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Short communication

Edwardsiella tarda DnaK: Expression, activity, and the basis for the construction of a bivalent live vaccine against *E. tarda* and *Streptococcus iniae*

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ABSTRACT

Edwardsiella tarda and *Streptococcus iniae* are important aquaculture pathogens that affect many species of farmed fish. In this study, we analyzed the expression, activity, and immunoprotective potential of *E. tarda* heat shock protein DnaK. We found that *dnaK* expression was upregulated under conditions of heat shock, oxidative stress, and infection of host cells. Recombinant DnaK (rDnaK) purified from *Escherichia coli* exhibited ATPase activity and induced protection in Japanese flounder (*Paralichthys olivaceus*) against lethal *E. tarda* challenge. On the basis of these results and our previous observation that a protective *S. iniae* antigen Sia10 which, when expressed heterogeneously in *E. coli* DH5α, is secreted into the extracellular milieu, we constructed a chimeric antigen by fusing DnaK to Sia10. The resulting fusion protein Sia10-DnaK was expressed in DH5α via the plasmid pTDK. Western blot analysis indicated that Sia10-DnaK was detected in the culture supernatant of DH5α/pTDK. When flounder were vaccinated with live DH5α/pTDK, strong protection was observed against both *E. tarda* and *S. iniae*. ELISA analysis detected specific serum antibody production in fish vaccinated with rDnaK and DH5α/pTDK. Taken together, these results indicate that rDnaK is an intrinsic ATPase with immunoprotective property and that Sia10-DnaK delivered by a live bacterial host is an effective bivalent vaccine candidate against *E. tarda* and *S. iniae* infection.

1. Introduction

Edwardsiella tarda is a Gram-negative bacterium belonging to the family of Enterobacteriaceae. It is a pathogen to both marine and freshwater fish and, upon infection, induces development of edwardsiellosis, a systematic disease that has caused heavy economic losses to aquaculture industries worldwide [1–3]. In China, *E. tarda* is known to infect a number of farmed fish species, notably flounder and turbot, which are principal economic species cultured in northern China [4,5]. In addition to aquatic animals, *E. tarda* is also a human pathogen and known to cause gastroenteritis and extraintestinal disorders [6]. Currently, control of *E. tarda* in aquaculture relies chiefly on the use of antibiotics in most countries including China. In recent years, experimental *E. tarda* vaccines in various types have been reported, most of which are monovalent vaccines targeted at *E. tarda* alone.

Bacterial DnaK/Hsp70 is a molecular chaperone that assists folding of nascent and misfolded proteins and facilitates protein translocation across membranes. As a result, DnaK plays an important role under both stressed and normal physiological conditions. DnaK is an intrinsically weak ATPase and in structure contains an ATPase domain at the N-terminal region, a substrate binding domain, and a C-terminal domain of unknown function [7,8]. In *Escherichia coli*, DnaK forms a chaperone machinery with two co-chaperones, i.e., DnaJ and GrpE, that regulate the activity of DnaK [9]. It is known that microbial heat shock proteins, such as DnaK and DnaJ, are potent immunostimulants that can activate mammalian immune systems [10]. In line with these observations, some heat shock proteins, including DnaK, are found to be immunoprotective when used as subunit vaccines [11–15]. Owing to their immunological qualities, heat shock proteins have been widely employed in vaccine design against various infectious diseases and cancers [16].

In this study, we characterized the DnaK from a pathogenic *E. tarda* strain isolated from diseased flounder. We found that expression of *dnaK* was positively regulated by certain stress signals and that recombinant DnaK was an active ATPase and a protective

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antigen. Based on these observations, we developed a DnaK-containing bivalent vaccine delivered by a live bacterial host against *E. tarda* and *Streptococcus iniae*, the latter being a Gram-positive pathogen that can infect a wide range of farmed fish [17].

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. tarda TX1 and *S. iniae* SF1 were isolated from diseased flounder [18,19]. *E. coli* BL21(DE3) and DH5α were purchased from Takara (Dalian, China). All strains were grown in Luria–Bertani broth (LB) at 37 °C (for *E. coli*) or 28 °C (for *E. tarda* and *S. iniae*).

2.2. Quantitative real-time reverse transcriptase-PCR (qRT-PCR) analysis of dnaK expression under stress conditions

For heat shock treatment, TX1 was grown in LB medium at 28 °C to an OD_{600} of 0.5, and the cells were then divided into two parts: the first part was cultured continuously at 28 °C while the second part was cultured at 37 °C (sub-lethal temperature). For H_2O_2 treatment, the cells were grown in LB medium to an OD_{600} of 0.5; half of the cell culture was removed and grown separately in LB medium supplemented with 1 mM H_2O_2, while the other half was grown in LB medium without H_2O_2. Total RNA was extracted from the cells harvested at different time points (5 min, 10 min, 30 min, and 60 min) after heat shock or H_2O_2 treatment and used for qRT-PCR analysis as described previously [20]. In all cases, 16S rRNA was used as a control. All data are given in terms of relative mRNA, expressed as means plus or minus standard errors of the means (SE).

2.3. qRT-PCR analysis of dnaK expression during infection

To examine *dnaK* expression during infection, flounder head kidney (HK) macrophages were prepared as described previously [21] and cultured in L-15 medium (Thermo Scientific HyClone, Beijing, China) in a 96-well culture plate (10^5 cells/well) at 25 °C. TX1 was cultured in LB medium to mid-logarithmic phase and resuspended in PBS to 10^8 CFU/ml. A portion of the bacterial cells was used for total RNA preparation with HP Total RNA (Omega Bio-Tek, USA). For cellular infection, HK macrophages were infected with TX1 bacterial suspension (10^7 CFU/well) for 0.5 h at 25 °C and washed 3 × with PBS. The cells were added with fresh medium and continued to incubate at 25 °C for 1 h, 2 h, 3 h, and 4 h respectively. At the end of each incubation, the cells were lysed with 1% Triton X-100 and used for total RNA extraction as described above. One microgram of total RNA was used for cDNA synthesis with the Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis of *dnaK* expression was performed as described above.

2.4. Plasmid and strain construction

To construct pEtDK, *dnaK* was amplified from *E. tarda* TX1 by PCR with primers F1 (5′- CCCGGGATGGGTAAAATCATTGGTATCGA- 3′, underlined, SmaI site) and R1 (5′-CCCGGGTTTTTTATCTTT-CACTTCTTCGAA- 3′, underlined, SmaI site). The PCR products were ligated with the TA cloning vector pBS-T (Tiangen, Beijing, China), resulting in pBSDK, which was digested with SmaI, and the fragment containing *dnaK* was retrieved and inserted into pET259 [20] at the SwaI site, resulting in pEtDK. To construct pTDK, the coding sequence of *sia10* was amplified by PCR with primers F5 (5′- CCCGGGCA-TATGAAAAAAATCGCAACTA -3′; underlined, SmaI and NdeI sites) and R6 (5′- GATATCTCCTCCGATATGAATATAATTATATGAGCT -3′; underlined, EcoRV site). The PCR products were inserted into the expression plasmid pBT3 [22] between the NdeI/EcoRV sites, resulting in plasmid pT3S10. pBSDK was digested with SmaI, and the *dnaK*-containing fragment was retrieved and inserted into pT3S10 at the EcoRV site, resulting in pTDK. In pTDK, Sia10-DnaK was expressed constitutively as a His-tagged fusion protein under the P_{trc} promoter. *E. coli* DH5α was transformed with pTDK, resulting in DH5α/pTDK. The control strain DH5α/pBT3 was similarly constructed by transforming DH5α with the backbone plasmid pBT3.

2.5. Purification of recombinant DnaK (rDnaK)

E. coli BL21(DE3) was transformed with pEtDK; the transformants were cultured in LB medium at 37 °C to mid-logarithmic phase, and expression of *dnaK* was induced by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After growing at 37 °C for an additional 5 h, the cells were harvested by centrifugation, and His-tagged rDnaK was purified under native conditions using nickel–nitrilotriacetic acid columns (GE Healthcare, Piscataway, NJ, USA) as recommended by the manufacturer. The purified protein was dialyzed in phosphate-buffered saline (PBS) and concentrated with Amicon Ultra Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with Coomassie brilliant blue R-250. The concentration of purified protein was determined using the Bradford method with bovine serum albumin as the standard.

2.6. Western blot

Western immunoblot analysis was performed as described previously [23].

2.7. ATPase assay

The ATPase activity of rDnaK was determined as described previously [24]. Briefly, rDnaK in different concentrations was incubated in Buffer I (10 mM Hepes, 10 mM $MgCl_2$, 20 mM KCl, 0.5 mM dithiothreitol, and 1 mM ATP) at 37 °C for 1 h, followed by adding trichloroacetic acid to 15% to stop the reaction. An equal volume of Buffer II (1% ammonium molybdate, 6% ascorbic acid, 2% sodium citrate, and 2% acetic acid) was added to the assay mixture, followed by incubation at 45 °C for 25 min. The release of inorganic phosphate was quantified by measuring absorbance at 660 nm. ATPase activity was presented as phosphate release normalized against a standard curve generated using KH_2PO_4.

2.8. Vaccination

For vaccination with rDnaK, the protein was resuspended in PBS to a concentration of 200 μg/ml and mixed at an equal volume with aluminum hydroxide as described previously [25]. As a control, PBS was also mixed similarly with aluminum hydroxide without rDnaK. Japanese flounder (average 11.8 g) were divided randomly into two equal-sized groups and injected intraperitoneally (i.p.) with 100 μl of rDnaK suspension (100 μg/ml) or PBS (control). At one month post-vaccination, 25 fish were taken from each group and challenged via i.p. injection with 100 μl TX1 that had been cultured as described above to an OD_{600} of 0.8, washed with PBS, and resuspended in PBS to 4×10^6 CFU/ml. The fish were maintained at 22 °C as described previously [25] and monitored for mortality for a period of 20 days. Dying fish were randomly selected for the examination of bacterial recovery from liver, kidney, and spleen. Relative percent of survival (RPS) was calculated according to the following formula: RPS = {1 − (%mortality in vaccinated fish/% mortality in control fish)} × 100 [26].