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Construction of a recombinant plasmid containing multi-copy CpG motifs and its effects on the innate immune responses of aquatic animals

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Abstract

Bacterial DNA and synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG dinucleotides (CpG motifs) have been shown to induce potential immune responses. In this study, we designed a recombinant plasmid containing multi-copy CpG motifs, and observed its effects on innate immune responses of fish and prawn. The results showed that such plasmid DNA, compared to the vacant vector, can highly induce the activation of head kidney macrophages and the proliferation of peripheral blood leukocytes in *Carassius auratus* and *Lateolabrax japonicus in vitro*, as well as the activity of humoral defense proteins and the antibacterial activity of haemolymph in *Litopenaeus vannamei in vivo*. It implies that the multi-copy CpG motifs harboured in plasmid could contribute to these innate immunostimulatory effects. Therefore, the study suggested that the plasmid containing multi-copy CpG motifs might have its potential application in improving host resistance to pathogen insults in aquaculture, and have its notable advantages of high efficacy, economical cost and application to a broad range of aquatic species. © 2007 Elsevier Ltd. All rights reserved.

Keywords: CpG motif; Recombinant plasmid; Innate immune responses; Crucian carp; Japanese seabass; Pacific white shrimp

1. Introduction

In commercial aquaculture, considerable effort is directed towards preventing the onset of disease caused by pathogens. One approach to prevention the use of immunostimulants which are naturally occurring compounds that modulate the immune system by increasing the host's defence to pathogens. Immunostimulants are also defined in view of recent discoveries of the pathogen-associated molecular patterns (PAMPs) including cell wall products such as lipopolysaccharide (LPS), glucans, alginate, and nucleic acids [1].

Recent research found that the immunostimulatory response observed in mammalian cells has been shown to arise by the way of the recognition of the unmethylated CpG dinucleotides within a certain base context (CpG motif) present in bacterial DNA or synthetic oligonucleotides (ODNs) [2,3]. B cells [2], macrophages [4], natural killer cells [3,5], and dendritic cells [6] subsequently identified as direct cellular targets of CpG motifs require Toll-like receptor

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9 (TLR9) in responses to CpG motifs [7]. In mice and human models, the above cells activated by CpG DNA secrete cytokines and chemokines that create a pro-inflammatory (IL-1, IL-6, IL-18 and TNF) and Th1-biased (IFN- γ , IL-12) immune milieu [2,4,8,9].

The immunostimulatory effects of CpG motifs recently reported on aquatic animals, are the activation of macrophages primarily [10,11] and leucocytes [12–15] in fish, the effect of prophenoloxidase (proPO) system of prawn haemocytes [16] and the increase of lysozyme, phenoloxidase (PO) activity in mussel haemolymph [17]. Multiple injections of CpG-ODNs in fish were shown to induce early, non-specific antibacterial [18] and antiviral [15] protection, indicating the potential of CpG-ODNs therapy in aquatic animals to improve resistance to pathogen insults. However, it is costly to obtain synthetic ODNs, which limited the universal use of CpG immunostimulants in aquaculture. In this paper, we reported the construction of a plasmid containing tandem CpG-ODNs (harbouring multi-copy CpG motifs), and the evaluation of its immunostimulatory effects on the innate immune responses to aquatic animals, Crucian carp, Japanese seabass and Pacific white shrimp.

2. Materials and methods

2.1. Fish and prawn

Crucian carp (*Carassius auratus*), weighing approximately 400 g each, Japanese seabass (*Lateolabrax japonicus*), weighing approximately 450 g each and Pacific white shrimp (*Litopenaeus vannamei*), weighing 10 ~ 15 g each, were obtained from hatchery of Zhejiang Institute of marine Fisheries (China). The Crucian carp were kept in recirculating aerated freshwater at 25 °C and the Japanese seabass were in seawater (water temperature 18 °C, salinity 30). Both were fed with commercial pellets at a ration of 0.7% of their body weight twice daily. Pacific white shrimp were kept in the recirculating aerated seawater (water temperature 25 °C, salinity 30), stocked at a density of 20–25 individuals per square meter, and fed with feed pellets, equivalent to 5% of their body weight, twice a day.

2.2. Design and synthesis of tandem ODNs

Ten ODNs (ODN 1681, 1669, 2133, 2102, 2143, 2006, 1826, 1670, 1668, 1651), which were previously used effectively in mammalian and aquatic lives [10-15] were designed to connect one another in series to form a CpG-rich fragment (226 bp) containing tandem ODNs (18 copies of CpG motifs) (Fig. 1). In addition, *XhoI* restriction site was arranged in the 3'-end of the tandem fragment. The designed sequence was chemically synthesized by a DNA synthesizer (ABI3400) and inserted into pMD18-T (Songon, China), and a plasmid 'pMD18-T/18CpG' was obtained.

2.3. Construction of the multi-copy tandem CpG-containing plasmid

The tandem fragment constructed in pMD18-T/18CpG was introduced into pYES2 according to the method of isocaudarner restriction ligation [19]. Briefly, the insert of the tandem fragment in pMD18-T/18CpG was cut out using *XhoI* (located in the tandem fragment) and *SaII* (located in pMD18-T), two isocaudarner restriction enzymes, which

1681	1669	2133
TTGGTTCGTCGTTTTGAC	JTTTTGTCGTTTTCGTCGTTT	GTCGTTTTGTCGTTTCGTCG 1
	JL	
2102		2143
TTTTGTCGTTTTGTCGTT	CCATGACGTTCCTGACGTTAC	CGATAACGTTGCCGGTGACG 1
الـــــــــــــــــــــــــــــــــــــ	ال	
2006	1826	1670
2006 TCCATGACGTTCCTGATG	1826 CTTCCATGACGTCCCTGATGC	1670 CTCGAG 226
2006 TCCATGACGTTCCTGATG	I826 TTCCATGACGTCCCTGATGC?	1670 CCTCGAG 226

Fig. 1. Description of tandem ODNs sequence. Ten ODNs were designed to connect one another in series to form a CpG-rich fragment (226 bp) containing 18 copies of tandem CpG motifs, shown by gray boxes. 'GACGTT', 'GTCGTT' motifs are optimal in mice and human, respectively [20]. 'TCGTCGTT' motif is found to enhance the immune effects in primates [3]. The above ODNs containing 'GACGTT', 'GTCGTT', 'AACGTT' or 'GACGTC' motifs have immunostimulatory effects in fish [10–15].

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