Volume 330, number 3, 265–269 FEBS 12979 September 1993

Extracellular secretion of STa heat-stable enterotoxin by *Escherichia coli* after fusion to a heterologous leader peptide

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Received 2 July 1993

The mature 19-amino acid STa heat-stable enterotoxin of *E. coli* has a preceding peptide of 53 amino acids which contains two domains called Pre (aa 1–19) and Pro (aa 20–53) sequences, proposed to be essential for extracellular toxin release by this host. The Pro sequence, however, has been proven not be indispensable for this process since Pro deletion mutants secrete STa. To find out if Pre and/or other unremoved natural STa flanking sequences are responsible for toxin secretion in those mutants we genetically fused mature STa directly to the leader peptide of the periplasmic *E coli* heat-labile enterotoxin B-subunit (LTB). Expression of this gene fusion resulted in extracellular secretion of biologically active STa by *E. coli* independently of natural STa neighboring genetic sequences. Moreover, these results suggest that STa might be able to gain access to the extracellular milieu simply upon its entry into the *E. coli* periplasm once guided into this compartment by the LTB leader peptide. To test if extracellular secretion in this fashion might be extended to other disulfide bond-rich small peptides, the 13 amino acid conotoxin GI and a non-enterotoxic STa-related decapeptide were cloned. None of the two peptides was found in culture supernatants, in spite of high structural homology to the toxin. Failure to be secreted most likely leads to degradation as peptides were also not detected in bacterial sonicates. We hypothesize that cysteine-rich peptides must have an amino acid length and/or number of disulfide bridges closer to those in STa for them to follow this toxin secretory pathway in *E. coli*.

Synthetic gene; Conotoxin GI; Precursor peptide; Enterotoxin B-subunit (*E. coli*), Cysteine-rich peptide

1. INTRODUCTION

Enterotoxigenic *Escherichia coliI* produces heat-stable enterotoxins classified according to their solubility in methanol as STa (or STI) and STb (or STII). The methanol soluble enterotoxin STa has been isolated from porcine, bovine and human isolates from bacteria associated with diarrhea [1]. In addition, several other heat-stable enterotoxins have more recently been identified from non-01 *V. cholerae* [2], from *V. cholerae* 01 [3] *Y. enterocolitica* [4], from *C. freundii* [5]. *E. coli* STa and non-01 V. cholerae ST, have a structural gene which includes a large precursor peptide of more than 50 amino acids preceding the mature enterotoxin active moiety of 19–20 amino acids including 6 cysteines engaged in intrachain disulfide bonds. There is a large extent of homology within the different enterotoxic domains in all members of the STa family, in contrast, the amino acid sequences of precursor peptides exhibit substantial heterogeneity. Two regions in the precursor peptide of STa can be distinguished which may be functionally different. According to a model, the first region of the precursor peptide, of around 20 amino acids, is thought to be involved in translocation to the periplasm of a fusion peptide composed of STa joined covalently to the carboxy end of the following 33 amino acids of the precursor peptide. This 33-amino acid peptide has been proposed to be essential for extracellular secretion of STa [6] but its role in this process has remained unidentified. That the peptide is not indispensable for secretion was recently shown by genetically deleting amino acid residues 20 to 53 (designated Pro sequence) to obtain active extracellular STa [7]. Upon removal of Pro the genetic information for the amino terminal 20 aa (designated Pre sequence) together with other natural STa flanking DNA sequences were left intact thus leaving open the possibility that either, or both, mediate extracellular secretion of STa. We here set out to explore that possibility by fusing an isolated synthetic STa gene directly after the gene for the heterologous periplasmic leader peptide of the *E. coli* heat-labile enterotoxin B-subunit (LTB). Effective expression and secretion of STa, as here shown, definitely rule out that Pre, Pro and other natural genetic sequences are essential for secretion of the toxin. To test if secretion of STa in this fashion was shared by structurally similar peptides conotoxin GI [8] and an STa-related decapeptide [9] were cloned with the same genetic procedures. Neither of the two peptides was secreted by *E. coli* suggesting that STa exits the bacterial cell via a selective secretion pathway which appears to discriminate even against structurally close relatives.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

E coli HB101 was used as the host strain in all constructions. Cultures were grown in LB broth [10]. Plasmid pJS7-11 which encodes an STa-CTB hybrid protein [11] was used to create the fusion of STa to the LTB leader peptide. Plasmid pJS18 was previously described as a vector employed to generate gene fusion proteins at the carboxy end of cholera toxin B-subunit [11] Plasmid pJS18 was used to clone the synthetic conotoxin GI gene as an *XmaI/Hin*dIII insert. All recombinant plasmids were introduced to HB101 by the CaCl transformation procedure [10] Extransformants were selected by growth on agar plates with ampicillin at a final concentration of 100 or 200 μg/ml.

2.2. *Genetic constructions*

Plasmid pJS7-11 was cut at its unique *Xma*I site located at the carboxy end of the gene encoding STa and an adapter introduced (see Fig. 1) which incorporated a stop codon after STa. The adapter was ligated to *Xma*I-digested pJS7-11 by adding it at a 50-fold molar excess over the vector plasmid and using T4-ligase as recommended by the manufacturers (Gibco-BRL). The junction between STa and CTB in pJS7-11 encodes for Pro, Gly and Asn [11], after insertion of the adapter codons for Pro and Gly were reconstituted but Asn was substituted by Tyr which in turn is followed by the stop codon. Therefore insertion of the adapter caused termination of translation of STa to leave a fusion peptide where STa is preceded by the LTB leader peptide. Because the adapter gave an altered reading frame of the CTB gene colonies could be screened for insertion by lack of production of CTB in GM1 ELISA tests [12]. The synthetic conotoxin GI gene was prepared for insertion by pairing equimolar concentrations of single-stranded complementary synthetic oligodeoxynucleotides with extensions compatible with *Xma*I and *Hin*dIII restriction ends Insertion of oligonucleotides to vector pJS18 gave plasmid pJSCon1 (see Fig 2). Paired oligodeoxynucleotides were added in a 50-fold molar excess over the vector plasmid and ligation carried out overnight using T4-ligase as described above. Insertion of the conotoxin GI synthetic gene resulted in elimination of the CTB mature gene in pJS18 allowing screening of recombinant colonies by absence of CTB production as described above. All constructs were confirmed to carry the desired insert first by restriction analysisand subsequently by DNA sequencing using the Sequenase Version 2 method (USB Laboratories).

2.3 *Detection of STa and conotoxin GI in animal assays*

The biological activity of STa was assayed in suckling mice as described [1]. Groups of three mice were inoculated intragastrically with culture supernatants or cell lysates obtained by sonication. The gut to carcass ratio was determined. Values higher than 0.080 were considered positive Purified STa at a maximal concentration of 20 ng/ml was used as a positive reference in the test. Approximate toxin concentrations were determined by comparison to the STa control of twofold serially diluted samples. Activity of conotoxin GI was determined by intraperitoneal injection of undiluted culture supernatants or total cell sonicates into young Balb/c mice essentially as previously reported [8]. Positive response to conotoxin in this test is evidenced by muscular paralysis after approximately 20 minutes following injection. Commercial conotoxin GI (Sigma Chemical Co.) was used as a positive control at 20 μg/kg of body weight. Samples were injected in 100 μl volumes and mice kept under surveillance until expected signs were observed.

2.4 *Detection of STa by ELISA*

Cell supernatants and total cell sonicates were assayed for the presence of STa on a competitive GM1 ELISA using a recombinant decapeptide-CTB protein as the coating agent [9] and STa specific monoclonal antibodies [13]. A positive sample was identified by binding inhibition of native STa to the well as followed by a decrease in absorbance at 492 nm after reaction with orthophenylenediamine substrate Release of STa to *E coli* supernatant as a result of cell lysis was ruled out by testing the same culture supernatants and cell sonicates for β-lactamase activity [14]. All β-lactamase activity was found in cell sonicates, as expected (98–100%), while negligible amounts were detected in the medium (0–2%)

3. RESULTS

3.1. *Secretion of STa by* E. coli *after cloning under the LTB leader peptide*

Plasmid pJS7–11 [11], encoding an STa-CTB fusion protein was used to obtain plasmid pJS11–21 (Fig. 1). In pJS7–11 the natural LTB leader peptide precedes STa which in turn precedes mature CTB, all in the same translational reading frame. In plasmid pJS7–11 there is a single *Xma*I site at the junction between STa and mature CTB (Fig. 1). This strategically positioned single *Xma*I site was exploited to introduce a synthetic adapter

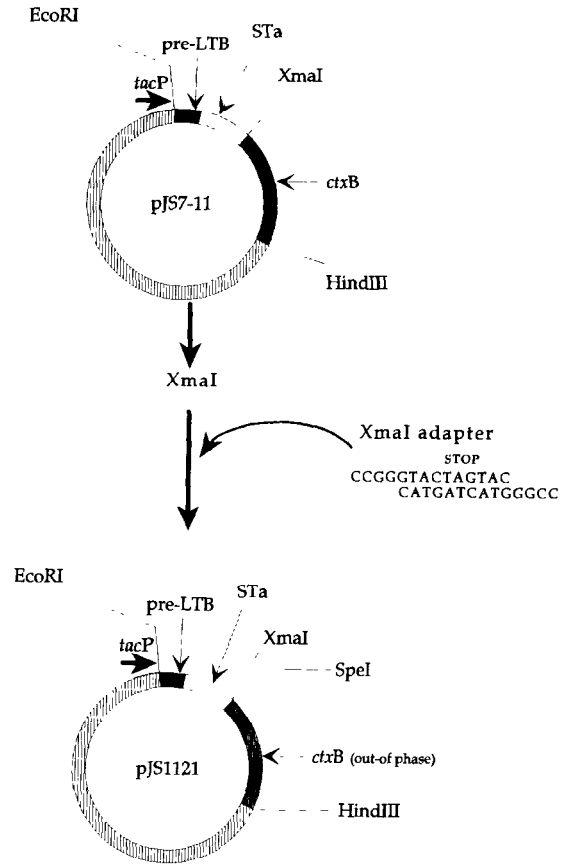

Fig. 1. Construction of plasmid pJS1121 encoding mature STa fused directly to the LTB leader peptide. The *Xma*I adapter (shown) was introduced in-frame so as to insert a stop codon (TAG) after the gene for STa in plasmid pJS7–11 [11]. Insertion of the adapter also resulted in incorporation of a unique *Spe*I site in the plasmid as shown. Plasmids are not drawn to scale Aproximate locations of unique relevant restriction sites and genes are shown. Pre-LTB designates the leader peptide for LTB. The gene encoding the B-subunit of cholera toxin is indicated (*ctx*B). The promoter and its direction of transcription is shown by the thick arrowhead. Both pJS7–11 and pJS1121 harbor a gene for resistance to ampicillin in the region symbolized by the larger shadowed portion of the plasmid maps but the presence of this gene it is not shown in the figure.

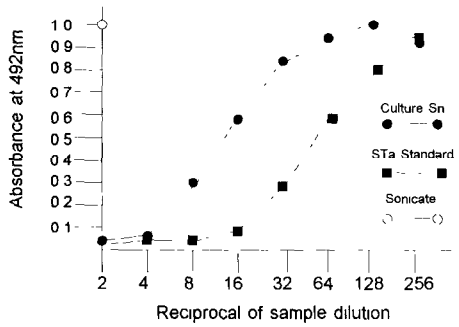

Fig. 2. Detection of STa in culture supernatants of HB101 (pJS1121) by a competitive ELISA [13]. Concentrations were determined by the reduction in absorbance at 492 nm as indicated. Each point in the curves is the average of three independent determinations. Cell sonicates gave full absorbance (ca 1.0) as they contained no STa. The standard curve was constructed using a solution of purified STa at a concentration of 1 μg/ml. For comparisons the sonicate cell pellet was diluted to the original culture volume

with a translational stop codon (TAG) between mature STa and CTB. The six nucleotides which determine the single XmaI site in pJS7–11 are situated in-frame thus coding for a Pro-Gly dipeptide extension after the terminal Tyr in mature STa. To avoid potential secretion interference of this foreign dipeptide extension at the carboxy terminal end we included a codon for Tyr (TAC) immediately after Pro-Gly so as to mimic the natural free carboxy Tyr in STa. Upstream regions in pJS7–11 were not manipulated so as to mantain expresion of the fusion peptide by the *tac*P promoter in the vector. Transformation of pJS11–21 into *E. coli* HB101 lead to production of STa by this host as determined by ELISA assays (Fig. 2). All STa reactive material was localized to the culture supernatant being undetectable in total washed cell lysates thus suggesting full secretion of the toxin by the recipient *E. coli*. Supernatant levels of STa produced by HB101(pJS11–21) were approximately 0.25 μg/ml (Fig. 2). These values were in nearly perfect accordance with toxin concentrations determined in culture supernatants by the suckling mice assay showing that secreted STa was fully biologically active.

3.2. *Cloning of conotoxin GI under the LTB leader peptide*

To determine if secretion of STa after cloning under the LTB leader peptide was shared by two peptides of related structure, i.e. small peptides with a compact structure and several intramolecular cysteine bridges, we undertook cloning of the 13-amino acid conotoxin GI under the same conditions as for STa. Only the mature sequence of the conotoxin GI has been reported so it is not yet known if this peptide is synthesized as a precursor form but this may not be unexpected since related neurotoxins, such as the so-called King Kong toxins, are synthesized in that form [15]. The mechanism which leads to their release has not been elucidated but it is clear that those precursors must undergo processing before release of the small active toxins, in an analogous fashion to processing of STa. Based on this and on significant structural and immunological homology of conotoxin GI with STa [16] we decided that cloning of conotoxin GI after the LTB leader peptide might behave similarly to STa fusions. To this end paired synthetic oligodeoxynucleotides, encoding the reported amino acid sequence for conotoxin GI [17], were joined to *Xma*I/*Hin*dIII-cut plasmid pJS18 to obtain plasmid pJSCon1 (Fig 3). After transformation of pJSCon1 into *E. coli* analysis of biological activity in the mouse model showed no conotoxin GI activity in culture supernatants. Similar results were obtained when tenfold concentrated supernatants were tested (data not presented). Assays of total cell sonicates also provided negative results indicating that the active peptide was not produced by this organism.

4. DISCUSSION

The nucleotide sequence of the first cloned STa gene was reported over 12 years ago [18]. This pioneer work demonstrated that STa was encoded in the form of an unusually long precursor peptide of 72 amino acids. That this unusual structure was functionally relevant and possibly had a role in secretion was further shown by other researchers [19]. Soon speculations followed that as being one of the few peptides secreted to the extracellular milieu by *E. coli* STa required to be synthesized as such a long precursor for secretion. However, no solid data on the role of this precursor was provided until recently when Okamoto and Takahara [7] duly showed that deletion of the intermediate 33 amino acid residues prior to mature STa (designated Pro sequence) did not prevent folding and secretion of the toxin by *E. coli*. Those findings have now been confirmed by others [20]. In Pro deletion mutants the so-called Pre-sequence [7] as well as sequences flanking the STa gene, including the natural STa promoter, have been left intact. Secretion of active STa in the absence of the deleted 33 amino acids has two implications: first, that the toxin may fold independently of the Pro sequence; and second, that secretion to the extracellular milieu can occur in the absence of the deleted 33 amino acids. The remaining native STa Pre sequence peptide and/or flanking sequences should then be assumed to be mediating folding and secretion of the toxin. We here addressed the question of whether an unrelated leader peptide can replace the native Pre sequence to lead to folding and secretion from *E. coli*, in total absence of the Pro peptide and other natural STa flanking DNA sequences. A priori it would not seem unnatural to assume that a small peptide, such as STa, with six closely located cysteines, all engaged in intramolecular bridges, might be thermodynamically favoured to acquire its final folding, inde-

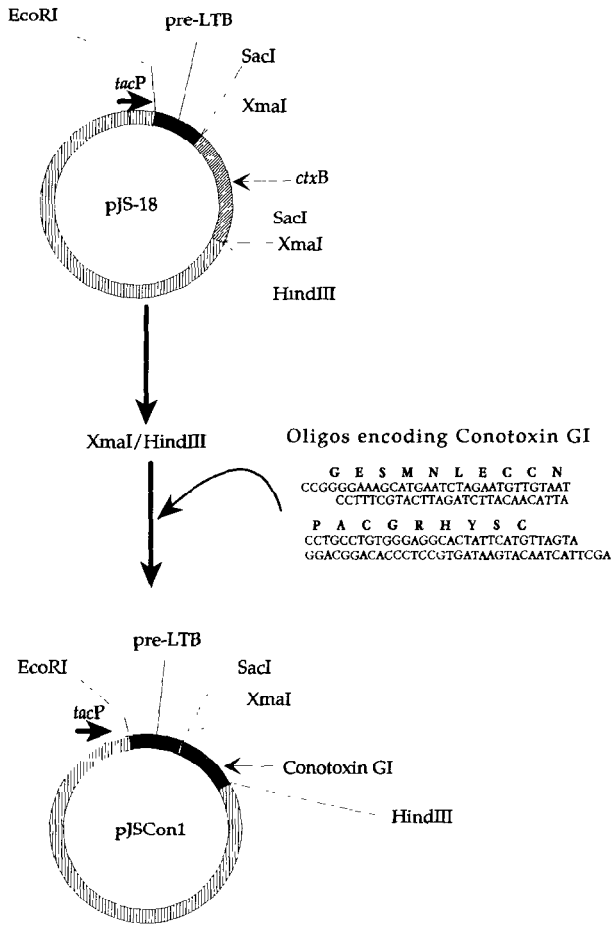

Fig. 3. Construction of plasmid pJSCon1 by cloning of a synthetic conotoxin GI gene into plasmid vector pJS-18 [11]. Aproximate locations of relevant unique restriction sites and genes are shown. Symbols for genes, promoters and positions of resistance gene are as in legend to Fig. 1. The DNA sequence of the synthetic conotoxin GI gene and encoded amino acids, symbolized by their single letter codes, are shown. Single-stranded DNA extensions shown are compatible with *Xma*I and *Hin*dIII restriction sites. Note that insertion of paired oligodeoxynucletides (oligos) leads to replacement of *ctx*B in pJS18 by the synthetic conotoxin GI gene to give plasmid pJSCon1.

pendently of preceding sequences, provided that it is in an appropriate cellular environment. In fact it was based on those a priori assumptions that we have previously undertaken fusions of mature STa to several different proteins. We have fused STa to the carboxy end of the A-subunit of the *E. coli* heat-labile enterotoxin [21], to the amino or carboxy ends of the B-subunit of cholera toxin [11], or to the carboxy end of *E. coli* outer membrane protein OmpC [22]. In all cases we obtained periplasmic, or membrane associated, hybrid proteins with properly folded STa joined covalently to the carrier proteins. Expression of active STa after joining of the isolated gene to the LTB leader peptide, as here reported, confirms that STa may attain its native conformation in the absence of its natural Pro and Pre sequences. Furthermore, secretion of STa as mediated by the LTB leader peptide may allow one to suggest that as long as STa is conveyed to the periplasm it may then go across the bacterial outer membrane on its own, perhaps due to its small, hydrophobic and compact nature. If that were the case other molecules with similar characteristics would be expected to behave in the same way. To test this hypothesis we cloned after the LTB leader peptide conotoxin GI, a 13-amino acid compact molecule with two intramolecular disulfide bridges, which has significant amino acid homology to STa [16]. As shown, secretion of conotoxin GI by E. coli did not take place under otherwise identical conditions, in clear contrast to STa. Retention of active conotoxin inside the cell was also ruled out by the absence of neurotoxic activity in cell sonicates, absence of activity here was probably due to intracellular proteolytic degradation. To investigate if lack of secretion was due to amino acid sequence differences we cloned in the same genetic position after the LTB leader peptide (data not shown) a decapeptide highly homologous to STa with the sequence CAELCCNPAC, which is identical to residues 5–14 in STa except for alanine number 2 which replaces cysteine number 6 in STa. No secretion of this decapeptide was observed either as determined by inhibition ELISA using appropriate monoclonal antibodies [23]. Since both, conotoxin GI and the decapeptide, are shorter than STa and have only 2 disulfide bridges, rather than three like in STa, one could propose that their lack of secretion is due to their smaller size and/or absence of one disulfide bond. The contention that neither conotoxin GI nor the decapeptide are able to acquire proper folding on their own and in consequence are not secreted may be fought by evidence that the decapeptide adopts an antigenic conformation, irrespective of its genetic fusion to either one of the two ends of the cholera toxin B-subunit peptide chain [11], just as observed for STa. On the other hand it is possible that STa amino acid residues missing in conotoxin and the decapeptide, other than the cysteines themselves, may be requisites sine qua non for secretion. Strict amino acid sequences compatible with extracellular secretion seem not likely as there is amino acid heterogeneity between the various toxic domains of the ST family of toxins [2]. Moreover, extracellular secretion in *E. coli* has also been reported for distantly related heat-stable enterotoxins [24]. This leaves with the appealing alternative that for the LTB leader peptide (or other bona fide leader peptides) to mediate extracellular secretion in *E. coli* of cysteine-rich peptides one simply requires to clone slighthly larger peptides, and/or peptides with three intrachain disulfide bonds. To answer this question we are currently cloning peptides which have two or three intramolecular disulfide bonds, with or without homology to STa, to examine their cellular location in *E. coli*. By this approach we hope to come to an understanding of the molecular mechanism for ex-

tracellular secretion of STa. If general secretion rules are found one could foresee engineering of *E. coli* for culture supernatant delivery of natural, or even man-designed, cysteine-rich peptides of medical, biochemical and pharmacological interest.

Acknowledgements: This work was supported by grants from the Swedish Agency for Research Cooperation with Developing Countries (SAREC) and from CONACYT, Mexico (Project 0672-M9109) J.S. is a member of the Sistema Nacional de Investigadores (SNI), Mexico. We gratefully acknowledege technical support by S. Johansson and G. Wiklund.

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