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CONSTRUCTION OF *ESCHERICHIA COLI* STRAIN PRODUCING CPG-ENRICHED RECOMBINANT PLASMID

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Introduction. Unmethylated CpG dinucleotides in particular sequence contexts (CpG motifs), which are present in bacterial (but not mammalian) DNA stimulate innate host defense mechanisms by activating Toll-like receptor 9 (TLR9) signaling pathways [1, 2]. Synthetic oligodeoxynucleotides (ODNs) containing CpG motifs have been shown to act as potent adjuvants that can induce T helper-1 (Th-1)-type immune responses by mimicking the effects of bacterial CpG DNA [3–5]. Synthetic CpG ODNs can improve immune responses to a variety of coadministered antigens, and therefore represent a promising new approach for improving vaccine efficacy [6, 7]. Recently, synthetic CpG ODNs have been shown to enhance the immunogenicity of various microbial and tumor antigens in humans [8].

ODNs with natural phosphodiester bonds (PO-ODNs) are susceptible to nuclease degradation in cells [9]. Therefore, in order to provide resistance to nuclease activity and ensure efficient uptake of CpG ODNs by cells, phosphorothioate backbone-modified CpG ODNs containing sulfur substituted for the non-bridging oxygen atoms (PS-ODNs) were developed [10]. However, several laboratories have shown that severe side effects occurred in PS-ODNs-treated mice [11]. Therefore, production of a potent immunostimulatory CpG PO-ODN that does not cause side effects is highly desirable for inducing a well-controlled immune response.

Another way of CpG DNA production is construction of CpG-enriched plasmids with subsequent cloning in *Escherichia coli* cells. It is known that CpG motifs present in the backbone of DNA vaccines play an important role in the development of immune responses, and increasing the number of the motifs can enhance immune responses to various antigens [12, 13]. Compared with synthetic ODNs, plasmids have more stable chemical properties and are economical in scaled-up production. Moreover, such CpG-containing plasmids are non-toxic for organisms as they lack unnatural phosphorothioate linkages. Hence, CpG-enriched plasmids may prove to be more useful than protein or peptide vaccine adjuvants.

By examining many possible base combinations, optimal CpG motifs for different species were defined. For activating human cells, the optimal motif is GTCGTT. It's also appears to be efficient in many other vertebrate species, including cow, sheep, cat, dog, goat, horse, pig, and chicken [8].

According to published data increasing CpG-motifs copy number enhance biological activity of CpG-DNA [13, 14]

Taking all aspects into consideration, the aim of the research was construction of *E. coli* strain producing recombinant plasmids enriched with multi-copy GTCGTT CpG motif.

Materials and methods. To construct multi-copy CpG fragments, two partially overlapping primers containing four CpG motifs were chemically synthesized (CpG-F and CpG-R). *Hind*III restriction sites were inserted at 5′ ends of the primers (the n*ucleobases* are in italic).

Primers for cloning of **GTCGTT**-motif:

CpG-F –

5′-*AGCTTC***GTCGTT**TT**GTCGTT**TT**GTCGTT**TT**GTCGTT***A*-3′**;**

CpG-R –

5′-*AGCTT***AACGAC**AA**AACGAC**AA**AACGAC**AA**AACGAC**G*A*-3′.

To anneal the primers 50 μL of the reaction mixture containing 500 pmol of each primer, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$ and 50 mM NaCl was heated at 95°C and slowly cooled down to 4°C. The products obtained were double stranded DNA molecules with sticky 3′ and 5′ complementary ends. After phosphorylation of 5′ ends with T4 polynucleotide kinase (Sileks, Russia) the products were ligated to each other. The fragments were separated using agarose gel electrophoresis and the ones with the highest size (900–1300 base pairs) where isolated and purified with MinElute Gel Extraction Kit (Qiagen, Germany) according to manufacturer recommendations. A-tailing of the purified CpG-containing fragments was performed by treating products with *Taq* DNA polymerase (Promega, USA) and mixture of deoxynucleoside triphosphates.

Purified double-stranded CpG polynucleotides were ligated into the T-vector prepared by digestion of plasmid pXcmkn12 (Cloning Vector Collection, Japan) with *Xcm*I restriction enzyme. A 10 μL aliquot of the ligation mixture was used to transform *E. coli* DH5α competent cells (Novagen, USA). Cells where grown on LB agar plates [15] with 100 μg/mL ampicillin.

Several obtained colonies were screened by polymerase chain reaction (PCR) for plasmid pXcmkn 12 enriched with CpG motifs using primers terminating the cloned fragments. Amplification by PCR was performed in a reaction mixture (30 μL) containing crude cell lysate as a DNA template, 5 pmol of each synthetic primer (5′-GTAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′), four deoxyribonucleoside triphosphates (0.2 mM each), $MgCl_2$ (2 мМ), $(NH_4)_2SO_4$ (17 mM), 67 mM Tris-HCl buffer (pH 8.3), 0.02% Tween 20 and 1 U of *Taq* DNA polymerase. The reaction was conducted for 30 cycles (30 s at 95°C, 30 s at 55°C and 1 min at 72°C). Amplification products were analyzed using agarose gel electrophoresis.

Plasmid with the longest inserted fragment (designated pCpG-KH11) was chosen for subsequent manipulations. *E. coli* DH5α cells containing target plasmid were grown at 37°C with orbital shaking at 200 rpm in 250 mL Erlenmeyer flasks with 50 mL of LB culture medium [15]. After 16 h of growth cells were harvested by centrifugation for 10 min at 12,000 g, washed once with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and slurried in the same buffer. Plasmids were isolated from obtained cell biomass using alkaline lysis method [15].

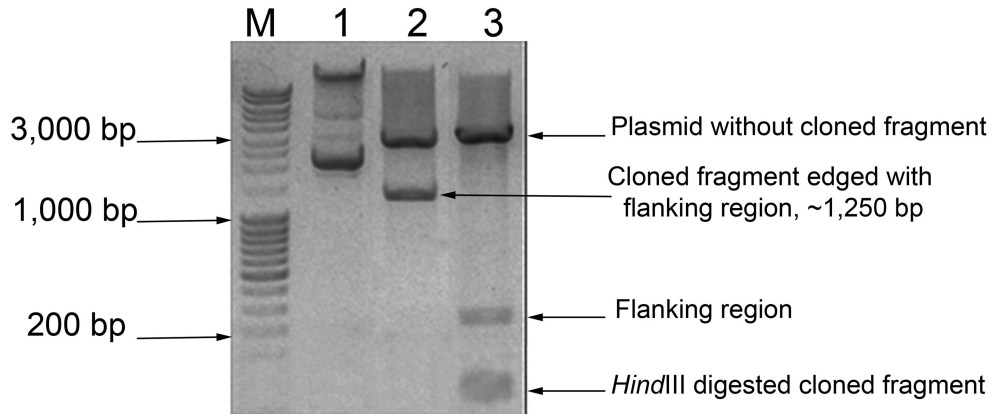
Restriction analysis of constructed plasmid was performed by its incubation with mixture of restriction nucleases *Nde*I and *EcoR*I. The presence of multi-copy CpG motifs in cloned fragment was affirmed by incubation of restriction products with *Hind*III restrictase.

The obtained plasmid pCpG-KH11 was introduced into *E. coli* strain BLR(DE3). The transformed cells were grown at 37°C with orbital shaking at 200 rpm in 250 mL Erlenmeyer flasks containing 50 mL of culture medium containing: 0,5% glycerol, 1 мМ $MgCl_2$, 1 мМ $MgSO_4$, 50 мМ Na_2HPO_4, 50 мМ KH_2PO_4, 25 мМ $(NH_4)_2SO_4$, 0,05% isoleucine, ampicillin (150 mg/L). Cultivation continued during 14–16 h. The obtained biomass was used for plasmid isolation by alkaline lysis method.

Results and discussion. Many new vaccines under development consist of rationally designed recombinant proteins that are relatively poor immunogens unless combined with potent adjuvants. There is only one adjuvant in common use in the U. S., aluminum phosphate or hydroxide (e. g. alum). This adjuvant, however, has significant limitations, particularly regarding generation of strong cell-mediated (T-cell) immune responses. In recent years, a novel adjuvant, JVRS-100, composed of cationic liposome DNA complexes (CLDC) has been evaluated for immune enhancing activity. The JVRS-100 adjuvant has been shown to elicit robust immune responses compared to CpG ODN and alum adjuvants, and it efficiently enhances both humoral and cellular immune responses [15]. Z. Quan et al. [17] and X. Guo et al. [18] demonstrated that plasmids containing CpG motifs could function as immune potentiators to porcine reproductive and respiratory syndrome killed virus vaccines. In view of these scientific data, the aim of the work was preparation of recombinant CpG-enriched plasmids for further priority use as vaccine adjuvants.

Our work resulted in construction of plasmid containing multi-copy GTCGTT CpG motif. The cloned motif is known to be the most effective in stimulation of the human immune system [8].

Characterization of constructed plasmid was performed by restriction analysis. Purified plasmid was incubated with mixture of restriction endonucleases *Nde*I and *EcoR*I which recognition sites terminated



Electrophoregram of restriction analysis of constructed plasmid. Line 1 is DNA molecular size markers; line 2 is purified plasmid pCpG-KH11; line 3 is pCpG-KH11 digestion products after treatment with *Nde*I and *Eco*RI; line 4 is pCpG-KH11 digestion products after treatment with *Nde*I, *Eco*RI and *Hind*III

the cloned fragment. The length of obtained fragment according to electrophoregram (Figure) was about 1,250 base pairs.

Multi-copy CpG fragment consists of tandem repetitions containing 4 CpG-motifs separated by *Hind*III restriction sites. To confirm structure of cloned fragment digestion products were additionally incubated with *Hind*III restrictase. Electrophoregram of pCpG-KH11 restriction analysis is shown in Figure.

The number of cloned CpG motifs was calculated by the following formula:

$$X = \frac{D - 286}{37} \cdot 4,$$

where D – length of the cloned fragment identified after *Nde*I and *Eco*RI digestion (1,250 bp); 286 – length of flanking region; 37 – length of 1 tandem repetition of CpG motifs (bp); 4 – number of CpG motifs in 1 tandem repetition.

By our estimates, the plasmid pCpG-KH11 carries 104 copies of GTCGTT motif, which is the greatest amount among the well-known analogs [19–21].

The resultant recombinant plasmid pCpG-KH11 was introduced into *E. coli* strain BLR(DE3). The transformed cells were grown at 37°C with orbital shaking at 200 rpm in 250 mL Erlenmeyer flasks containing 50 mL of previously described culture medium. The recovered cell biomass was used for isolation and purification of target plasmid. It was shown that the plasmid was produced at a relatively high level (about 7.7 mg/L), so that the constructed recombinant strains can be successfully used for biotechnological production of CpG-enriched plasmids.

Conclusions. The completed work resulted in construction of the plasmid pCpG-KH11 carrying the greatest amount (104 repetitions) of GTCGTT CpG motif among the well-known analogs. The cloned motif is considered to be the most effective in stimulation of the human immune system, according to literature data. Using methods of genetic engineering, strain *E. coli* CpG-KH11 producing plasmid pCpG-KH-11 has been obtained. The strain is potent source of CpG-enriched plasmid and it makes it possible to produce about 7.7 mg of target plasmid DNA per 1 L of culture medium.

It is suggested that the constructed strains may be used in biotechnological production of pharmaceutically valuable CpG DNA.

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Summary

As a result of current work the plasmid pCpG-KH11 carrying 104 copies of CpG motif GTCGTT has been constructed. The cloned motif is considered to be the most effective in stimulation of the human immune system. The constructed plasmid carries the greatest amount of CpG-motifs among the well-known analogs. Using methods of genetic engineering *E. coli* CpG-KH11 strain has been obtained. The strain is potent in pCpG-KH11 production and makes it possible to produce about 7.7 mg of target plasmid DNA per 1 L of culture broth.