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Oral delivery of *Bacillus subtilis* spores expressing cysteine protease of *Clonorchis sinensis* to grass carp (*Ctenopharyngodon idellus*): Induces immune responses and has no damage on liver and intestine function

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ABSTRACT

Clonorchis sinensis (*C. sinensis*) is a fish-borne trematode. Human can be infected by ingestion of *C. sinensis* metacercariae parasitized in grass carp (*Ctenopharyngodon idella*). For induction of effective oral immune responses, spores of *Bacillus subtilis* (*B. subtilis*) WB600 were utilized as vehicle to delivery *Cs*CP (cysteine protease of *C. sinensis*) cooperated with CotC (*B.s*-CotC-CP), one of coat proteins, to the gastrointestinal tract. After routine culture of 8–12 h in LB medium, *B. subtilis* containing CotC-*Cs*CP was transferred into the sporulation culture medium. SDS-PAGE, western blotting and the growth curve indicated that the best sporulation time of recombinant WB600 was 24–30 h at 37 °C with continuous shaking (250 rpm). Grass carp were fed with three levels of *B.s*-CotC-CP (1×10^6, 1×10^7, and 1×10^8 CFU g^{-1}) incorporated in the basal pellets diet. The commercial pellets or supplemented with spores just expressing CotC (1×10^7 CFU g^{-1}) were served as control diet. Our results showed that grass carp orally immunized with the feed-based *B.s*-CotC-CP developed a strong specific immune response with significantly ($P < 0.05$) higher levels of IgM in samples of serum, bile, mucus of surface and intestinal compared to the control groups. Abundant colonization spores expressing *Cs*CP were found in hindgut that is conducive to absorption and presentation of antigen. Moreover, *B. subtilis* spores appeared to show no sign of toxicity or damage in grass carp. Our cercariae challenge experiments suggested that oral administration of spores expressing *Cs*CP could develop an effective protection against *C. sinensis* in fish body. Therefore, this study demonstrated that the feed-based recombinant spores could trigger high levels of mucosal and humoral immunity, and would be a promising candidate vaccine against *C. sinensis* metacercariae formation in freshwater fish.

1. Introduction

The clonorchiasis caused by *Clonorchis sinensis* (*C. sinensis*) is a long chronic infection disease in definitive hosts, often accompanied by a broad range of hepatic and bile duct symptoms, such as cholangitis, cholecystitis, bile duct obstruction, cholelithiasis, and even cholangiocarcinoma and hepatic cirrhosis [1–3]. Clonorchiasis is mainly prevalent in Asian countries and regions, such as China, South Korea, northern Vietnam, and Russia [3]. It is estimated that

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more than 200 million people are at risk of infection with *C. sinensis* and over 15 million are infected globally, of which approximately 13 million infection people come from China [4,5]. The global burden of clonorchiasis is about 275, 370 disability-adjusted life years (DALYs), and nearly 5, 591 people died from the infection every year, so that severe disease burdens, medical and economic problems have been brought to low- or middle-income countries of Asia [6]. It's quite difficult to eliminate spread of *C. sinensis* by controlling definitive hosts, because of in addition to humans, cats, dogs, and other piscivorous animals can serve as reservoir hosts for *C. sinensis* [3,7]. Blocking the transmission of *C. sinensis* infection in intermediate hosts (e.g., freshwater fish) might be an effective way to control clonorchiasis.

C. sinensis is one of the well-known fishborne zoonotic trematodes. Freshwater fish or shrimp can act as the second intermediate hosts for development of *C. sinensis* metacercariae. Humans and other mammals get infection mainly because of eating raw or inadequately cooked freshwater fish containing infective metacercariae [4,8]. Currently, more than 100 species of freshwater fish can serve as the second intermediate hosts of *C. sinensis*, among which most belong to the Cyprinidae family. However, the common edible and economic fish (e.g., *Ctenopharyngodon idellus*, *Carassius auratus*, and *Hypophthalmichthys nobilis*) is the most important cause of human or mammalian infection with *C. sinensis* [1,8,9]. So it's necessary to limit and reduce the metacercariae encysted edible fish into the market or restaurant. Safe and effective vaccines are very critical for controlling pathogens or diseases and promoting healthy development of the aquaculture industry.

Different immunization routes have been applied in cultured fish, including immersion, injection (e.g., intra-peritoneal and intra-muscular), and oral treatment. Overall, compared with immersion and injection, candidate antigens directly incorporated in or adhered to the feed is a preferable route since it's needle-free, no size limitation, lower cost, and more convenient for farmer operation [10,11]. However, soluble or crude antigens will generate poor immune responses due to easily degraded by gastric acid and various proteolytic enzymes in digestive tract [12]. New carriers or adjuvants are therefore, essential. So that antigens can reach the lymphoid tissue of the hindgut without destruction. Currently, *Bacillus* spores have been utilized as natural safety probiotics in humans and animals, or in feed supplements that improve the health of digestive, enhance the immunity and also improve the antagonism to pathogens of the host [13–15]. Moreover, *Bacillus subtilis* (*B. subtilis*) spore has been proved to be an idea oral vaccine delivery system to present heterologous antigens to the gastrointestinal tract. The characteristics of long-term storage, extreme robustness (e.g., resistant to environments of strong acid, high temperature and dessication), and gene operability guarantee that the *B. subtilis* spore as the feasibility of the oral vaccine [15,16]. Nowadays, many researchers are interested in the application of *B. subtilis* probiotics in aquaculture, whether it is in the form of dietary supplementation or vaccine delivery vehicle [17–19].

Cysteine proteases play numerous indispensable roles in the physiology and pathology of parasitic organisms, in addition to general functions of proteolytic processing, which may be closely related to egg hatching, larval migration, excystment/encystment, exsheathing, nutrition acquisition, parasite immunoevasion, and so on [20–22]. Parasite cysteine proteases possess good stability and unusual immunogenicity, and have been exploited as promising chemotherapeutic, serodiagnostic markers and vaccine candidates [20,21,23,24]. Cysteine protease from *C. sinensis* (*Cs*CP) is a vital component present in excretory-secretory products (ESPs) of both metacercariae and adult worm, and is critical to the life cycle and pathogenicity of *C. sinensis* [20,21,25]. *Cs*CP as indispensable endogenous protease of various life stages of *C. sinensis* is probable an intrinsic metacercarial excystment/encystment factor [20,25,26]. Therefore, *Cs*CP must be a potential vaccine candidate for intervention of metacercariae formation in fish body and inhibition of metacercariae excystment in duodenum of definitive host.

In our previous study, the platform of heterogenous antigens delivery based on *B. subtilis* spore has been successfully established and confirmed to be valid and feasible for further oral immunization trails [27–29]. We previously constructed the recombinant spores expressing abundant fusion protein of CotC-*Cs*CP, and maintained its good immunogenicity of *Cs*CP on spores [30]. In the present study, we explored the optimum inducing conditions of spore germination and analyzed mucosal and humoral immunological effects triggered by feeding grass carp with *B. subtilis* spores expressing CotC-*Cs*CP (*B.s*-CotC-CP) or only CotC (*B.s*-CotC). In addition, we evaluate the effects of the oral immunization with *B.s*-CotC-CP or *B.s*-CotC on the liver and intestine functions of fish.

2. Materials and methods

2.1. Bacterial strains and plasmids

The *B. subtilis* strain of WB600 was routinely stored in our lab. The coding sequence of CotC containing promoter (380 bp, accession number X05680) was amplified from genomic DNA of *B. subtilis* by PCR using primers CotC-F and CotC-R1 or -R2 (the primers sequences were shown in Table 1). The coding sequence of *Cs*CP (930 bp, accession number JN655695) was amplified from *C. sinensis* genome with primers *Cs*CP-F and *Cs*CP-R (Table 1). The fusion gene of CotC-*Cs*CP was constructed by PCR with primers CotC-F and *Cs*CP-R. All the employed primers were listed in Table 1. The PCR product of CotC or CotC-*Cs*CP was then cloned into *E. coli*/*B. subtilis* shuttle vector pEB03 (Fig. 1A and B). The recombinant plasmids were confirmed by DNA sequencing. The confirmed plasmids were transformed into *E. coli* DH5α (Promega, USA) for colony formation and subsequently electro transformed into *B. subtilis* WB600 (WB600-pEB03-CotC and WB600-pEB03-CotC-*Cs*CP) [30]. Schematic diagram of recombinant protein (CotC-*Cs*CP) expression on spores of WB600 was shown in Fig. 1C. Recombinant *Cs*CP (r*Cs*CP) was purified from *E. coli* BL21 (DE3) harboring the recombinant plasmid of pET-28a (+)-*Cs*CP as previously described [21].

2.2. Culture, sporulation and identification of WB600-pEB03-CotC-CsCP

WB600-pEB03-CotC-*Cs*CP was grown on spectinomycin ($Spec^+$) resistance Luria Bertani (LB) agar plates (pH 7.0, 37 °C) for 10–12 h. Colonies picked from the cultured plates were then sub-cultured into 5 ml and 150 ml liquid LB medium ($Spec^+$, 100 μg ml^{-1}), respectively, at 37 °C with continuous shaking (250 rpm). The optical density (OD) values at 600 nm were measured every two hours until 24 h. The *B. subtilis* in end of logarithmic growth phase was inoculated into 500 ml non-resistance sporulation medium at a ratio of 1:100. 1 L of Difco Sporulation Medium (DSM) contained 8 g Difco™ Nutrient Broth (BD, USA), 1 g KCl, 0.25 g $MgSO_4$, 0.002 g $MnCl_2 \cdot 4H_2O$, 1 μM $FeSO_4$, and 0.5 mM $CaCl_2$. Cultured bacterium liquid was collected every 6 (or 12) hours intervals till 72 h to measure the absorbance at 600 nm and obtain growth curve. Additionally, the samples of sporulated *B. subtilis* at different time points (0 h, 6 h, 12 h, 18 h, 24 h, 30 h, 36 h, 42 h, 48 h, 54 h, 60 h and 72 h) were subjected to 12.0% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting to observe the expression of CotC-*Cs*CP [28–30]. Grayscale analysis (ImagePro Plus software, IPP) was employed to compare the expression levels.