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# Identification and analysis of a CpG motif that protects turbot (*Scophthalmus maximus*) against bacterial challenge and enhances vaccine-induced specific immunity

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ABSTRACT

Oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs in certain contexts are known to be immunostimulatory in vertebrate systems. CpG ODNs with immune effects have been identified for many fish species but, to our knowledge, not for turbot. In this study, a turbot-effective CpG ODN, ODN 205, was identified and a plasmid, pCN5, was constructed which contains the CpG motif of ODN 205. When administered into turbot via intraperitoneal (i.p.) injection, both ODN 205 and pCN5 could (i) inhibit bacterial dissemination in blood in dose and time dependent manners, and (ii) protect against lethal bacterial challenge. Immunological analyses showed that in vitro treatment with ODN 205 stimulated peripheral blood leukocyte proliferation, while i.p. injection with ODN 205 enhanced the respiratory burst activity, chemiluminescence response, and acid phosphatase activity of turbot head kidney macrophages. pCN5 treatment-induced immune responses similar to those induced by ODN 205 treatment except that pCN5 could also enhance serum bactericidal activity in a calcium-independent manner. To examine whether ODN 205 and pCN5 had any effect on specific immunity, ODN 205 and pCN5 were co-administered into turbot with a *Vibrio harveyi* subunit vaccine, DegQ. The results showed that pCN5, but not ODN 205, significantly increased the immunoprotective efficacy of DegQ and enhanced the production of specific serum antibodies in the vaccinated fish. Further analysis indicated that vaccination with DegQ in the presence of pCN5 upregulated the expression of the genes encoding MHC class IIα, IgM, Mx, and IL-8 receptor. Taken together, these results demonstrate that ODN 205 and pCN5 can stimulate the immune system of turbot and induce protection against bacterial challenge. In addition, pCN5 also possesses adjuvant property and can potentiate vaccine-induced specific immunity.



## 1. Introduction

It is known that bacterial DNA can stimulate the immune systems of vertebrate, partly due to the presence in the former of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides within certain base contexts [1–3]. In bacterial and viral genomes, the CpG motifs are prevalent and unmethylated, whereas in vertebrate the CpG motifs are suppressed, methylated, and flanked by bases with features that are different from those in bacterial DNA [4–6]. Hence, these CpG motifs can serve as pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) of vertebrate immune system. Numerous studies have shown that synthetic oligodeoxynucleotides (ODNs) containing CpG motifs (CpG ODNs) can mimic bacterial CpG dinucleotides and produce various immune effects, including activation of natural killer (NK) cells and monocytes/macrophages [7–9], stimulation of lymphocyte proliferation [2,10], and enhancement of antigen presentation [11]. The immune response induced by CpG is mediated through the Toll-like receptor 9 (TLR9), a PRR expressed on cells such as B cells, dendritic cells, and macrophages [12–14]. Synthetic CpG ODNs of different forms differ in immunological property [15]. For example, the A-class ODNs, which are constructed on phosphodiester backbones, promote the activation of NK cells but not B cells, whereas B-class ODNs, which are constructed on phosphorothioate backbones, are effective at B cell activation [6,16]. In addition to the effects on innate immune response, some CpG ODNs also possess adjuvant property and can modify specific immune responses induced by antigens during vaccination [17,18].

The effectiveness of CpG ODNs as immunostimulants depends on the sequence and structure characteristics of the ODNs and on the animal species in which the CpG ODNs are tested. It is observed that species specificity exists where recognition of and

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response to CpG ODNs are concerned [19]. To date CpG ODNs with immunopotentiating properties have been identified for a number of fish species, including rainbow trout (*Oncorhynchus mykiss*) [20–24], Atlantic salmon (*Salmo salar* L.) [25], common carp (*Cyprinus carpio* L.) [26–29], olive flounder (*Paralichthys olivaceus*) [30,31], grass carp (*Ctenopharyngodon idellus*) [32], chinook salmon (*Oncorhynchus tshawytscha*) [33], and catfish (*Ictalurus punctatus*) [34]. However, it is largely unclear whether the immune effects of these CpG ODNs are species-specific. The immune responses induced by fish CpG ODNs are varied, ranging from stimulating macrophage activation and leucocyte proliferation to enhancing bactericidal activity and cytokine secretion [16]. Some of the fish CpG ODNs are known to be immunoprotective and possess adjuvant properties [16].

Turbot is an economically valued fish species that has been cultured extensively in Europe, South America, and Asia. In recent years, disease outbreaks caused by microbial pathogens such as *Edwardsiella tarda* and *Vibrio* [35] have become a severe problem faced by turbot industries worldwide. Therefore, the need for the development of effective means of disease control is urgent. To our knowledge, no turbot-effective CpG ODNs have been reported. The aim of this study was to identify CpG ODNs that can elicit immune responses in turbot to the effect of inhibiting bacterial infection and potentiating vaccine-induced specific immunity.

## 2. Materials and methods

### 2.1. Bacterial strain and growth conditions

*E. tarda* TX1 and *V. harveyi* T4 are fish pathogens that have been reported previously [36,37]. *Escherichia coli* DH5α was purchased from Takara (Dalian, China). All strains were cultured in Luria–Bertani broth (LB) medium [38] at 37 °C (for *E. coli*) or 28 °C (for TX1 and T4).

### 2.2. CpG ODNs

Ten of the CpG ODNs used in this study have been reported previously: 2133 [21], 2143 [21], 1668 [23], A [26], B [26], 1826 [32,33], 2006 [32], 1670 [21,25], 1677 [25], and 1679 [25]. The sequences and immunological properties of these CpG ODNs are summarized in the review by Carrington and Secombes [16]. In addition to the above CpG ODNs, ODN 203 (5′-GATCTCGCTCGCTCGCCTAT-3′; underlined, CpG motif), ODN 205 (5′-GATCGCGTGCGTGCGTCTAT-3′; underlined, CpG motif), and ODN 205 M (fx1; nucleotides that differ from ODN 205 are boxed), a derivative of ODN 205 in which the CpG motif is destroyed by site-directed mutagenesis, had also be used in this study. All the ODNs were constructed on a phosphorothioate backbone and synthesized by Sangon (Shanghai, China). The synthesized ODNs were solubilized in sterile deionized water and stored at −20 °C.

### 2.3. Fish

Turbot (*Scophthalmus maximus*) were purchased from a local fish farm and acclimatized in the laboratory for 2 weeks before experimental manipulation. Fish were maintained at ∼19 °C in aerated seawater and fed daily with commercial dry pellets. Before experiments, fish were randomly sampled for the examination of bacterial recovery from blood, liver, kidney, and spleen. Fish were considered healthy only when no bacteria could be detected from any of the examined tissues.

**Table 1**
Primers used in this study.

| Primers | Sequences (5′ → 3′) | Target gene |
|---|---|---|
| EFurF20 | GCAAGATCCTACGTGCCAT | TX1 *fur* |
| EFurR113 | GTCCAGGCAGATCAGGT | |
| VFurF3 | TACTCAACCAATTCGATGAT | T4 *fur* |
| VFurR6 | GTACTTAGCGGCGATTTCTT | |
| ActF | TGAACCCCAAAGCCAACAGG | β-Actin |
| ActR | AGAGGCATACAGGGACAGCAC | |
| C3F | GGTACAACTTCAACAACAACAACAA | C3 |
| C3R | AGCGTAGTACAGCGACACCATT | |
| IgMF | GCAGCAAAACTGTGACTCTAAATG | IgM |
| IgMR | CAGTAGTCAAAGATCCACCCCAT | |
| IL1F2 | AGGTGGAGGACAAAAGCAGTCT | IL-1β |
| IL1R2 | TGATGTACCAGTTGGGGAAGC | |
| MHCIF | CAAAGTCAACATTGGAACCCTAA | MHC Iα |
| MHCIR | CCCATTCACAGCCGTACATCA | |
| MHCIIF | GTCTCAACATTCCCTATCCCAACA | MHC IIα |
| MHCIIR | GCTCCTCCACATCCCAGATTC | |
| MxF | GGCATCACTAGGGTGGCTGTA | Mx |
| MxR | CCAGGCTGATAGTTTCTTGCTTT | |
| NKEFF2 | TACCATGAAGATTCCCCTTGTG | NKEF |
| NKEFR2 | CAATGCCATCGTCCTCCTTT | |
| TCRF | GTGGAGCAAAACCAAATCAACA | TCR-α |
| TCRR | CCGGCTTCACAGCACAGTAGTA | |
| TLR3F | GCCATTTATGGAAGCAGGAAG | TLR3 |
| TLR3R | CCAGAAAGACCAGGATCAGCAC | |
| TNFαF | TGAGGCAAATCAGCAGCAAT | TNF-α |
| TNFαR | GCCTTGACCGTTCTTCCACT | |
| IL8RF | GGCTCAGCAAAGACTCGCA | IL-8R |
| IL8RR | CCCGTTGATGACAAACCTCC | |

### 2.4. Bacterial recovery from fish blood

Bacterial recovery from fish blood was performed as described previously [36]. Briefly, fish were sacrificed with an overdose of tricaine methanesulfonate (Sigma, St. Louis, MO, USA), and blood was taken under aseptic conditions. The blood was plated on LB agar plates, and the plates were incubated at 28 °C for 48 h. The colonies emerged on the plates were enumerated. For *E. tarda* TX1 recovery, the genetic nature of the colonies was verified by PCR analysis using primers EFurF20 and EFurR113 (Table 1), which are specific to the *fur* gene of TX1. For *V. harveyi* T4 recovery, the genetic nature of the colonies was verified by PCR analysis using primers VFurF3 and VFurR6 (Table 1), which are specific to the *fur* gene of T4. PCR products were randomly selected and verified by DNA sequencing.

### 2.5. Effect of CpG ODNs on bacterial infection

Turbot (∼7 g) were divided randomly into 13 groups and administered intraperitoneally (i.p.) with each of the 12 CpG ODNs (2133, 2143, 1668, A, B, 1826, 2006, 1670, 1677, 1679, 203, and 205) at 0.2 μg/fish or phosphate buffered saline (PBS) as a control. At 48 h post-administration, the fish were challenged with *E. tarda* TX1. Bacterial recovery from blood was determined at 12 h post-challenge as described above. The effect of ODN 205 M was similarly examined.

### 2.6. Analysis of the antibacterial effect of CpG ODN 205 in relation to dose and time

To examine the effect of dose on the antibacterial property of ODN 205, turbot (∼7 g) were divided randomly into four groups and administered i.p. with PBS (control) or ODN 205 at the concentrations of 0.008 μg, 0.04 μg, and 0.2 μg per fish, respectively. The fish were challenged with TX1 and examined for bacterial recovery as above. To examine the effect of time on the antibacterial property of ODN 205, turbot (∼7 g) were divided randomly into six groups; three groups (A, B, and C) were injected i.p. with PBS (con-