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PCR-MEDIATED CLONING AND EXPRESSION OF THE GENE FOR THE B-SUBUNIT OF *VIBRIO* *CHOLERA*E TOXIN ISOLATED RECENTLY IN IRAN

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ABSTRACT

Knowing the nucleotide sequence of the cholera toxin operon, we designed oligonucleotide primers for its PCR amplification from local clinical isolates of *V. cholerae*. The resulting amplification product was cloned in a common pUC18 vector. Subsequently, a part of this operon encoding the cholera toxin B-subunit (CTB) was reamplified and cloned between the *Bam*HI and *Eco*R1 sites of the same vector to create a recombinant plasmid pR18CTB. Temperature-controlled expression of the target protein was achieved by supplementing pR18CTB with a DNA fragment which contained a strong promoter *PR* and the gene for a heat-sensitive repressor *cl857* of bacteriophage lambda from an expression vector pCQV2. When induced, the constructed plasmid pSCTB18 provided for the production of recombinant CTB secreted into the periplasmic space in a yield of about 3mg per liter of bacterial culture, as revealed by GM1-ELISA.

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INTRODUCTION

Cholera remains a health threat in much of the developing world. It has been estimated that more than 150,000 people, both children and adults, die from cholera each year.¹⁻³

The pathogenicity of *V. cholerae* is largely due to the secretion of a toxic protein molecule. The enterotoxin of *V. cholerae* is the prototype of a group of bacterial exoproteins that are capable of stimulating adenylate cyclase and increasing intracellular levels of cyclic AMP in eukaryotic

cells.⁴⁻⁸ In amino acid sequence, the cholera toxin (CT) is 80% homologous to the heat-labile toxin (LT) produced by enterotoxigenic *E. coli* strains causing traveller's diarrhea.⁷

CT and LT are hexameric proteins with an AB₅ structure. The central A subunit (27 kDa) responsible for the toxic activity consists of two polypeptide chains (22 kDa and 5 kDa) linked by a single disulfide bridge. CTB is a pentamer of five identical beta-chains (11.7 kDa) arranged in a ring-like configuration.⁹ CTB is responsible for the binding of the toxin to its receptor, GM1 ganglioside.^{7,9,10}

B-Subunit Gene Expression of *V. cholerae* Toxin

The cholera toxin (*ctx*) genes are arranged in an operon within a larger cholera toxin (*CTX*) genetic element, which is composed of a core (containing *ctxAB* [5], the toxin encoding genes *zot* [11] and *ace* [12], and a colonization factor gene, *cep* [13]) as well as two or more flanking copies of a directly repeated sequence, RS1, which contain at their termini a 17-bp end-repeat sequence.

CTB is a safe and effective oral immunizing agent which, in a large field trial, has been shown to afford protection against both cholera and diarrhea caused by enterotoxigenic *E. coli*.¹⁴⁻¹⁶ Moreover, CTB is a good carrier protein and an effective adjuvant which can stimulate the important SIgA production when administered with other antigens.¹⁷⁻²² It can be also used as a receptor blocking and receptor modulating agent for short-term prophylaxis of cholera and *E. coli* diarrhea.^{23,26} At present major difficulties in large scale production of CTB are: 1) risks associated with work with pathogenic *V. cholerae*, and 2) complexity and high expense of the purification procedure. Because of this, the production of arCTB is a topical problem of modern biotechnology.

In this paper we report the construction of a recombinant plasmid pSCTB18 which provides for temperature-controlled production of rCTB in *E. coli* cells.

MATERIALS AND METHODS

Bacterial strains, plasmids and DNA manipulations

E. coli strains XL1-blue and JM105 used as the hosts were cultured in LB medium supplemented with 100 µg/mL ampicillin to propagate plasmids and stored at -70°C in the same medium containing 15% (v/v) glycerol.

A common pUC18 vector was used for initial cloning of the CT- and CTB-encoding sequences.²⁴ A pCQV2 expression vector was used as a source of the 1.4 - kilobase pairs fragment containing a strong promoter *PR* and the gene for a heat-sensitive repressor *ci857* of bacteriophage lambda.²⁸ Bacterial transformations and plasmid DNA purification were performed as described by Sambrook et al.²⁹ Restriction endonucleases, T4 DNA ligase and *Taq* DNA polymerase were purchased from Gibco-BRL. The enzymes were used under conditions recommended by a supplier. Ganglioside GM1, the reference CTB and other chemicals were obtained from Sigma.

Chromosomal DNA was isolated from a recent local clinical isolate of *Vibrio cholerae* O1 (*E1 Tor* biotype) by the miniprep method of Owen et al.³⁰ with minor modifications.

Polymerase chain reaction (PCR) was carried out in a Pharmacia LKB-Gene ATAQ controller under standard buffer conditions using oligonucleotide primers prepared on an Applied Biosystems DNA synthesizer model 360B. Amplification products were analyzed by agarose gel electrophoresis, and the wanted DNA fragments were isolated

ISOLATED CTB	ATGATTAAAT	TAAAATTTGG	TGTTTTTTTT ³⁰
CTB OF 569B	ACAGTTTTAC	TATCTTCAGC	ATATGCACAT ⁶⁰
	GGAAACACCTC	AAAATATTAC	TGATTTGTGT ⁹⁰
	GCA GAATCAC	ACAACACACA	AATATATACG ¹²⁰
	CTAAATGATA	AGATATTTTC	GTATACAGAA ¹⁵⁰
	TCTCTAGCTG	GAAAAAGAGA	GATGGCTATC ¹⁸⁰
	ATTACTTTTA	AGAATGGTGC	AATTTTTC ²¹⁰
	GTAGAAGTAC	CAAGTAGTCA	ACATATAGAT ²⁴⁰
	TCACAAAAAA	AAGCGATTGA	AAGGATGAAG ²⁷⁰
	GATACCCTGA	GGATTGCATA	TCTTACTGAA ³⁰⁰
	GCTAAAGTCG	AAAAGTTATG	TCTATGGAAT ³³⁰
	AATAAAACGC	CTCATGCGAT	TGCCGCAATT ³⁶⁰
	AGTATGGCAA	AT ³⁷²	

Fig. 1. Nucleotide sequence of the CTB gene in plasmid pSCTB18. The antisense strand is shown from 5' to 3'. For the mature B subunit sequence, differences from the published nucleotide sequence of B subunit from strain 569B are shown beneath the nucleotide sequence. The signal sequences are underlined.

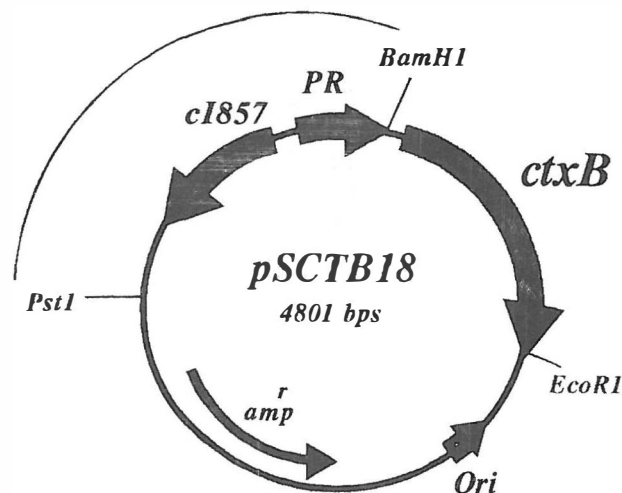


Fig. 2. Map of pSCTB18.

from the low gel temperature agarose (Sigma, type VII).

DNA sequencing was performed by the dideoxy chain termination technique of Sanger et al.³¹

Double diffusion in gel analysis by Ouchterlony method was performed using a polyclonal immune serum raised against standard CTB.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analyses were carried out as described by Sambrook et al.²⁹ Subsequent to electrophoretic separation in a 15% SDS

MAb D15-8

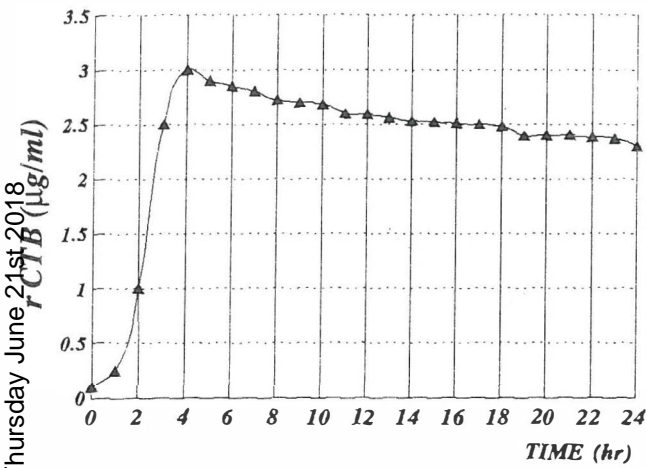


Fig. 3. Dependence of accumulated rCTB on the induction time. Recombinant CTB was measured with GM1-ELISA calibrated against standard CTB from *V. cholerae* 569B.

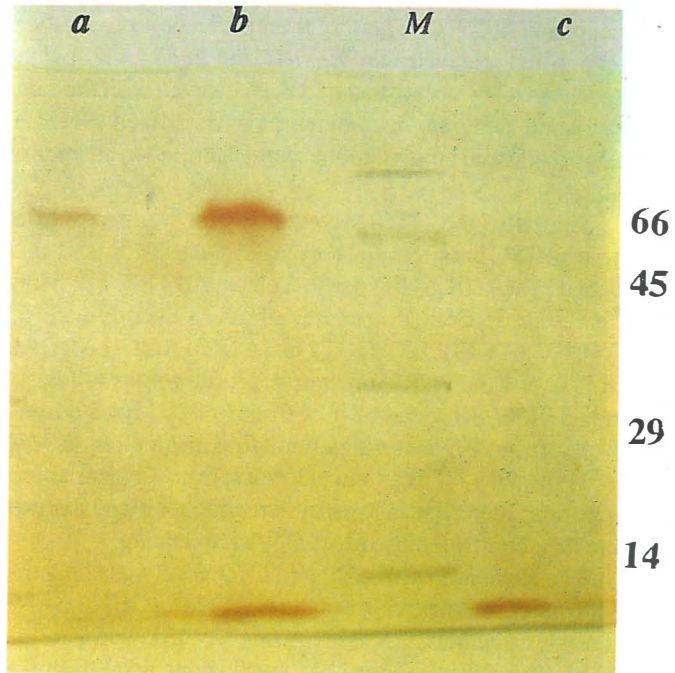
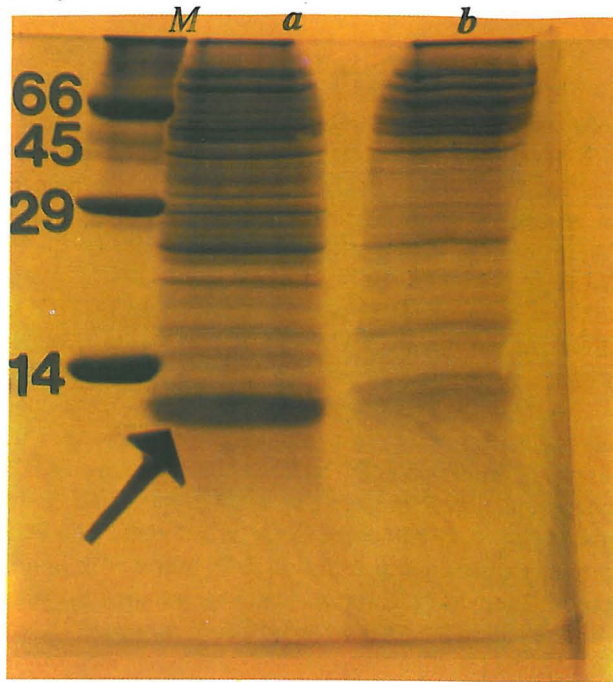


Fig. 4. SDS-PAGE and immunoblot analyses of partially purified rCTB. Panel A) Gel stained with Coomassie brilliant blue. Extracts from *E. coli* cells containing pSCTB18 after (lane a) and before (lane b) the heat-shock induction. M: molecular weight markers, lysozyme (14 KDa), carbonic anhydrase (29 KDa), ovalbumin (45 KDa), bovine albumin (66 KDa). Arrow points to the band corresponding to monomer of rCTB. Panel B) Western blot analysis of rCTB. lane a: reference CTB without boiling. rCTB without (lane b), and with (lane c) boiling, the upper and lower bands correspond to the pentameric and monomeric forms of CTB, respectively. M; markers are the same as in panel a.

Laemmli, proteins were transferred to a nitrocellulose BA85 (Schleicher & Schuell) using the semi-dry system (Novablot transfer, Pharmacia) during 2h at 10V. The membrane was blocked with 1.5% BSA for 1h. After washing, the primary anti-CTB antibody was added and incubated for 3 h at 37°C. After 5-6 washes with PBS/Tween 20 the antimouse-IgG Fc-specific HRP-conjugated antibody (Sigma) was added and incubated for 2 h at 37°C. After washing, the 3,3'-diamino-benzidine (DAB) substrate was added and stained protein bands were visualized in 10-15 min.

Recombinant CTB was detected and measured by the GM1-ganglioside enzyme-linked immunosorbent assay (GM1-ELISA)³²⁻³⁵ using an anti-CTB monoclonal antibody (kindly provided from the Pasteur Institute, Paris). The microtiter plate wells were coated with 5 µg of ganglioside GM1 type III in 100 µL of 0.05 mol/L carbonate buffer, pH=9.6 for 6 h at room temperature and kept at 4°C for later use. Coated plates were first washed with three changes of PBS, and blocked with 1% BSA (W/V) in PBS for 1h at 37°C. One hundred µL of serial sample dilutions per well was added and incubated at 37°C for 1 h. Then plates were washed four times with PBS containing 0.05% Tween 20, and 100 µL of anti-CTB MAb per well were added. The plates were incubated for 1.5 h at 37°C, and after extensive washing with PBS/Tween 20 the goat-antimouse IgG Fc-specific peroxidase conjugate (Sigma) was added into each well. The plates were incubated for 1.5 h at 37°C, washed, and a chromogenic substrate, orthophenylenediamine (OPD) in citrate buffer was added. The absorption at a wave length 492 nm (A492) was measured on an ELISA reader.

RESULTS AND DISCUSSION

Construction of a CTB-expressing plasmid

Knowing the nucleotide sequence of the cholera toxin gene *CtxAb*,^{36,37} we designed a pair of oligonucleotide primers that annealed to opposite ends of the CT-coding region. The 5'-terminal (forward) primer was 5'-CACGGATCCTGTTGGTGATGAGCGTTATCGC and the 3'-terminal (reverse) primer was 5'-CACGAATTCTGCCGCGCCACATAATACGCACT with the first 9 nucleotides in each case being extrinsic to the target sequence and creating the underlined unique *Bam*H1 and *Eco*R1 restriction endonuclease sites, for ease of the following cloning. With this pair of primers, a 1685-bp fragment containing the CT-coding region was prepared by a 35-cycle amplification from 0.1 µg of *V. cholerae* DNA as template. The PCR temperature profile was as follows: 1.5 min at 94°C, 1 min at 65°C, 2 min at 72°C for the first 5 cycles; then 30 cycles with alternating incubations at 94°C for 1 min and 72°C for the first 5 cycles; then 30 cycles with alternating incubations at 94°C for 1 min and 72°C for 3 min, ending with one 5-min incubation at 72°C. The target amplification product was gel purified, cleaved with *Bam*H1 and *Eco*R1, and ligated into the pUC18 vector that had been cleaved with the same restriction enzymes to yield a plasmid named pR18CT. Correct plasmid constructions were identified by restriction analysis.

Similarly, the CTB-encoding region was reamplified from pR18CT as template by a 23-cycle PCR and then cloned in pUC18 giving rise to a plasmid pR18CTB. In this case the forward primer had a sequence 5'-CACGGATCCTAGAGCTTGGAGGGAGGGAAGAGCCGT, and the PCR temperature profile was as follows: 1 min at 94°C, 30 sec at 65°C, 30 sec at 72°C for the first 3 cycles; then 20 cycles with alternating incubations at 94°C for 1 min and 72°C for 1 min. Correct plasmid constructions were first identified by restriction endonuclease analysis, and then confirmed by direct DNA sequencing.

The sequence of the isolated CTB gene was compared with the reported sequence of the CTB^{7,16,36-9} (GENE BANK accession number: X00171), using DNASIS software and the result is given in Fig. 1. As can be concluded from Fig. 1, the CTB coding sequences for both the classical and *E1 Tor* biotypes are almost identical. Some minor differences can be noticed in CTB genes between biotypes. Three mutations have occurred during evolution, in such a way that nucleotide 115 and 203 change in a T↔C and nucleotide 223 change in a A↔G fashion. These variations are not silent, so that amino acids 18, 47 and 54 of the CTB chain differ: the sequence of the isolated *E1 Tor* CTB chain possesses respectively Tyr, Ile and Ser residues, whereas the 569B strain contains His, Thr and Gly at the corresponding positions.

Dams et al.³⁹ reported for amino acid 54 a Gly residue,

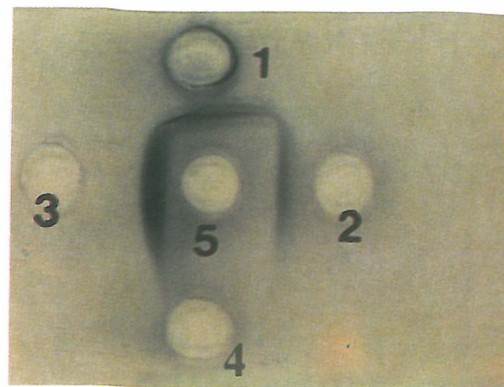


Fig. 5. Ouchterlony immunodiffusion analysis of rCTB. Wells 1) cell extract of XL1-blue containing pSCTB18; 2) standard CT from *V. cholerae* 569B; 3) standard CTB; 4) cell extract of XL1-blue containing pUC18 (negative control); 5) polyclonal anti-CTB serum.

for *E1 Tor* strains 2125 and 62746. Mekalanos et al.³⁶ found for amino acid 54 a Ser residue. Brickman et al. reported a sequence identical to the 2125 sequence published by Mekalanos. The other *E1 Tor* strains have the same sequences as 2125. At present it is reasonable to assume that these amino acids are not directly involved in an important structural or functional role of CTB. No variation of the derived sequence from the one already reported³⁶ was detected. The gene isolated in our study shows more than 97% homology with the reported 569B classical biotype.

For the purpose of expressing the CTB-encoding sequence, a 1472-bp *Pst*I-*Bam*H1 fragment containing a strong promoter *PR* and the gene for a heat-sensitive repressor *C1857* of bacteriophage lambda was recloned from an expression vector pCQV2 to pR18CTB resulting in the target plasmid named pSCTB18 (Fig. 2). The plasmid so produced was tested for ability to provide for a heat-inducible production of rCTB in transformed *E. coli* cells.

Preparation and analyses of rCTB

E. coli XL1-blue containing pSCTB18 was cultured in LB supplemented with ampicillin (100 µg/mL) at 30°C with good aeration to midexponential phase (A600 approximately 0.8). Then bacteria were heat-induced by adding an equal volume of the medium preheated to 65°C and allowed to continue growing at 42°C for 1-24 h. Aliquots were taken at regular intervals to test lysates for rCTB by GM1-ELISA. To do this, cells harvested by centrifugation (5000 rpm, 3 min) were once washed with a cold PBS, resuspended in an equal volume of 50 mM Tris-HCl, pH 7.2/1 mM EDTA/1 mM phenylmethylsulfonyl fluoride and disrupted by sonication with a Branson model W-350 set at maximum output (five 30-sec pulses). After the cell debris was removed by ultracentrifugation (30,000 rpm, 30 min), rCTB was precipitated by adding sodium hexametaphosphate to 2.5 mg per mL and adjusting the pH to 4.5 with concentrated

HCl. The pellet was collected by centrifugation, resuspended in PBS, and dialyzed against the same buffer. Alternatively, cells were subjected to an osmotic shock for extracting only the periplasmic proteins as described elsewhere.

As is seen in Fig. 3, *E. coli* cells harbouring pSCTB18 produce rCTB which can be released by osmotic shock, indicating that it is secreted into the periplasmic space. The maximum accumulation level (about 3 mg per liter of culture) is reached 4-5 h after the heat-shock. It is worthy of note that for a given expression system, the use of an alkaline shift in the pH of the growth medium can result in the production of over 15-fold more protein than when using a temperature shift.⁴⁰

Coomassie-stained SDS-PAGE comparing rCTB with the reference CTB demonstrated that both proteins have the same electrophoretic mobility (Fig. 4A).

The *in vitro* immunological properties of the recombinant and reference CTBs were further compared by means of the immunoblot, double immunodiffusion and GM1-ELISA analyses. Western blotting with MAb D15-8 revealed bands at the positions expected for the pentameric and monomeric forms of the native CTB (Fig. 4B).

The immunoprecipitation bands in the Ouchterlony analysis showed reactions of complete fusion between rCTB, native CTB and CT, with no evidence of spur formation in any direction, indicating that the recombinant protein is immunologically indistinguishable from the native CTB (Fig. 5).

The ability of the rCTB to recognize and react with GM1-ganglioside was compared with native CTB by GM1-ELISA, which revealed an unchanged receptor binding affinity. Clearly, the ability to oligomerize and to bind to GM1-ganglioside are important for this protein to be selected out of the intestinal milieu and to induce an immune response in the Peyer's patches.

The results presented here indicate conclusively that the rCTB secreted by the plasmid-bearing *E. coli* strains is functional, has the correct conformation and can efficiently bind to the GM1-ganglioside receptor. These properties are of fundamental importance for vaccine development. Hence the constructed plasmid pSCTB18 provides the necessary background for both the large-scale production of CTB and genetic fusion of foreign antigens to it for the purpose of developing diverse hybrid vaccines.

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