Recombinant system for overexpression of cholera toxin B subunit in *Vibrio cholerae* as a basis for vaccine development

(cholera vaccine/hybrid protein/gene fusion vector/immunogenic carrier/cholera toxin epitopes)

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ABSTRACT We have constructed an overexpression system in which the gene encoding the B subunit of cholera toxin (CTB) was placed under the control of the strong tacP promoter in a wide host range plasmid. Recombinant nontoxigenic classical and El Tor *Vibrio cholerae* strains of different serotypes harboring this plasmid excreted 10- to 100-fold higher amounts of CTB than any other wild-type or recombinant strain tested and may therefore be useful killed oral vaccine strains. The manipulations to place the CTB gene under tacP also included, by design, the introduction of single enzyme restriction sites for gene fusions to the CTB amino terminus. Cloning into these sites allows construction of CTB-derived hybrid proteins carrying various putative vaccine peptide antigens.

Cholera and diarrhea caused by enterotoxigenic *Escherichia coli* are important causes of morbidity and mortality in many developing countries. *Vibrio cholerae* of serogroup 01 and enterotoxigenic *E. coli* may induce diarrhea when multiplying in the gut of infected individuals by releasing cholera toxin (CT) or heat-labile enterotoxin (LT), respectively. These toxins are very similar structurally and functionally and are composed of two types of subunits, a single copy of A (CTA or LTA), which is responsible for activation of the adenylate cyclase in the intestinal cell of the host, and a pentamer of B (CTB or LTB), which binds the respective holo toxin to its intestinal receptor (1, 2).

CTB is an effective oral immunizing agent, which in a large field trial has been shown to afford protection against both cholera and enterotoxigenic *E. coli*—caused diarrhea. This has made CTB as such an important component, together with killed whole vibrios, of an oral cholera vaccine (3, 4). Moreover, CTB has attracted much interest recently as an immunogenic carrier for various other peptide or carbohydrate antigens (5, 6) and has also been used as a receptor-blocking and receptor-modulating agent for short term prophylaxis of cholera and enterotoxigenic *E. coli* diarrhea (7, 8).

These findings have now emphasized the need to increase the yields of CTB for large-scale production, ideally avoiding at the same time the drawback in currently used preparation methods of having to purify the CTB protein from active toxin. To facilitate vaccine development based on use of CTB, we have constructed an overexpression system in which the CTB gene is under the control of the strong tacP promoter in a wide host range plasmid. Recombinant *V. cholerae* of different biotypes and serotypes carrying this plasmid produced increased amounts of CTB, which was secreted to the cell culture medium. Overexpression of CTB may now aid its purification by simplified means and assist in preparing improved killed oral cholera vaccine strains. In addition, the overexpression system developed allows for gene fusions at the CTB amino terminus, facilitating the construction of peptide-bearing immunogenic hybrid proteins.

**MATERIALS AND METHODS**

**Gene Fusions and DNA Sequencing.** The unphosphorylated oligodeoxynucleotides used to join the *Sac* I 3' end of the LTB leader to the 5' *Nde* I of CTB were purchased as single strands from the Department of Immunology, Biomedical Centre, University of Uppsala. After pairing, the oligonucleotides had *Sac* I and *Nde* I compatible single-stranded extensions and could be joined directly to *Sac* I/HindIII restricted pMMB68 (9, 10). Ligation was performed overnight at 4°C with T4 ligase and with a 10-fold molar excess of oligonucleotide to plasmid. To the ligation mixture a purified *Nde* I/HindIII fragment from plasmid pCVD30 containing the CTB gene (11) was added in equimolar amounts with respect to vector plasmid and the ligase reaction continued at 4°C for 18 hr. The ligated DNA was subsequently transformed into competent *E. coli* HB101 cells with selection for ampicillin resistance (100 µg/ml). To verify that the predicted sequences had been generated after cloning, the hybrid gene was subcloned into M13 and sequenced by the dideoxynucleotide method (12).

**Transfer of Plasmids to *V. cholerae* and Induction by Isopropyl β-D-Thiogalactopyranoside (IPTG).** Plasmid pJS162 containing the CTB gene under the control of tacP (Fig. 1) was transferred by conjugation from a helper *E. coli* strain (13) to the El Tor *V. cholerae* 01 JBK70 (14), using polymyxin B (50 units/ml) and ampicillin (100 µg/ml) for counterselection of the donor. When the plasmid was transferred to classical strains, rifampicin derivatives of them were first isolated. Selection for the recipient vibrio strains in this instance was done with rifampicin (50 µg/ml) and ampicillin (100 µg/ml). For induction with IPTG, cultures were grown at 37°C to the desired optical density (A600) in LB broth supplemented with antibiotics and then IPTG was added to the final concentrations needed. Growth was continued for 4 hr and the cells and culture supernatants were separated by centrifugation. Cell pellets were gently washed and resuspended in 1 original vol of cold phosphate buffered saline (pH 7.2) to be broken by two 30-sec sonic bursts with a miniprobe (Branson Sonifier).

**Immunosay of CTB.** Detection of recombinant CTB was carried out by the monosialoganglioside (GM1) ELISA (15) using anti-CTB monoclonal antibodies, which do not cross-react with LTB (16). Samples tested were either culture supernatants or sonicated cell pellets. Quantitation of recombinant CTB was made against a standard curve established for concurrently tested purified CTB from strain 569B (kindly provided by Institut Merieux, Lyon, France).

Abbreviations: aa, amino acid(s); CT, cholera toxin; CTA, CT A subunit; CTB, CT B subunit; GM1, monosialoganglioside; IPTG, isopropyl β-D-thiogalactopyranoside; LTB, B subunit of *Escherichia coli* heat-labile enterotoxin.
Purification of CTB and Amino Terminus Determination. The recombinant CTB was purified from a culture supernatant of JBK70 carrying plasmid pJS162 by affinity chromatography on a lyso-GM1 ganglioside Spherosil column (17). Purified CTB was subjected to determination of the amino-terminal residue as described by Von Bahr-Lindström et al. (18).

NaDodSO₄/PAGE. Purified recombinant CTB was electrophoresed in NaDodSO₄/13.5% polyacrylamide slab gels and the proteins were stained with Coomassie blue. To test for association of CTB into oligomers, samples were treated just prior to electrophoresis with 0.1% NaDodSO₄ sample buffer for 5 min at room temperature or subjected to boiling.

Association with CTA in Vitro. Purified CTA (prepared from 569B CT; List Biological Laboratories, Campbell, CA) was mixed with purified recombinant or 569B CT to give a total protein concentration of 200 μg/ml (19) and in the molar CTA/CTB ratio normally found in intact CT. After mixing, samples were acidified with 0.2 M glycine buffer (pH 2.7) and then neutralized by dialysis overnight against several changes of 0.2 M Tris buffer (pH 8.0). The neutralized samples were then tested with subunit-specific monoclonal antibodies by GM1 ELISA. The amounts of 569B CTA that associated then tested with subunit-specific recombinant CTB using untreated homologous CT as a reference. Antibodies to CT were assayed using purified 569B CTA (40 mg/ml in physiological phosphate buffer supplemented with 10 mg of bovine serum albumin per ml), and 0.1 ml of the mixture was then tested for residual toxicity by intradermal injection.

RESULTS

Placement of the CTB Gene Under tacP. The DNA encoding the LTB leader has a single EcoRI restriction site at its 5’ end located just upstream of the ribosome binding site that was used to insert the LTB gene after the tacP promoter (10). To profit from this strategically located EcoRI site (which is missing in the CTB gene) for expression of CTB from the same promoter, we fused genetically the mature CTB protein to the leader peptide of LTB in pMMB68. The CTB gene from pCVD30 (originating from V. cholerae strain 0395, classical Ogawa) has an Nde I site at the position for amino acid (aa) 18 of the leader peptide, while the LTB gene has a Sac I recognition sequence at the beginning of the mature protein. Fusion of the CTB gene by its 5’ Nde I end to the 3’ Sac I end in the LTB gene, via an ad hoc synthetic linker (Fig. 1A), leads to substitution of the LTB leader peptide by that in LTB. The resulting pJS162 plasmid (Fig. 1B) contained the hybrid CTB gene as an EcoRI/HindIII downstream of the tacP promoter.

Analysis of the CTB Gene Sequence. Sequencing of the hybrid CTB gene in plasmid pJS162 confirmed the sequence reported for the LTB leader peptide (12) and showed a high degree of overall homology with previously reported El Tor and classical CTB mature sequences (23–25). A comparison between our recombinant CTB (from a V. cholerae 0395 classical strain) and those other sequences is presented in Fig. 2.

Expression of CTB from tacP in V. cholerae. Conjugal transfer of pJS162 to the toxin-deleted JBK70 V. cholerae strains followed by induction with IPTG led to production of 40–50 μg of CTB per ml, of which >95% was secreted into the culture medium (Fig. 3). Expression of CTB to levels up to 50–100 μg/ml was also achieved in several toxin- or CTA-deleted classical and El Tor strains (Table 1). This represented a marked overexpression of CTB in comparison with the levels produced by many wild-type or recombinant V. cholerae strains examined, including the hypertoxigenic 569B strain that is currently used for purification of CTB for vaccine production. The recombinant CTB could then readily be purified in high yield from the culture supernatants of either of these strains using receptor-specific affinity chromatography on lyso-GM1 ganglioside (17).

Characterization of Recombinant CTB Protein. (i) Amino terminus determinations. Cleavage of the precursor peptide of the recombinant CTB would have been naturally predicted to take place at either one or both of the original leader peptide recognition sites in LTB or CTB. Identification of the first amino acid in the purified protein gave both tyrosine and alanine residues. This and the fact that the recombinant CTB was slightly larger than native CTB as determined by NaDodSO₄/PAGE (Fig. 4) suggested that proteolytic processing of the leader peptide had taken place between the glycine encoded by the linker (position +1) and tyrosine (position −4) as well as between this latter amino acid and alanine at −3 (Fig. 2). When the remainder of the treated CTB was subjected again to amino terminus determination alanine and histidine residues were now identified, confirming the
postulated cleavage positions and providing evidence that the recombinant CTB carried short peptide extensions at its amino terminus consisting of either 3 or 4 amino acids.  

(ii) Receptor and CTA subunit recognition. The presence of the few extra amino acids in the CTB did not affect its recognition of the GM1 receptor. The binding affinity for plastic-coated GM1 ganglioside was compared for the recombinant CTB and 569B CTB using the GM1 ELISA and no difference was revealed (data not shown). Retention of high binding affinity for GM1 ganglioside was in fact also taken advantage of in the purification of CTB as described above. The recombinant CTB was also unaffected in its ability to both oligomerize and associate with CTA, as shown by the high percentages of holotoxin recovered after acidification and neutralization of the mixed subunits (Table 2).  

(iii) Immunological properties of recombinant CTB. To determine whether the extra amino acids in CTB affected its immunoreactivity or even resulted in the appearance of new epitopes, we prepared rabbit antisera against recombinant and 569B CTB. The anti-CTB titers in GM1 ELISA were similar (range, 4 × 10^6 to 1 × 10^8) for anti-recombinant and anti-569B CTB as tested with either of the two CTB preparations as solid-phase antigen. Likewise, the neutralizing antitoxin titers did not differ, the sera against either recombinant or 569B CTB being able to completely neutralize 2 ng of CT at a concentration of 1 × 10^-4. Ouchterlony immunodiffusion tests performed with the purified recombinant CTB or 569B CTB and their corresponding rabbit immune sera as reactants showed immunoprecipitation bands of coalescence without any "spurs," indicating complete "identity" between the recombinant and native CTB (Fig. 5).
Table 1. Overexpression of CTB from the tacP promoter in V. cholerae

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype*</th>
<th>CTB, µg/ml</th>
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<tbody>
<tr>
<td>Expressing CTB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>from tacP promoter</td>
<td></td>
<td></td>
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<tr>
<td>JBK70 (pJS162)</td>
<td>El Tor Inaba</td>
<td>75</td>
</tr>
<tr>
<td>JS1569 (pJS162)</td>
<td>Classical Inaba</td>
<td>75</td>
</tr>
<tr>
<td>JS1395 (pJS162)</td>
<td>Classical Ogawa</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>CTA- CTB'</td>
<td></td>
</tr>
<tr>
<td>Expressing CT or CTB from own promoter</td>
<td></td>
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</tr>
<tr>
<td>569B</td>
<td>Classical Inaba</td>
<td>1.28</td>
</tr>
<tr>
<td>CVD103 (569B derivative)</td>
<td></td>
<td>0.88</td>
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<tr>
<td></td>
<td>CTA- CTB'</td>
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All strains were grown for the same periods at 30°C. Strains carrying pJS162 were grown to A600 = 1.0 before addition of IPTG to 100 µg/ml, after which incubation was continued for 4 hr. Strains JS1569 and JS1395 are rifampicin-resistance derivatives from strain CVD103 and CVD101(11)(0395 CTA- derivative), respectively. The levels of CTB produced by these latter strains in the absence of IPTG were <0.5 µg/ml and therefore did not contribute significantly to the values in the presence of the inducer.

*Nonfunctional (CTA-) or functional (CTA+) CTA gene and/or nonfunctional (CTB-) or functional (CTB+) CTB gene. Strains were prepared by gene deletion (11).

DISCUSSION

The demonstration that oral immunization with CTB provides protection against cholera and enterotoxigenic E. coli diarrhea in humans (3, 4) and the evidence in experimental systems that CTB is also an effective immunogenic carrier for various other peptide or carbohydrate vaccine candidate antigens (5, 6) have emphasized the need to develop means to produce high levels of this protein. We describe here a system in which using recombinant DNA technology we have achieved overexpression of CTB by placing the CTB structural gene under the control of the strong tacP promoter. Various V. cholerae strains harboring the wide host range plasmid (pJS162) containing the tacP-directed CTB overexpression system expressed 10- to 100-fold higher levels of CTB than any other wild-type or recombinant strains tested.

The reason for the efficient expression of CTB attained by us in contrast with previous attempts using the same promoter (25) has not been established. The removal of the CTB DNA upstream of the Nde I site used for cloning may have eliminated sequences interfering with high expression by affecting transcription (28) or translation (29). A positive effect on translation by the LTB ribosome binding site (Shine–Dalgarno sequence) seems unlikely since its complementarity to the 16S rRNA 3' end is less than that of the original CTB Shine–Dalgarno sequence (23). Although high expression of CTB in V. cholerae has been considered possible only when directed from the original ctx promoter and in the presence of ancillary regulatory elements (25), our demonstration of CTB overexpression by tacP suggests that control by other strong foreign promoters might also be feasible. Indeed, we have preliminary data showing high levels of CTB expression in V. cholerae determined by the T7 RNA polymerase-dependent promoter (30) (unpublished data).

Sequencing of the CTB gene in plasmid pJS162 showed a high degree of homology with published CTB sequences but also revealed some interesting dissimilarities. DNA sequences of cloned CTB genes from several V. cholerae El Tor strains are available (23–25), whereas only the CTB protein sequence for the 569B classical strain has

Fig. 4. PAGE of boiled and unboiled samples of CTB. Equal amounts of recombinant CTB protein (lanes 1 and 2) or reference 569B CTB (lanes 3 and 4) were electrophoresed in a NaDodSO4/13.5% polyacrylamide gel after treatment in sample buffer for 5 min at 100°C (lanes 1 and 3) or at room temperature (lanes 2 and 4). A molecular mass marker (Bio-Rad) with the approximate sizes of protein standards (kDa) is shown (lane MW). The slower migration of the recombinant CTB as compared to the 569B CTB is only slightly noticeable when examined as the monomers (Bm) but is more obvious in the oligomeric (pentameric) forms (Bp).

Fig. 5. Ouchterlony double-diffusion in gel analysis of 569B CTB (wells B and F) and recombinant CTB (wells G and D) reacted with rabbit antisera (wells A and E, anti-recombinant CTB; well C, anti-569B CTB).
been described (26, 27). Among the four (24) or five (25) aa reported to differ between the 569B CTB protein sequence and the various El Tor CTB DNA sequences, Asp-70 has been proposed to determine an El Tor-specific antigen epitope (24). We found that in our CTB sequence aa 70 and 22 were both aspartic acid as in the El Tor CTBs, while His-18, Thr-47, and Gly-54 (Tyr-18, Ile-47, and Ser-54 in the El Tor CTB) were as in 569B CTB. This suggests that Asn-70 (or Asp-70) plays no role in defining classical (or El Tor) specific CTB epitopes. His(Tyr)-18, Thr(Ile)-47, and Gly(Ser)-54, on the other hand, might be relevant determinants for such epitopes, but before any conclusions can be drawn there is a need to sequence at least a few additional classical CTB genes to determine possible internal variability among classical strains. The strategy followed to replace genetically the CTB leader peptide by that in LTB to achieve overexpression from tacP produced a hybrid leader peptide in which the original leader peptidase cleavage sites in LTB and CTB were both present. Processing of the leader peptide occurred at two places between these sites. As a result, the recombinant mature CTB carried an extension of 3 or 4 amino acids at its amino-terminus. These short peptide additions, however, did not cause any change in the immunological reactivity or other properties of recombinant CTB. Thus, as compared with native 569B CTB our recombinant CTB exhibited a normal ability to form pentamers and to associate with native CTA as well as unchanged GM1 ganglioside receptor binding affinity. Furthermore, the immunological analyses demonstrated that recombinant CTB was identical with the reference 569B CTB in its immunological determinants and in its capacity to give rise to anti-toxin antibodies. Overexpression of CTB as reported here should now facilitate the production of this protein for use as a protective immunogen in oral vaccination against cholera and LT-caused E. coli diarrhea. It is a particular advantage from the point of both polarization and standardization of CTB as a vaccine component that overexpression can be achieved by V. cholerae strains that stably, through genetic deletion, lack the ability to produce cholera holotoxin and/or its A subunit. In addition, because overexpression of CTB was attained in nontoxicigenic El Tor and classical V. cholerae 01 of either Inaba or Ogawa serotypes, it should be possible to prepare vaccine analogous to the oral B subunit-whole cell cholera vaccine recently field tested in Bangladesh (3) by simply growing and inactivating a combination of these CTB-hyperproducing strains. As mentioned, CTB has also attracted much interest recently as an immunogenic carrier for other vaccine relevant antigen epitopes. Hybrid derivatives of CT and LT enterotoxins for immunoprotection have been made by chemical coupling (refs. 5, 31, and 32; C. Czerkinsky, M. W. Russell, N. Lycke, M. Lindblad, and J.H., unpublished data) or by genetic fusion (33, 34). To create the possibility of obtaining CTB-derived hybrid proteins by gene fusion as an extra bonus of the tacP-directed overexpression system, we arranged the constructions so as to have adjacent single Sac I and Sma I recognition sequences at the amino terminus of the CTB gene. Use of these restriction sites allows linkage of diverse peptide antigens to CTB for immunoprotection studies against, e.g., viral, bacterial, and parasitic diseases. The accessibility of this approach has recently been demonstrated by the fusion and expression of a synthetic DNA sequence encoding a nontoxic decapetide antigen, derived from the E. coli heat-stable enterotoxin, to the pJ5162 CTB gene (35). We thank Susanne Johansson for skillful technical assistance, Mats Lake (Kabiogen, Stockholm) for help in the DNA sequencing, and M. Lindblad and the Department of Clinical Chemistry, University of Göteborg, for determination of the CTB amino terminus. We also thank B. E. Uhlin for useful discussions and J. B. Kaper for providing various V. cholerae and E. coli strains. This work was financially supported by the Swedish Medical Research Council and the World Health Organization.