat.

One of the most successful approaches to the control of infectious disease is to take advantage of natural and acquired host immunity. In this respect, vaccines constitute the most cost-effective tool for disease prevention. Shoemaker et al. (2005b) and Bebak et

* Corresponding author: ariascr@auburn.edu

Received July 17, 2009; accepted March 9, 2010
Published online May 13, 2010

al. (2009) developed a modified-live rifampicin-resistant mutant (commercially available as AQUA-VAC-COL) that provided good protection against columnaris under laboratory conditions. However, development of second-generation vaccines (e.g., recombinant) should be explored.

Recombinant vaccines are those where genes for target antigens are inserted into a vector that expresses the immunogenic protein in large amounts. After purification, these immunogenic proteins are administered to the host as subunit vaccines. These proteins provide some advantages over first-generation vaccines, such as increased safety, less antigenic competition (since only a few components are included in the vaccine), better control over vaccination dose, equal exposure of all vaccinated fish, ability to target the vaccines to the site where immunity is required, and ability to differentiate vaccinated animals from infected animals. Identification of protective antigens is critical in the process of developing recombinant vaccines. Unfortunately, very little information is available on potential immunogenic targets in *F. columnare* (Zhang et al. 2006). Some proteins successfully used as subunit vaccines against other fish bacterial pathogens include heat shock proteins (HSPs; Wilhelm et al. 2006), outer membrane proteins (Mao et al. 2007), a ribosomal protein (Crump et al. 2007), and a fibronectin-binding protein (Pasnik and Smith 2005). Among them, bacterial HSPs are known to play an important role in activating the immune system during infection (Zugel and Kaufmann 1999; Wallin et al. 2002). Several HSPs, including HSP-60, HSP-70, GroEL, DnaK, and DnaJ, have been successfully used as subunit vaccines in mice (Zugel and Kaufmann 1999; Leclerq et al. 2002; Sagi et al. 2006; Delpino et al. 2007).

The aim of this study was to identify and clone the HSP DnaJ from *F. columnare* and to evaluate its potential as a subunit vaccine against columnaris disease in channel catfish.

Methods

Bacterial strains.—Four *F. columnare* strains were used in the study: the type strain (ATCC [American Type Culture Collection] 23463) and three additional strains (ARS-1, ALG-00–530, and LSU) originally isolated from channel catfish with columnaris disease. The strains ATCC 23463 and ARS-1 belong to genomovar I, while the strains ALG-00–530 and LSU belong to genomovar II (Arias et al. 2004). Genomovar II has been shown to be more virulent for channel catfish than genomovar I, although the difference in their virulence mechanisms is unknown (Shoemaker et al. 2008). All strains were routinely grown in Shieh broth (Shoemaker et al. 2005a) at 28°C under continuous shaking. Total DNA was extracted by using the DNeasy Tissue kit (Qiagen, Valencia, California) in accordance with the manufacturer's instructions.

Amplification and cloning of DnaJ.—Primers against *F. johnsoniae dnaJ** (GenBank accession number ZP_01247507) were designed and used to amplify the *dnaJ** gene from *F. columnare*. The forward primer sequence was 5′-ATGAAAAAA-GATTTTTA-3′, and the reverse primer sequence was 5′-TTATGAAGAACATATCTT-3′. Amplification of *dnaJ** from all *F. columnare* strains was carried out in an MJ Research PCR thermocycler (Bio-Rad, Valencia, California) under standard polymerase chain reaction (PCR) conditions (Sambrook et al. 1989). Briefly, 100 ng of *F. columnare* DNA, 20 pmol of each primer, 15 μL of 2× PCR premix buffer H (Epicentre Biotechnologies, Madison, Wisconsin), and 1 unit of *Taq* DNA polymerase (enzyme code 2.7.7.7, IUBMB 1992; Promega, Madison, Wisconsin) were used in a total volume of 50 μL. Thermocycling conditions were as follows: (1) 5 min at 94°C; (2) 40 cycles of 60 s at 94°C, 90 s at 32.5°C, and 60 s at 72°C; and (3) a 10-min final extension at 72°C. The PCR products were resolved in 1% agarose gel and visualized under ultraviolet light after staining with ethidium bromide. The expected size bands were excised from the gel and purified using the Geneclean III kit (Qbiogene, Irvine, California). The DNA was cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, California) and transformed into chemically competent *Escherichia coli* cells (strain TOP10) (Invitrogen). *Escherichia coli* cells were grown in continuous shaking at 37°C in Difco Luria–Bertani (LB) broth (Becton, Dickinson, and Company, Sparks, Maryland) with ampicillin. The presence of the correct inserts was confirmed by restriction and sequence analysis. Clone CRII-FcolJ-530 from strain ALG-00–530 was selected for protein expression. Comparisons between *F. columnare dnaJ** sequences and those from *F. johnsoniae* and *F. psychrophilum* were carried out using BioNumerics version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium).

Expression constructs.—To clone *dnaJ** into the pQE30 bacterial expression vector (Invitrogen), the original sequence had to be modified by altering the original primers as follows. The *dnaJ** start codon was removed to avoid competition with the vector start codon, and *Bam*HI and *Hin*dIII sites were added to the forward and reverse primers, respectively. The new PCR product was again cloned into the pCRII-TOPO vector and transformed into TOP10 cells for sequencing confirmation. Clone CRII-BH-FcolJ was selected.

The modified *dnaJ** sequence was purified from this construct by restriction analysis, agarose electrophoresis, and purification using the Geneclean III kit. The modified sequence was then ligated into the pQE30 previously restricted with *Bam*HI and *Hin*dIII. After sequencing confirmation, *E. coli* clone QE30-FcolJ was selected for expression studies.

Expression and purification of recombinant proteins.—Expression and purification of the DnaJ protein were performed according to the QIAexpressionist manual (Qiagen). A colony of the pQE30-FcolJ *E. coli* clone was cultured overnight in 50 mL of LB broth with ampicillin (100 μg/mL) and kanamycin (30 μg/mL) at 37°C and 225 revolutions/min. One flask containing 200 mL of fresh LB broth with antibiotics was inoculated with 10 mL of the overnight culture and incubated for 1 h under the same culture conditions. Recombinant protein expression was induced by adding isopropyl thiogalactoside (IPTG) at a 1-mM final concentration. To determine the peak of recombinant protein production, the induced *E. coli* cells were sampled at 30 min and at 1, 2, 3, and 4 h. A crude protein extraction was carried out, and total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and stained as described below. The highest yield of induced protein was observed at 4 h postinduction (data not shown). For subsequent experiments, 4 h postinduction cells were pelleted by centrifugation at 4,000 × gravity (*g*) for 20 min and resuspended in 16 mL of lysis buffer B (100-mM Na_2HPO_4, 10-mM tris-HCl, and 8-M urea, pH 8). Cells were lysed by sonication on ice. The lysate was centrifuged at 10,000 × *g* for 25 min at room temperature to pellet the cell debris.

The DnaJ protein in the supernatant was purified by nickel–nitrilotriacetic acid (Ni–NTA) affinity chromatography (Qiagen). This resin matrix binds 6xHis-tagged proteins with high specificity. The 6xHis affinity tag is part of the pQE30 expression vector, and the DnaJ was cloned so that the tag was connected to the 5′ end of the protein. Four milliliters of 50% Ni–NTA resin were added to the culture supernatant. The mixture was incubated for 30 min at room temperature with shaking (200 revolutions/min). The lysate–resin mixture was then loaded into four purification columns (5 mL/column), and the flow through was collected for analysis. The resin was washed twice with 4 mL of washing buffer C (same as lysis buffer, pH 6.3). Fractions were collected for SDS-PAGE analysis. Elution was performed using two buffers (D and E) of the same composition as the lysis buffer but with reduced pH (5.9 and 4.5, respectively). Two 1-mL elutions were carried out with buffer D, and four 1-mL elutions were carried out with buffer E. All fractions were collected for SDS-PAGE analysis and protein quantification. Qualitative analysis of all the fractions from the purification process was carried out in 12% SDS-PAGE gels (Bio-Rad). Gels were stained with the GelCode Blue Stain Reagent (Pierce Biotechnology, Rockford, Illinois). Quantification of the eluted fractions was performed via the 2-bicinchoninic acid (BCA) protein assay (Smith et al. 1985).

Fish and rearing conditions.—Pathogen-free channel catfish ($n = 304$ fish; mean weight = 6 g) were randomly divided into sixteen 57-L glass tanks (19 fish/tank). Ten additional fish from the stock source were necropsied, bacteriologically examined, and found to be negative for *F. columnare*. Tanks were kept under recirculating conditions with a water flow rate of 0.5 L/min; temperature was maintained at 25°C, and salinity was approximately 3 g/L. Dissolved oxygen (supplied by air stones to each tank), temperature, and water quality parameters were monitored throughout the experiment and maintained at normal values conducive for growth (temperature = 27 ± 1°C [mean ± SE], alkalinity = 80 mg/L, hardness = 40 mg/L, salinity = 0.1‰, pH = 7.8 ± 0.2; ammonia and nitrite levels were undetectable). Fish were subjected to a photoperiod of 12 h light : 12 h dark and were fed once daily with Aquamax Grower 400 (Purina Mills, Inc., St. Louis, Missouri) to apparent satiation. Fish were allowed to acclimate for 10 d prior to immunization.

Immunization studies.—Prior to immunization, one fish was randomly selected from each tank and was bled (as described below) to obtain a baseline for serum titer against *F. columnare*. Four immunization treatments were conducted in channel catfish by intraperitoneal injection. Four replicates (tanks) were used per treatment. Before injection, fish were anesthetized by immersion in tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, Washington) at a concentration of 100 mg/mL of water. Treatment A consisted of injecting purified DnaJ (at 12 μg/fish) emulsified in Freund's complete adjuvant (50:50; Sigma, St. Louis, Missouri), with a final volume of 100 μL/fish. Treatment B consisted of injecting induced *E. coli* clone QE30-FcolJ lysate at 100 μL/fish (approximately 10^7 sonicated cells/fish). These cells were actively expressing the recombinant DnaJ protein and were sonicated with an Ultrasonic Model 1000L sonicator equipped with a Model 40TL needle probe (Ultrasonic Power, Inc., Freeport, Illinois); sonication was conducted in five cycles of 30 s at 60 W and 20 KHz on an ice bath. Treatment C consisted of injecting uninduced *E. coli* clone QE30-FcolJ lysate at 100 μL/fish (processed as for treatment B; these cells were not producing the recombinant DnaJ protein). For

treatment D (control), fish were injected with 100 μL of sterile phosphate-buffered saline (PBS). No subsequent booster injections were given. Serum was collected at 28 d postimmunization to check for antibody titers. Blood samples were taken from the caudal vein of 4 fish/tank (16 fish/treatment) and were allowed to clot at room temperature for 1 h and then overnight at 5°C. Samples were centrifuged at 15,000 × *g* for 5 min, and serum was collected and stored at −80°C.

Challenge studies.—At 4 weeks postimmunization, channel catfish (60 remaining fish/treatment, 15 fish/tank) were challenged with *F. columnare* ALG-00–530 by bath immersion at 4×10^7 colony-forming units/mL for 20 min. At this point, the aquarium system was changed from recirculation to a flow-through design, maintaining the same water flow rate and temperature; however, the salinity was reduced to less than 10 mg/L, which is normal for water in this well. The *F. columnare* strain ALG-00–530 was chosen because of its high virulence in channel catfish (Shoemaker et al. 2008). For 30 d postchallenge, the animals were monitored daily to assess morbidity and mortality. Typical columnaris signs were observed in moribund and dead animals, and the bacterium was recovered and identified in gills and skin lesions from 12 fish (3 fish/tank) by following standard protocols (AFS-FHS 2004). In addition, isolated cultures were typed according to the methods of Olivares-Fuster et al. (2006) to ensure that they were the same strain used for infection.

Enzyme-linked immunosorbent assay.—Antibody responses in fish from each treatment were evaluated for the presence of specific immunoglobulin against DnaJ using an indirect enzyme-linked immunosorbent assay (ELISA; Shoemaker et al. 2003). Purified DnaJ was diluted at a 10-μg/mL concentration in carbonate coating buffer (pH 9.6), and the solution was used to coat plates (100 μL/well). Plates were incubated at 4°C overnight, washed five times with washing buffer (PBST: PBS at pH 7.4 and 0.05% Tween 20), and blocked with 1% bovine serum albumin in carbonate buffer for 30 min at room temperature. Plates were washed again. Dilutions (1:10–1:5,120) of serum samples made in PBST were added to individual wells (100 μL/well), incubated for 30 min at room temperature, and washed five times with PBST. Wells containing only PBST were present in each plate and tested in the same manner.

Primary antibody was detected using the mouse monoclonal E-8 anti-channel catfish immunoglobulin M (IgM; Klesius 1990) at a 1:5,000 dilution in PBST. After 30 min of incubation at room temperature, the plates were washed five times with PBST, and peroxidase-conjugated AffiniPure rabbit anti-mouse immunoglobulin G (a tertiary antibody solution; Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) was added at a 1:10,000 dilution in PBST. After 15 min of incubation at room temperature, the plates were washed five times with PBS, and 50 μL of Ultra-TMB substrate (Pierce) were added to each well. After 15 min of incubation at room temperature, 50 μL of stop solution (2-M sulfuric acid) were added. The absorbance at 450 nm was then recorded with a microplate reader (Model HT2; Anthos Labtec Instruments, Eugendorf, Austria), and each serum sample was compared against the control wells. The ELISA titer was defined as the reciprocal of the dilution with an optical density at least two times the negative control.

Western blotting.—Antibody specificity against DnaJ was assayed by Western blot. Four sera were selected (one from each treatment) on the basis of the ELISA readings. Each serum was tested against purified DnaJ and the total proteins extracted from both the noninduced and induced *E. coli* cultures. Briefly, protein samples were resolved on 12% SDS-PAGE gels and then electrotransferred to polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 1 h. After blotting, membranes were incubated overnight with a 1:500 dilution of channel catfish serum collected from immunized fish. After washing, membranes were incubated for 1 h with E-8 anti-channel catfish IgM (1:500; Klesius 1990). The E-8 monoclonal antibody was detected by incubation with conjugated goat anti-mouse immunoglobulin (1:5,000) for 1 h. Membranes were visualized with Opti-4CNTM substrate (Bio-Rad) by following the manufacturer's recommendations.

Statistical analysis.—A one-way analysis of variance was used to make contrast of treatments regarding mean antibody response; this was followed by an evaluation of differences in least-squares means. The generalized linear model procedure (PROC GENMOD) in the Statistical Analysis System (SAS) version 9.1 (SAS Institute, Cary, North Carolina) was used to fit a logistic regression model (with correlated errors to account for the clustering related to the common tank effect) on postchallenge survival. Significant differences between treatments were determined, and differences in least-squares means were assessed. All statistical analyses were carried out using SAS. Differences were considered significant at *P*-values of 0.05 or less.

Results

*Cloning and Sequence Analysis of Flavobacterium columnare dnaJ**

Genes coding for DnaJ protein were amplified from all four *F. columnare* strains. Sequences from the

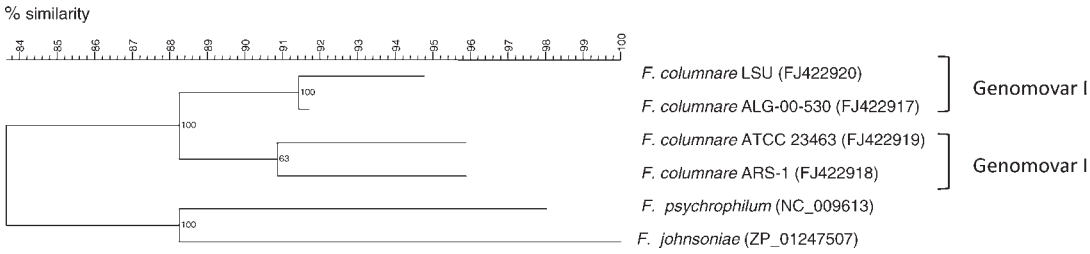

FIGURE 1.—Maximum likelihood (ML) tree based on *Flavobacterium* spp. *dnaJ** sequences (GenBank accession numbers are shown in parentheses). The tree shown is a consensus bootstrap tree based on 1,000 resampled ML trees. Bootstrap values for each branch are indicated. Genomovar ascription for *F. columnare* strains is specified.

ATCC 23463, ARS-1, ALG-00–530, and LSU strains were deposited into GenBank (accession numbers FJ422919, FJ422918, FJ422917, and FJ422920, respectively). A maximum likelihood tree based on *dnaJ** sequences is shown in Figure 1. Sequence similarity among *F. columnare* genomovars was above 90%. The *dnaJ** sequence identity within the *F. columnare* species was 88%. Interestingly, *F. johnsoniae* and *F. psychrophilum dnaJ** sequences also shared an 88% similarity, while *F. columnare* sequences clustered with the *F. johnsoniae* and *F. psychrophilum* sequences at 84%. Similar results were obtained when translated protein sequences were used in the analysis (data not shown). The *dnaJ** sequences from *F. psychrophilum* and *F. johnsoniae* shared a higher level of amino acid sequence similarity with each other than with *F. columnare*. As was expected, a clear division between *F. columnare* genomovars was found by either nucleic acid or protein sequence comparisons.

Expression and Purification of Recombinant DnaJ

Plasmid constructs were confirmed by restriction analysis and sequencing, and selected clones were stored at −80°C. Proper IPTG-induced expression of the recombinant DnaJ was analyzed. The time course study showed that optimal DnaJ expression from the *E. coli* QE30-FcolJ clone occurred at 4 h after induction. Specificity of the purification procedure is shown in Figure 2. Two bands very similar in size were expressed at high levels with the expected DnaJ molecular weight of about 40 kilodaltons (kDa). Based on these results, the second fraction eluted with buffer D (lane 8 in Figure 2) showed the highest yield of recombinant DnaJ and was used for the rest of the experiments. Two unexpected additional bands of approximately 20 and 15 kDa were also induced and co-purified. No proteins were detected by SDS-PAGE when uninduced *E. coli* cultures were subjected to the purification process (data not shown). Quantification of the purified DnaJ recombinant protein using the BCA assay resulted in DnaJ concentrations ranging from 50 to 100 μg/mL.

Recombinant Protein Antibody Response

Antibody titers against recombinant DnaJ were measured by ELISA, and results are summarized in Table 1. Mean baseline titers of fish sampled before immunization were less than 10 optical density units (OD). Fish immunized with purified DnaJ showed a significantly elevated titer against the recombinant protein when compared with the control group. Fish immunized with *E. coli* extracts expressing DnaJ also showed a positive antibody response against DnaJ. Uninduced *E. coli* cultures did not induce immunoreactivity against purified DnaJ and were not statistically different from the control group. Antibody specificity against DnaJ was confirmed by Western blots (data not shown). The 40-kDa band was highlighted after blotting using sera against purified DnaJ and against

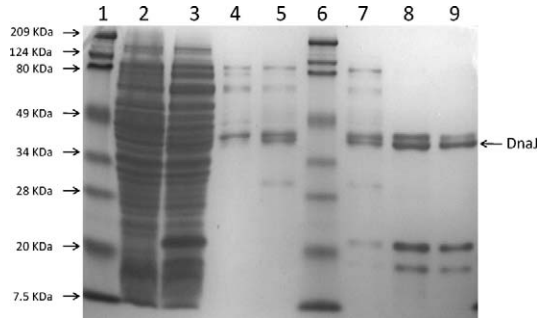

FIGURE 2.—Expression and purification of the *Flavobacterium columnare* DnaJ protein in *Escherichia coli* (kDa = kilodaltons). Lane 2 shows total protein extraction of uninduced *E. coli* clone QE30-FcolJ. Lane 3 represents total protein extraction of isopropyl thiogalactoside-induced *E. coli* clone QE30-FcolJ. Lanes 4 and 5 show serial elution fractions of induced *E. coli* cells after washes with buffer D (described in Methods). Lanes 7–9 depict the serial elution fractions of induced *E. coli* cells after washes with buffer E. Lanes 1 and 6 represent molecular standards.

TABLE 1.—Mean enzyme-linked immunosorbent assay antibody titers against *Flavobacterium columnare* DnaJ produced by channel catfish at 21 d postimmunization with recombinant DnaJ, expressed as OD mean (± SD). Different lowercase letters indicate significant differences between treatments at *P*-values less than 0.05 (Student's *t*-test was used to determine differences between groups).

Treatment	*N*	Range of titers	Mean (± SD)
Control	16	2	2 ± 0.0 z
DnaJ	16	3,840–5,120	3,840 ± 1,280 y
Induced-*Escherichia coli*	16	640–2,560	1,440 ± 560 x
Uninduced-*Escherichia coli*	16	10–80	38 ± 35 z

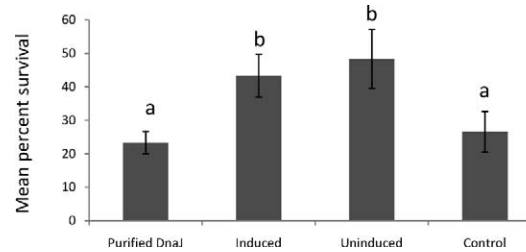

FIGURE 3.—Mean (±SE) percent survival in channel catfish immunized with purified recombinant *Flavobacterium columnare* DnaJ protein, induced *Escherichia coli* clone QE30-FcolJ lysate (expressing the recombinant DnaJ), uninduced *E. coli* clone QE30-FcolJ lysate, or sterile phosphate-buffered saline (control) and challenged with *F. columnare* strain ALG-00–530 at 4 weeks postimmunization. Comparison between treatments was made with a logistic regression. Significant differences between treatments ($P < 0.001$) are indicated by different letters.

induced *E. coli*. In addition to the 40-kDa band, two smaller bands (the ones that co-purified along with the DnaJ) were also detected by Western blot. No bands were observed with control and uninduced sera.

Bacterial Challenge

At 4 weeks postimmunization, channel catfish were challenged with *F. columnare* virulent strain ALG-00–530. Results from that challenge are summarized in Figure 3. Mean survival for the DnaJ-vaccinated group was 23% and statistically similar to that of the control group (26%; $P = 0.114$). Fish vaccinated with *E. coli* extracts had survival rates of 43% for the induced-*E. coli* group and 48% for the uninduced-*E. coli* group; both of these groups showed a significantly higher percent survival than the control group ($P = 0.034$ and 0.020, respectively).

Discussion

Heat shock proteins are highly conserved molecules distributed among all organisms; HSPs are expressed constitutively in cells under normal conditions but are upregulated in response to stressful stimuli, such as heat or cold shock, ultraviolet radiation, or oxidative stress. The HSPs act as chaperones helping with protein folding, maintaining protein complexes and structures, and facilitating protein transport across cell membranes. From an immunological point of view, HSPs have attracted interest since they were identified as powerful microbial antigens in a wide spectrum of infections (Wallin et al. 2002). Several studies have used HSPs as vaccines in other animal models, with significant protection results obtained against a variety of microbes (Zugel and Kaufmann 1999). In fish, Wilhelm et al. (2006) reported protection against *Piscirickettsia salmonis* from use of a HSP recombinant vaccine, and Sudheesh et al. (2007) recognized HSP-60 and HSP-70 as highly immunogenic proteins in *F. psychrophilum*. In this study, we identified and cloned the DnaJ (HSP-40) from *F. columnare* by using primers derived from the known *F. johnsoniae* genome. This was possible due to the highly conserved nature of HSPs among bacterial species. Specifically, *dnaJ** sequences have been used in phylogenetic studies and have proven to be a powerful marker in delineating members of the family Enterobacteriaceae (Nhung et al. 2007). The percentage of sequence similarity among the three *Flavobacterium* spp. *dnaJ** sequences analyzed was higher between *F. johnsoniae* and *F. psychrophilum* than between either of those species and *F. columnare*. Interestingly, all four *F. columnare* strains analyzed presented different *dnaJ** sequences, with intraspecies variation as high as 12%. Two consensus sequences were delineated and were correlated with genomovar ascription. The clear division of *F. columnare* isolates into at least two genomovars has been well documented by our group and others (Triyanto and Wakabayashi 1999; Arias et al. 2004; Thomas-Jinu and Goodwin 2004; Darwish and Ismaiel 2005; Olivares-Fuster et al. 2006, 2007; Shoemaker et al. 2008), but the dissimilarity found in a highly conserved housekeeping gene such as *dnaJ** further suggests a division within the species.

Expression and purification of DnaJ in *E. coli* were successful, and highly purified protein was recovered from the extracts. The DnaJ was expressed predominantly as inclusion bodies, but solubilization was straightforward. However, two bands that were very close in size appeared at the predicted 40-kDa size. In addition, two highly induced proteins of a much smaller size were co-purified during the extraction protocol. All of these proteins were present only after induction and, based on the specificity of the purification process, seemed to contain the 6xHis tag. One possible explanation for the presence of the extra

bands is the production of truncated DnaJ by *E. coli*. It has been shown that *F. psychrophilum* genes have a strong codon bias at the third position and that 19 codons seen in *F. psychrophilum* are considered to be rare in *E. coli* (Shah 2007). This could explain the inefficient expression and truncated proteins obtained when expressing *Flavobacterium* spp. genes in a phylogenetically distant species such as *E. coli*.

Intraperitoneal injection with recombinant DnaJ elicited a humoral immune response against the protein. In a similar study by Plant et al. (2009), immunization of rainbow trout *Oncorhynchus mykiss* with recombinant HSP-70 resulted in highly elevated antibody titers against this antigen. In our work, titers against DnaJ were lower than those reported from HSP-70-immunized rainbow trout (OD mean titer $>$ 3,000 in our study compared to $>$45,000 in the study by Plant et al. 2009) but higher than titers obtained in channel catfish when whole *F. columnare* cells were used as antigens (Shoemaker et al. 2003). It has been shown that in fish, antibody titers do not always correlate with protection (Ellis 1999). However, Shelby et al. (2007) proved that passive immunization of channel catfish with anti-*F. columnare* whole-cell lysates resulted in significantly reduced mortalities when fish were challenged with *F. columnare*. Their results strongly suggest that specific antibodies in serum are involved in the protective immune response against columnaris disease in channel catfish. Moreover, it has also been shown that cutaneous antibodies against *F. columnare* are produced in fish infected with *F. columnare*. This constitutes a significant step in understanding the acquired immunity against this bacterium since *F. columnare* is typically associated with the external surfaces of fish.

Despite the strong immune response against DnaJ obtained for vaccinated channel catfish, this humoral response did not confer protection against columnaris disease. Similarly, no protection was observed against *F. psychrophilum* when HSP-60 and HSP-70 were used as antigens (Plant et al. 2009). The lack of protective effect when using HSPs as vaccines is not specific for fish as it has also been reported in mammals. Noll and Autenrieth (1996) failed to achieve protection against *Yersinia enterocolitica* after immunizing mice with purified HSP-60. Likewise, Leclerq et al. (2002) detected a strong immune response against the HSP GroEL from *Brucella abortus* in mice, but no significant level of protection was observed after challenge.

Our data showed a protective effect when *E. coli* lysates were used. However, no difference in protection between induced (containing *F. columnare* recombinant DnaJ protein) and uninduced cultures was observed; therefore, the positive effect was apparently due to *E. coli* antigens and not to the recombinant DnaJ. It is likely that the *E. coli* lipopolysaccharide (LPS) or endotoxin conferred some protection against columnaris since LPS is a key virulence factor in Gram-negative bacteria. It has been reported that immunization of channel catfish with *E. coli* lysates offered a certain degree of protection against *Edwardsiella ictaluri* infections (Tyler and Klesius 1994). Although the LPS from *F. columnare* does not resemble the typical ladder-like pattern usually obtained from enterobacteria (Zhang et al. 2006), it is likely that the most conserved part of the LPS, the lipid A, presents enough similarity between phylogenetically distant bacteria to confer cross-protection. Other antigens expressed by *E. coli*, such as adhesins and hydrolytic enzymes, may present epitopes similar to those occurring in *F. columnare*.

In summary, our results showed that the use of the HSP DnaJ as recombinant vaccine does not confer protection against columnaris disease in channel catfish; therefore, additional antigens should be investigated as vaccine candidates for *F. columnare*. However, this study demonstrated that immunization with purified recombinant proteins elicited a specific humoral immune response in channel catfish. In addition, our data suggest that careful attention should be paid to expression of *F. columnare* proteins in an *E. coli* system due to possible codon bias.

Acknowledgments

We thank Chris Sayles (Auburn University) and Paige Mumma (U.S. Department of Agriculture [USDA], Agricultural Research Service [ARS]) for their technical support. We are grateful to Mark Carpenter (Department of Mathematics and Statistics, Auburn University) for his advice on data analysis. This research was funded by a USDA-ARS–Auburn University Specific Cooperative Agreement (Prevention of Diseases of Farmed Raised Fish) and USDA-ARS Current Research Information System Project Number 6420-32000-022–00D. Reference to trade names does not imply endorsement by the U.S. Government.

References

AFS-FHS (American Fisheries Society Fish Health Section). 2004. Suggested procedures for the detection and identification of certain finfish and shellfish pathogens. American Fisheries Society, Bethesda, Maryland.

Altinok, I., and J. M. Grizzle. 2001. Effects of low salinities on *Flavobacterium columnare* infection of euryhaline and freshwater stenohaline fish. Journal of Fish Diseases 24:361–367.

Arias, C. R., T. L. Welker, C. A. Shoemaker, J. W. Abernathy,

and P. H. Klesius. 2004. Genetic fingerprinting of *Flavobacterium columnare* isolates from cultured fish. Journal of Applied Microbiology 97:421–428.

Bebak, J., M. Matthews, and C. A. Shoemaker. 2009. Survival of vaccinated, feed-trained largemouth bass fry (*Micropterus salmoides floridanus*) during natural exposure to *Flavobacterium columnare*. Vaccine 27:4297–4301.

Crump, E. M., J. Burian, P. D. Allen, S. Gale, and W. W. Kay. 2007. Identification of a ribosomal L10-like protein from *Flavobacterium psychrophilum* as a recombinant vaccine candidate for rainbow trout fry syndrome. Journal of Molecular Microbiology and Biotechnology 13:55–64.

Darwish, A. M., and A. A. Ismaiel. 2005. Genetic diversity of *Flavobacterium columnare* examined by restriction fragment length polymorphism and sequencing of the 16S ribosomal RNA gene and the 16S-23S rDNA spacer. Molecular and Cellular Probes 19:267–274.

Decostere, A., F. Haesebrouck, G. Charlier, and R. Ducatelle. 1999. The association of *Flavobacterium columnare* strains of high and low virulence with gill tissue of black mollies (*Poecilia sphenops*). Veterinary Microbiology 67:287–298.

Delpino, M. V., S. M. Estein, C. A. Fossati, P. C. Baldi, and J. Cassataro. 2007. Vaccination with *Brucella* recombinant DnaK and SurA proteins induces protection against *Brucella abortus* infection in BALB/c mice. Vaccine 25:6721–6729.

Ellis, A. E. 1999. Immunity to bacteria in fish. Fish and Shellfish Immunology 9:291–308.

Hawke, J. P., and R. L. Thune. 1992. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. Journal of Aquatic Animal Health 4:109–113.

IUBMB (International Union of Biochemistry and Molecular Biology). 1992. Enzyme nomenclature 1992. Academic Press, San Diego, California.

Klesius, P. H. 1990. Effect of size and temperature on the quantity of immunoglobulin in channel catfish, *Ictalurus punctatus*. Veterinary Immunology and Immunopathology 24:187–195.

Leclerq, S., J. S. Harms, G. M. S. Rosinha, V. Azevedo, and S. C. Oliveira. 2002. Induction of a Th1-type of immune response but not protective immunity by intramuscular DNA immunisation with *Brucella abortus GroEL* heat-shock gene. Journal of Medical Microbiology 51:20–26.

Mao, Z., L. Yu, Z. You, Y. Wei, and Y. Liu. 2007. Cloning, expression and immunogenicity analysis of five outer membrane proteins of *Vibrio parahaemolyticus* zj2003. Fish and Shellfish Immunology 23:567–575.

Nhung, P. H., K. Ohkusu, N. Mishima, M. Noda, M. M. Shah, X. Sun, M. Hayashi, and T. Ezaki. 2007. Phylogeny and species identification on the family Enterobacteriaceae based on DnaJ sequences. Diagnostic Microbiology and Infectious Disease 58:153–161.

Noll, A., and I. B. Autenrieth. 1996. Immunity against *Yersinia enterocolitica* by vaccination with *Yersinia* HSP60 immunostimulating complexes or *Yersinia* HSP60 plus interleukin-12. Infection and Immunity 64:2955–2961.

Olivares-Fuster, O., J. L. Baker, J. S. Terhune, C. A. Shoemaker, P. H. Klesius, and C. R. Arias. 2007. Host-specific association between *Flavobacterium columnare* genomovars and fish species. Systematic and Applied Microbiology 30:624–633.

Olivares-Fuster, O., C. A. Shoemaker, P. H. Klesius, and C. R. Arias. 2006. Molecular typing of *Flavobacterium columnare* isolates by single stranded conformation polymorphism analysis. FEMS Letters in Microbiology 269:63–69.

Pacha, R. E., and E. J. Ordal. 1970. Myxobacterial diseases of salmonids. Pages 243–257 *in* S. F. Snieszko, editor. Symposium on diseases of fishes and shellfishes. American Fisheries Society, Special Publication 5, Bethesda, Maryland.

Pasnik, D. J., and S. A. Smith. 2005. Immunogenic and protective effects of a DNA vaccine for *Mycobacterium marinum* in fish. Veterinary Immunology and Immunopathology 103:195–206.

Plant, K. P., S. E. LaPatra, and K. D. Cain. 2009. Vaccination of rainbow trout, *Oncorhynchus mykiss* (Walbaum), with recombinant and DNA vaccines produced to *Flavobacterium psychrophilum* heat shock proteins 60 and 70. Journal of Fish Diseases 32:521–534.

Sagi, S. S. K., P. Paliwal, A. Bansal, C. Mishra, N. Khan, S. R. Mustoori, G. Ilavazhagan, R. C. Sawhney, and P. K. Banerjee. 2006. Studies on immunogenicity and protective efficacy of DnaJ of *Salmonella typhi* against lethal infection by *Salmonella typhimurium* in mice. Vaccine 24:7135–7141.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shah, D. 2007. Effects of codon usage bias on recombinant expression of *Flavobacterium psychrophilum* proteins in *Escherichia coli*. Flavobacterium 2007 Workshop, Shepherdstown, West Virginia.

Shelby, R. A., C. A. Shoemaker, and P. H. Klesius. 2007. Passive immunization of channel catfish, *Ictalurus punctatus* (Rafinesque), with anti-*Flavobacterium columnare* sera. Diseases of Aquatic Organisms 77:143–147.

Shoemaker, C. A., C. R. Arias, P. H. Klesius, and T. L. Welker. 2005a. Technique for identifying *Flavobacterium columnare* using whole-cell fatty acid profiles. Journal of Aquatic Animal Health 17:267–274.

Shoemaker, C. A., P. H. Klesius, and J. Evans. 2005b. Modified live *Flavobacterium columnare* against columnaris disease in fish. U.S. patent 6,881,412.B1. April 12, 2005.

Shoemaker, C. A., O. Olivares-Fuster, C. R. Arias, and P. H. Klesius. 2008. *Flavobacterium columnare* genomovar influences mortality in channel catfish (*Ictalurus punctatus*). Veterinary Microbiology 127:353–359.

Shoemaker, C. A., R. A. Shelby, and P. H. Klesius. 2003. Development of an indirect ELISA to detect humoral response to *Flavobacterium columnare* infection of channel catfish, *Ictalurus punctatus*. Journal of Applied Aquaculture 14:43–52.

Shotts, E., and C. Starliper. 1999. Flavobacterial diseases: columnaris disease, cold-water disease, and bacterial gill disease. Pages 559–576 *in* P. Woo and D. Bruno, editors. Fish diseases and disorders, volume 3, viral, bacterial and fungal infections. CABI Publishing, New York.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia,

F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150:76–85.

Sudheesh, P. S., B. R. LaFrentz, D. R. Call, W. F. Siems, S. E. LaPatra, G. D. Wiens, and K. D. Cain. 2007. Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and a non-virulent strain of the fish pathogen *Flavobacterium psychrophilum*. Diseases of Aquatic Organisms 74:37–47.

Thomas-Jinu, S., and A. E. Goodwin. 2004. Morphological and genetic characteristics of *Flavobacterium columnare* isolates: correlations with virulence in fish. Journal of Fish Diseases 27:29–35.

Triyanto, K., and H. Wakabayashi. 1999. Genotypic diversity of strains of *Flavobacterium columnare* from diseased fishes. Fish Pathology 34:65–71.

Tyler, J. W., and P. H. Klesius. 1994. Protection against enteric septicemia of catfish (*Ictalurus punctatus*) by immunization with the R-mutant, *Escherichia coli* (J5). American Journal of Veterinary Research 55:1256–1260.

USDA (U.S. Department of Agriculture). 2003a. Part I: reference of fingerling catfish health and production practices in the United States. USDA National Animal Health Monitoring System, Fort Collins, Colorado.

USDA (U.S. Department of Agriculture). 2003b. Part II: reference of food size catfish health and production practices in the United States. USDA National Animal Health Monitoring System, Fort Collins, Colorado.

Wakabayashi, H. 1993. Columnaris disease. Pages 23–39 *in* V. Inglis, R. J. Roberts, and N. R. Bromage, editors. Bacterial diseases of fish. Blackwell Scientific Publications, London.

Wallin, R. P. A., A. Lundqvist, S. H. More, A. von Bonin, R. Kiessling, and H.-G. Ljunggren. 2002. Heat-shock proteins as activators of the innate immune system. Trends in Immunology 23:130–135.

Wilhelm, V., A. Miquel, L. O. Burzio, M. Rosemblatt, E. Engel, S. Valenzuela, G. Paranda, and P. D. T. Valenzuela. 2006. A vaccine against the salmonid pathogen *Piscirickettsia salmonis* based on recombinant proteins. Vaccine 24:5083–5091.

Wise, D. J., L. H. Khoo, and J. S. Terhune. 2002. The epidemiology of bacterial diseases in food-size channel catfish. Journal of Aquatic Animal Health 14:263–272.

Zhang, Y., C. R. Arias, C. A. Shoemaker, and P. H. Klesius. 2006. Comparison of lipopolysaccharide and protein profiles between *Flavobacterium columnare* strains from different genomovars. Journal of Fish Diseases 29:657–663.

Zugel, U., and S. H. E. Kaufmann. 1999. Role of heat shock proteins in protection from and pathogenesis of infectious diseases. Clinical Microbiology Reviews 12:19–39.