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Large-Scale Production of *Vibrio cholerae* Toxin B Subunit for Use in Oral Vaccines

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By systematically manipulating promoter and ribosome binding structures, plasmid copy number and the structure of the cholera toxin B (CTB) subunit gene, we were able to develop a plasmid expression system that, when used in conjunction with an optimized growth medium, provided yields of CTB approaching one gram per liter. The CTB protein which was secreted to >95%, could readily be purified from the growth medium of a *V. cholerae* production strain and was shown to be immunologically indistinguishable from previously used vaccine preparations of native or recombinant CTB.

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Infection by *Vibrio cholerae* (serogroup O1) or enterotoxigenic *Escherichia coli* remains an important cause of disease and mortality in many developing countries. In both cases diarrhoea is induced by bacterial toxins released from the pathogenic organisms adhering to and multiplying in the small intestine. The cholera toxin (CT) produced by *V. cholerae* O1 and the heat-labile enterotoxin of *E. coli* (LT) are closely related both structurally and functionally[1,2]. The structure of both CT and LT and their biological activities have been extensively studied. Each consists of a single A subunit (CTA or LTA) responsible for the activation of adenylate cyclase in the intestinal cells of the host, and a pentamer of identical B subunits (CTB or LTB) that binds the holotoxin to its GM1 ganglioside receptor[3].

Protective immunity against cholera can be provided by locally produced intestinal IgA antibodies directed against either bacterial antigens such as the O1 lipopolysaccharide or CT (and then more specifically against the CTB pentamer)[4]. When present simultaneously the antimicrobial and antitoxin antibodies cooperate synergistically in their protective action. Based on this, an oral vaccine has been developed consisting of CTB together with killed whole cells of *Vibrio cholerae* O1[5]. In addition to protecting against cholera[6,7] this vaccine, specifically through its CTB component, has been shown to induce a substantial level of protection against diarrhoea caused by LT producing *E. coli* (ETEC)[8,9]. Based on this finding, a prototype oral vaccine against *E. coli*-induced diarrhoea has recently been developed containing CTB and killed CFA fimbriated *E. coli*[10].

Until recently CTB for vaccine purposes has been prepared by chemical isolation from CT produced in relatively high yield by a particular wild-type strain of *V. cholerae*[11]. However, despite its oligomeric composition, CTB has properties that make it attractive from the point of view of over-expression as a recombinant protein. Firstly, even in the absence of CTA, pentamers that retain the ability to bind GM1 and are non-toxic can assemble from B subunit monomers. Furthermore the assembled CTB pentamers are secreted from *Vibrio* strains in the same way as the holotoxin[11,12].

We have recently described the scaled-up production of recombinant CTB based on an LTB/CTB gene fusion described by Sanchez and Holmgren[13]. Despite being identical to native CTB in terms of immunogenicity and allowing for much easier purification due to the absence of any toxic A subunit, the recombinant CTB product has limitations; yields remain only moderately higher than the optimized production achieved from CT obtained from *V. cholerae* and the product contains either three or four additional amino acids due to a repeated signal peptididase recognition sequence, that results in inconsistent processing.

The present work describes the construction of plasmids that allow the accumulation of a homogeneous recombinant CTB that is essentially identical to the native product, and that is produced at significantly higher yields than previously reported for assembled product in either *E. coli* or *V. cholerae*.

Results

Construction of expression vectors. Briefly, a native *ctx*B gene was cloned into pUC19 (ref. 14) as an XbaI/HindIII fragment taken from pCVD30 (ref. 15). Using the resulting plasmid, pML-CTB1, synthetic oligonucleotides replaced the region encoding CTA2 and the CTB signal peptide with a SacI site at the junction between the signal peptide sequence and the mature protein such that the gene could be fused with the LTB signal peptide sequence using a naturally occuring SacI site at the corresponding position (Fig. 1). This gave a CTB gene that carried alanine rather than threonine at position +1 of the mature protein. The added oligonuculeotide also introduced a 50bp deletion that was subsequently repaired by substitution of an SspI/HindIII fragment with one from an undeleted clone after the modified DNA had been transferred to an expression vector carrying the LTB RBS and signal peptide under the control of the leftwards promoter of phage lambda (λP_L). The final plasmid, pML-LCTBλ2 shown in Figure 1, allowed inducible expression of recombinant CTB in a suitable *E. coli* host strain carrying the temperature-sensitive λcI857 repressor gene such as N4830-1 (ref. 21).

For maximal production of CTB in *V. cholerae*, it was considered that a constitutive expression system was preferable to an inducible one. For this reason the modified *ctx*B gene from pML-LCTBλ2 was retained under the control of the previously used *tac* promoter from plasmid pKK223-3 (ref. 16). This gave pML-LCTB*tac* which was subsequently modified to pML-LCTB*tac*1 by removal of a 1.3kbp BamHI/PvuII fragment from the vector. In both *E. coli* and *V. cholerae* these constructs produced reproducibly high levels of CTB when compared to previously described expression systems. However, to further improve productivity, the copy number of the plasmid was increased. pML-LCTB*tac*1 was digested with EarI and the fragment carrying the origin of replication and the *bla* gene was replaced with the equivalent fragment from pUC19. This resulted in the high copy number plasmid pML-LCTB*tac*2 (Fig. 1).

CTB production from recombinant plasmids: Constitutive expression in *V. cholerae*. Expression in *V. cholerae* results

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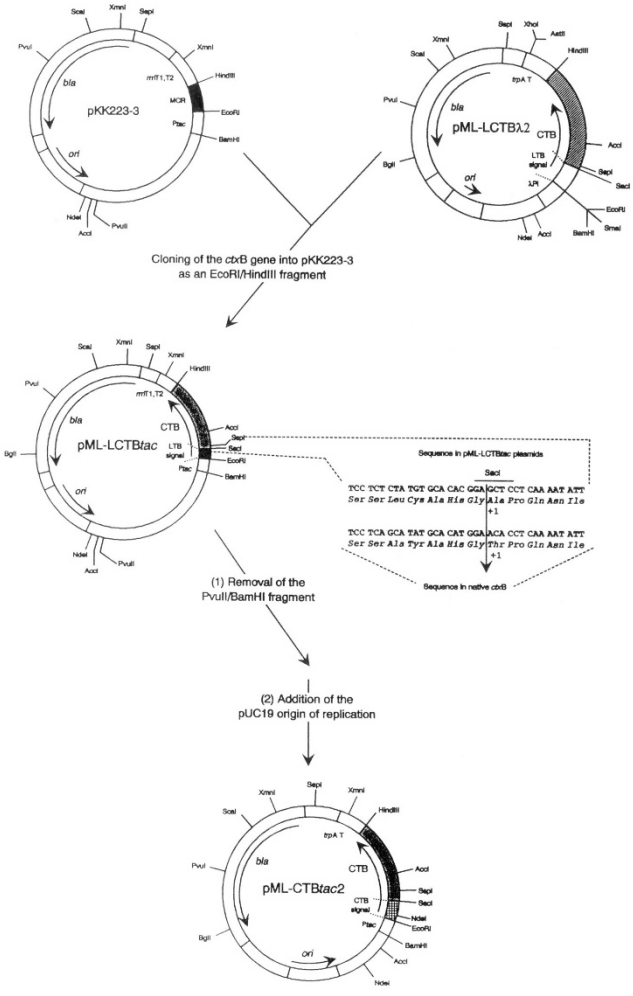

FIGURE 1. Construction of expression vectors for expression of CTB in *Vibrio cholerae*. The modified *ctx*B gene was cloned into pKK223-3 resulting in pML-LCTB*tac*. This was subsequently modified by the removal of a BamHI/PvuII fragment (pML-LCTB*tac*1) and replacement of the origin of replication with that from pUC19 giving pML-LCTB*tac*2 which is identical in size and restriction map with pML-LCTB*tac*1. Sequences of the *ctx*B gene surrounding the signal peptide cleavage site and the modifications effected by insertion of oligonucleotides as shown. The DNA sequence of the gene from the proline codon at position +2 of the mature protein is identical to that given elsewhere[13] as are the LTB RBS and signal peptide sequences upto and including the SacI site. The bold vertical arrow indicates the peptide cleavage site as determined by N-terminal sequencing of purified CTB products. The CTB sequence was altered to change the amino acid at position +1 to Alanine in order to retain a unique SacI site.

in accumulation of CTB in the growth medium. It was found that a modified syncase medium[17] gave the best results in terms of CTB yield and also allowed cultures to reach a high optical density. Typical accumulation patterns for strain JS1569 carrying pML-LCTB*tac* and pML-LCTB*tac*2 grown in 5L fermentors with double concentration syncase medium are shown in Figure 2(A) and 2(B). In cultures carrying pML-LCTB*tac*2 it can be seen that the majority of the product accumulates during late exponential and stationary phases when the sucrose carbon source has been exhausted. Further extension of the fermentation time results in modest increases in the total amount of CTB obtained (~650mg/l after 52h) but for production on a large scale shorter fermentations are normally desirable; after 24 hours the CTB level under the described conditions has reached around 600mg/l. Subsequent modifications to the growth medium (see Experimental Protocol) have increased CTB yields to nearly 1g/l (Fig. 2C) after extended incubation. However, yields in strains carrying pML-LCTB*tac*2 could not be maintained in the absence of selective pressure and ampicillin was required at a concentration of at least 100μg/ml. In contrast, the plasmid pML-LCTB*tac* derived from the expression vector pKK-223-3 (ref. 16) produced 20–40% of the level of CTB obtained from pML-LCTB*tac*2 but retained productivity in the absence of ampicillin (Fig. 2A).

Following growth to stationary phase the cells do not lose their capacity to produce CTB. Cultures have been harvested and resuspended in an equal volume of fresh medium. In the absence of significant growth, CTB can continue to accumulate to concentrations equivalent to those found in the original culture (data not shown).

Characterization of recombinant CTB. *Protein analysis.* The composition of protein products was determined by N-terminal sequencing. This confirmed that the recombinant CTB was consistently processed at the predicted cleavage site giving rise to a product carrying an alanine at position +1 in place of the native threonine (see Fig. 1). Coomassie stained SDS-PAGE gels comparing samples of different purified recombinant CTBs with native CTB isolated from *Vibrio cholerae* 569B[22] demonstrated that the new recombinant protein migrated in exactly the same manner as the native protein whereas the previous heterogeneous preparations gave somewhat diffuse bands (Fig. 4).

In vitro epitope studies. The *in vitro* immunological properties of rCTB obtained from *Vibrio cholerae* carrying pML-LCTB*tac*2 were compared with those of rCTB expressed from the *ctx*B gene of Sanchez and Holmgren and native CTB isolated from *Vibrio cholerae* strain 569B[22] using Ouchterlony immunodiffusion and GM1-ELISA epitope mapping tests.

For the Ouchterlony analyses polyclonal immune sera were raised in rabbits against each of the different CTB preparations. These sera were then used in double diffusion tests against the different CTB preparations as shown in Figure 4 to reveal any possible epitope differences between the CTB proteins. The immunoprecipitation bands formed showed reactions of complete fusion with no evidence of spur formation in any direction indicating that the different CTB proteins are immunologically indistinguisible.

In the GM1-ELISA epitope mapping tests the different CTB proteins, after binding to plastic-adsorbed GM1 ganglioside in ELISA microtiter wells, were tested for their reactivity with a panel of seven monoclonal antibodies recognizing different epitopes in the native CTB(569B). Each of the monoclonal antibodies reacted essentially identically with the different CTB proteins, thus supporting the conclusion from the Ouchterlony analyses that the proteins are immunologically indistinguisible.

In vivo immunogenicity. The *in vivo* immunogenic properties of rCTB produced from *V. cholerae* carrying pML-LCTB*tac*2 were compared with those of rCTB obtained from the previously described construct of Sanchez and Holmgren and native CTB(569B) by testing antibody responses in rabbits and mice after parenteral and peroral immunizations, respectively.

In rabbits, immune sera were raised by immunizing rabbits three times subcutaneously with the different CTB proteins and the immune sera were then assayed for anti-CTB antibodies by GM1-ELISA and for cholera toxin neutralizing antibodies by the CHO cell toxicity assay. As shown in Table 1, both the GM1-ELISA and neutralizing antibody titres were very similar after immunization with the different CTB proteins. Furthermore, whilst the GM1-ELISA and neutralizing antibody titres presented in Table 1 were demonstrated using CTB and cholera toxin from strain 569B (Classical *V. cholerae* O1) the same

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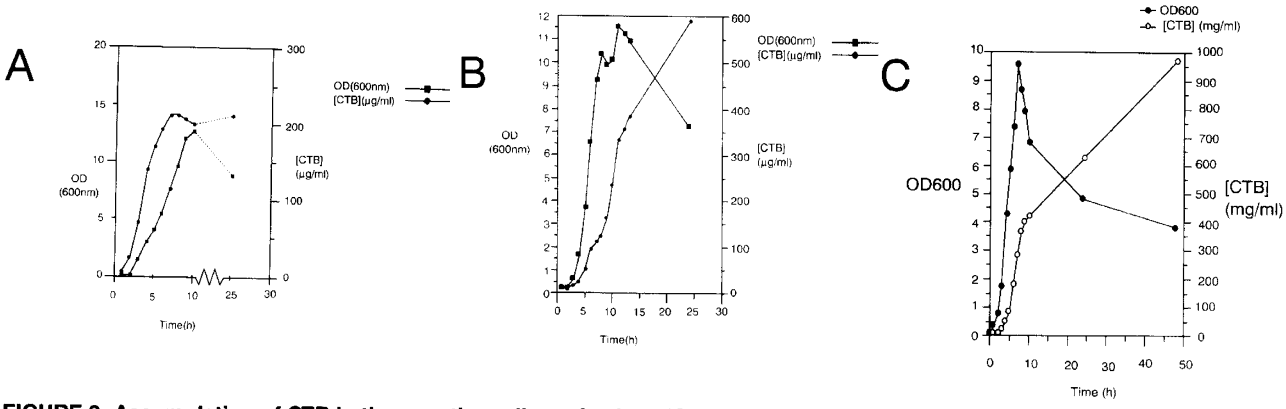

FIGURE 2. Accumulation of CTB in the growth medium of cultures of *Vibrio cholerae* carrying (A) pML-LCTB*tac* and (B) pML-LCTB*tac*2. Both cultures were grown in 5L 2x syncase medium but cultures carrying pML-LCTB*tac* were grown in the absence of antibiotic whereas cultures with pML-LCTB*tac*2 contained 100µg/ml ampicillin. (C) Accumulation of CTB from *V. cholerae* JS1569 carrying pML-LCTB*tac*2, in modified syncase as described in the Experimental Protocol in the presence of 100µg/ml ampicillin during growth in a 5L fermentor. After 48h the accumulated CTB usually ranges from 0.9–1.0mg/ml according to Mancini determinations.

results were obtained for the different sets of rabbit immune sera using CTB and cholera toxin from clinically more relevant *V. cholerae* O1 strains; both Classical and El tor strains isolated in Bangladesh during the decade from 1980, a recent El tor isolate from Peru and most recently the toxin from Bangladesh isolates of the new cholera epidemic serogroup "O139" (data not shown).

Likewise, as tested in mice the peroral immunogenicity of the different types of CTB protein was similar. The specific serum antibody levels and the frequency of specific IgA anti-CTB secreting cells in mesenteric lymph nodes after two oral immunizations did not differ significantly in response to two peroral immunizations with the different CTB proteins. Furthermore, in other animals rested for 6 months after the initial oral

TABLE 1. Immunogenicity in rabbits and mice of rCTB derived from *V. cholerae* JS1569 carrying pML-LCTB*tac*2 as compared with rCTB derived from the same strain carrying a derivative of pJS162 (24) and native CTB from *V. cholerae* 569B.

Immunization	Immune Response	
Rabbits, subcutaneous immunizations with:	GM1-ELISA titre (log10)*	Neutralizing titre (log10)*
- rCTB(pML/358)	6.2 (6.0–6.5)	4.4 (4.1–4.7)
- rCTB(pJS/213)	5.7 (5.4–5.9)	4.1 (3.8–4.4)
- CTB(569B)	5.8 (5.4–6.0)	4.1 (3.8–4.4)
Mice, peroral immununizations with:	GM1-ELISA titre (log10)*	IgA ASC / 107 MLN cells**
Two doses of		
- rCTB(pML/358)	5.2 (4.9–5.5)	3.5 (3.0–3.8)
- rCTB(pJS/213)	5.1 (4.8–5.4)	3.3 (3.0–3.7)
- CTB(569B)	5.0 (4.8–5.3)	3.2 (3.0–3.3
Same + single boost after 6 months		
- rCTB(pML/358)	6.1 (5.9–6.4)	3.9 (3.5–4.0)
- rCTB(pJS/213)	5.9 (5.8–6.2)	3.6 (3.4–4.0)
- CTB(569B)	6.0 (5.8–6.3)	3.8 (3.3–4.2)

*Antibody titres shown represent geometric means and in parentheses ranges in groups of 3–4 rabbits and 4–5 mice, respectively.
**Number of specific anti-CTB antibody-secreting cells (ASC) per 10^7 mesenteric lymph node (MLN) cells represent geometric means and in parentheses ranges in groups of 4–5 mice.

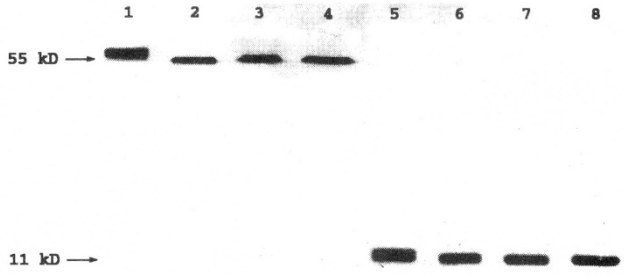

FIGURE 3. SDS-PAGE (15%) gel comparing CTB from different sources. Unboiled samples containing: (1) 1µg rCTB produced from *Vibrio cholerae* JS1569 carrying a plasmid derived from Sanchez and Holmgren[13]; (2) 1µg native CTB isolated from *Vibrio cholerae* 569B; (3) 1µg rCTB obtained from *Vibrio cholerae* JS1569 carrying pML-LCTB*tac*1; (4) 5µl sample of crude culture supernatant after overnight growth of *Vibrio cholerae* JS1569 carrying pML-LCTB*tac*2. Wells 5 to 8 are loaded in the same way as 1 to 4 respectively and show the dissociation of pentameric CTB after boiling in SDS.

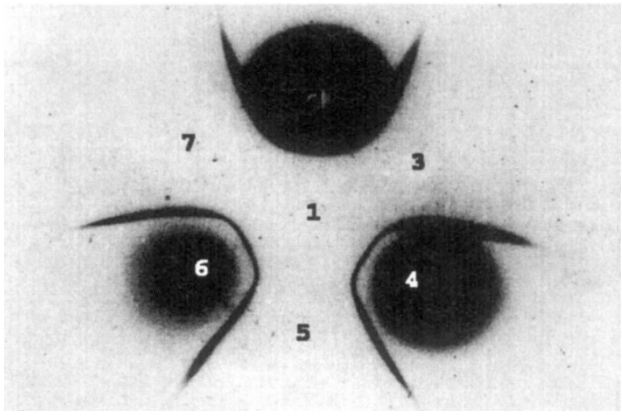

FIGURE 4. Ouchterlony immunodiffusion analysis of rCTB(pML-CTB*tac*1) (well 1), rCTB[13] (well 5) and native CTB(569B) (wells 3 and 7) diffusing against antisera against the different CTB proteins (in well 4 anti-rCTB (pML-LCTBtac1), in well 6 anti-rCTB[13] and in well 2 anti-CTB(569B)). Immunoprecipitation bands show complete fusions between the the different CTB proteins against each of the different antisera.

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immunizations and then given a single oral boost the specific serum and mesenteric lymph node antibody responses were also very similar indicating that the ability to induce immunologic memory did not differ between the CTB proteins tested (Table 1).

Discussion

CTB is an important component of a recently developed and licensed oral cholera vaccine and is also part of a vaccine against enterotoxigenic *E. coli* diarrhoea now undergoing clinical testing. The need for these vaccines, if they can be produced at an affordable price to the people in the countries where these diseases are endemic, is hundreds of millions of doses per year. Since the oral dose of CTB in these vaccines is 1mg, it represents the limiting component in terms of amounts available and costs. Improved ability to produce CTB will thus have direct positive consequences on vaccine production and cost-effectiveness.

The overexpression system described by Sanchez and Holmgren[13] represented a first step in the achievement of this goal by avoiding the cumbersome purification of CTB from the holotoxin and providing a modest increase in the amount produced (from ~30g to 100g per 1000L fermentor). The system described in the present study represents a significant improvement by providing almost a 10-fold increase in CTB per fermentation volume of a more homogenous composition.

The *tac* promoter-based vectors used in *V. cholerae* have, by alteration of a combination of variables, been demonstrated under favourable growth conditions, to allow the accumulation of large amounts of CTB in the growth medium. It was found that highest levels of expression were obtained with the high copy-number plasmid pML-LCTB*tac*2, when the growth medium was modified to contain less carbohydrate carbon source and more caesamino-acids. However, *Vibrio cholerae* strains with this plasmid, which yield ~1g/l of CTB, require ampicillin in the medium to maintain optimium production. Although downstream processing can effectively remove antibiotic residues from the purified product, work is in progress to construct similar plasmids with alternative markers and appropriate host strains that will remove the need for antibiotic selection.

From the outset it was proposed to use the combination of a powerful promoter and efficient RBS to optimize the expression of the cloned *ctx*B gene. It was found however, that physiological constraints imposed by the toxicity of CTB to the host cells required the use of a less efficient arrangement. Plasmids that were predicted to give very high constitutive levels of CTB were unstable in *E. coli*. Initially, no transformants could be isolated that yielded plasmids that gave the predicted restriction patterns or produced CTB. Only when ligated DNA was electroporated directly into *V. cholerae* were colonies readily isolated that both gave the correct restriction digests and produced large amounts of CTB. It was concluded that this instability in *E. coli* was due to toxicity of CTB at the concentrations that would be reached within the periplasmic space. The ability of *Vibrio* strains to secrete CTB maintains intracellular concentrations at non-toxic levels, but even in *V. cholerae* the combination of optimum signals for CTB expression proved to be unstable when placed in a high copy number plasmid. It is thus apparent that there are limitations to the use of *E. coli* as an intermediate in the construction of vectors for the production of CTB.

From the vaccine development point of view it is important that the recombinant CTB is immunologically indistinguishable from the B subunit moiety of the cholera toxin produced by the newly described epidemic serogroup O139 now rapidly spreading and at least at present, outcompeting *V. cholerae* O1 as a cause of severe cholera disease in India and Bangladesh[18,19]. We have recently sequenced the *ctx*B gene of *V. cholerae* O139 isolates and found it to be identical to that of El tor strains (M. Lebens, unpublished data). In accordance with this we have found that rabbit antisera against our recombinant CTB neutralizes the O139 toxin to the same extent as toxin produced by O1 strains (this study) and can also prevent the the development of experimental cholera (fluid accumulation) in rabbit intestinal loops infected with O139 *V. cholerae* (J. Holmgren, manuscript in preparation).

Experimental Protocol

Bacterial strains and plasmids. The strains of *E. coli* used in the present study were HB101 (ref. 20) and N4830-1, a *Gal*K$^-$ P1 transductant of N4830 (ref. 21) (obtained from Pharmacia (Sweden) AB). *Vibrio cholerae* strain JS1569 was used as host for CTB expression plasmids. This is a rifampcin resistant, *ctx*A-deleted derivative of classical strain 569B[22] obtained by selection from CVD103 (ref. 23). Strains were maintained on L-agar plates supplemented with the appropriate antibiotic (ampicillin, 100μg/ml). For long-term storage cell suspensions in L-broth containing 15% glycerol were kept at −80°C. All strains except N4830-1, which was grown at 30°C, were grown at 37°C.

DNA manipulation. All procedures for the isolation of plasmid DNA and its subsequent manipulation were essentially as described by Sambrook et al[24]. Synthetic oligonucleotides were prepared by Syntello Vaccine Development AB (Göteborg, Sweden.) using an Applied Biosystems DNA synthesizer. Restriction endonucleases and other enzymes for modification of DNA were obtained either from Boehringer Manheim (Sweden) AB or Pharmacia (Sweden) AB as were starting plasmids used in the construction of the expression vectors. *E. coli* was transformed with purified plasmid or ligated DNA after treatment with rubidium chloride and calcium chloride. *Vibrio cholerae* strain JS1569 was subjected to electroporation as follows: 200μl from an overnight culture grown at 37°C was used to inoculate 20 ml of fresh L-broth. The resulting culture was grown with vigorous shaking for 1.25h at 37°C or until the OD_{600} had reached 0.5 after which it was chilled on ice for 10min. The cells were spun down in sterile oak-ridge tubes at 7000rpm for 15min in a Beckman J21 centrifuge. The pellet was immediately resuspended in an equal volume of ice-cold solution containing 1mM calcium chloride and 1mM magnesium chloride. Cells were recentrifuged and resuspended in 200μl ice-cold solution of 0.5mM calcium chloride and 0.5mM magnesium chloride. The resulting cell suspension was used directly for electroporation. Electroporation was carried out on 50μl of cell suspension at 2.5kV and 25μF using a Biorad Gene Pulser fitted with a pulse controller set at 200Ω. DNA was added in a maximum volume of 1.5μl which allowed the electroporation of DNA taken directly from ligation reactions. Following electroporation 1ml of fresh L-broth was added to the cell suspension and the total volume was incubated at 37°C without shaking for at least 1h before spreading onto L-agar plates supplemented with the appropriate antibiotic selection. With plasmids prepared on cesium chloride gradients the described procedure gave 10^8 to 10^9 transformants/μg DNA. These efficiencies were not adversely affected by common manipulation procedures such as DNA extraction from agarose gels that severely reduce transformation efficiency in *E. coli*.

CTB: determination of concentrations and isolation of protein. CTB concentrations in cell extracts after induction of *E. coli* carrying appropriate recombinant plasmids were determined by GM1 ELISA as described previously[25]. Higher concentrations obtained from culture supernatants of *Vibrio cholreae* were determined by the Mancini immunodiffusion method[26]. CTB was extracted from the medium of *Vibrio cholerae* cultures by precipitation with sodium hexametaphosphate. Cells were removed from culture supernatants by centrifugation (7000rpm for 30min). Sodium hexametaphosphate was added to a final concentration of 2.5mg/ml and allowed to dissolve completely. The pH of the resulting solution was adjusted to 4.5 and proteins were left to precipitate overnight. The precipitate was spun down and redissolved in a minimal volume of phosphate buffered saline pH7.2 (PBS). Not all the precipitated material redissolved. The resulting suspension was dialyzed extensively against PBS and was finally centrifuged at 45,000rpm for 30min to remove undissolved material. This procedure considerably concentrated the CTB and gave a product usually about 70% pure. As a final purification step giving CTB at a purity of greater than 95% the material could be concentrated by ultrafiltration through an Amicon YM10 filter and subjected to gel filtration through a sephadex G100 column.

Culture of *Vibrio cholerae* for CTB production. For large-scale production of CTB a 5L bench-top fermentor (New Brunswick Scientific, Edison USA) was used. Fermentation of *Vibrio cholerae* was performed at 37°C in double concentration or modified syncase with high aeration (5L/min) and stirring (500rpm). The fermentor was inoculated with a total of 160ml from four 40ml overnight cultures grown at 37°C in 250ml Ehrlenmeyer flasks containing the same medium. Samples were taken during the fementation for determination of CTB concentrations and pH, and optical density (600nm) were monitored. For optimal fermentation yields the following modified syncase medium was used: Casamino acids; 20g/l, sucrose; 2.5g/l, NH4Cl; 1.18g/l, Na2SO4; 0.089g/l, Na2HPO4.2H2O; 6.27g/l, K2HPO4.3H2O; 6.27g/l, MgCl2.6H2O;

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0.0042g/l, MnCl2.4H2O; 0.004g/l, FeCl3.6H2O; 0.005g/l[17].

Immunization of rabbits and serological methods. Immunizations of rabbits and analyses of generated antisera were done according to procedures described previously[13].

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