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## Extracellular DsbA-insensitive Folding of *Escherichia coli* Heat-stable Enterotoxin STa *in Vitro*\*

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To study the folding of human *Escherichia coli* heat-stable enterotoxin STh, we used the major protein subunit of CS31A fimbriae (ClpG) as a marker of STh secretion and a provider of a signal peptide. We established that STh genetically fused to the N or C terminus of ClpG was able to mobilize ClpG to the culture supernatant while still retaining full enterotoxigenicity. These features indicate that the STh activity was not altered by the chimeric structure and suggest that spatial conformation of STh in the fusion is close to that of the native toxin, thus permitting recognition and activation of the intestinal STh receptor *in vivo*. In contrast to other studies, we showed that disulfide bond formation did not occur in the periplasm through the DsbA pathway and that there was no correlation between DsbA and secretion, folding, or activity. This discrepancy was not attributable to the chimeric nature of STh since there was no effect of *dsbA* or *dsbB* mutations on secretion and activity of recombinant STh from which ClpG had been deleted. Periplasmic and lysate fractions of *dsbA*<sup>+</sup> and *dsbA*<sup>−</sup> cells did not have any STh activity. In addition, the STh chimera was exclusively found in an inactive reduced form intracellularly and in an active oxidized form extracellularly, irrespective of the *dsbA* background. Subsequently, a time course experiment in regard to the secretion of STh from both *dsbA*<sup>+</sup> and *dsbA*<sup>−</sup> cells indicated that the enterotoxin activity (proper folding) in the extracellular milieu increased with time. Overall, these findings provide evidence that STa toxins can be cell-released in an unfolded state before being completely disulfide-bonded outside the cell.

*Escherichia coli* heat-stable enterotoxin STa (also referred to as STI) is responsible for serious and sometimes fatal diarrheal disease in both humans and domestic animals (1). STa causes intestinal fluid secretion by binding to its cellular receptor, transmembrane guanylyl cyclase C (2), thus activating guanylyl cyclase C and leading to an increase in intracellular mucosal cGMP. This increase in cGMP adversely affects electrolyte flux in the intestine; sodium absorption is inhibited, and chloride secretion is stimulated. These ion flux changes result in the secretory diarrhea characteristic of enterotoxigenic *E. coli* infection (3, 4). The receptor binding and enterotoxic properties of STa have been mapped to a highly conserved 13-amino acid

C-terminal core sequence essential for toxicity and the heat-stable nature of the toxin. Six cysteine residues are present within this domain, and the three intramolecular disulfide bonds formed between the cysteine residues are necessary for toxicity of the molecule (5). The two species of STa, STp and STh, from porcine (or bovine) and human strains of enterotoxigenic *E. coli*, respectively, are typical extracellular peptides consisting of 18 (STp) or 19 (STh) amino acid residues (6, 7). STa is synthesized in the cytoplasm as a 72-amino acid precursor consisting of pre-, pro-, and mature STa regions (8, 9). The precursor is translocated across the inner membrane by the Sec-dependent export pathway (10); the 19-amino acid leader peptide is removed by signal peptidase; and the generated 53-amino acid pro-STa intermediate is delivered to the periplasm. The natural pre- and prosequences are dispensable for STa secretion (8). Mature STa without the prosequence may be able to gain access to the extracellular milieu upon its entry into the *E. coli* periplasm, once guided into this compartment by a heterologous periplasmic leader peptide (11). Conflicting observations have been reported on the mechanism of secretion of the toxin from the periplasm to the outside of the cell, making this mechanism poorly understood. Indeed, some authors found that the pro-STa region is cleaved in the periplasmic space, where the thiol-disulfide oxidoreductase DsbA (14) directly catalyzes the formation of the disulfide bonds of STa, and that *dsbA* mutation causes a marked reduction in STa activity (12, 13). In contrast, other investigators hypothesized that pro-STa can exit to the extracellular milieu and that STa is disulfide-bonded outside the cell, making the role of DsbA in STa folding unclear (9, 15). Such disagreement may be explained by the small size of the STa molecule and the escape velocity with which it is released into the extracellular milieu and thus by the difficulty in detecting and quantifying the intermediates in the different cellular compartments. In addition, STa is poorly antigenic and not immunogenic and reacts unexpectedly in conventional protein treatments such as staining, trichloroacetic acid precipitation, and electrophoresis (16), thus limiting progress in the elucidation of the STa secretion pathway. For these reasons, a number of attempts to develop genetic fusions between STa and heterologous carrier proteins have been made (11). Unfortunately, in most cases, the resulting chimeras were periplasmic or membrane-associated, and no STa activity was detected in the extracellular milieu. In contrast, in this work, we have shown that fusions containing various STa peptides at the N or C terminus of ClpG, the major protein subunit of *E. coli* CS31A fimbriae (17), result in the extracellular secretion of ClpG-STa hybrids with high enterotoxin activity. We used ClpG as a marker to facilitate STa detection in secretion and folding studies. We analyzed the effects of *dsbA* and *dsbB* mutations on the secretion and enterotoxigenicity of STa chimeras and determined the redox state of STa, both inside and outside of *dsbA*<sup>+</sup> and *dsbA*<sup>−</sup> *E. coli* cells.

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Disulfide bond formation during translocation of STa across the outer membrane was also examined.

#### EXPERIMENTAL PROCEDURES

**Bacterial Strains, Plasmids, Oligonucleotides, and Media**—The bacterial strains and plasmids used in this study are presented in Table I. Bacteria were grown at 37 °C in LB broth or LB agar with or without the following antibiotics: ampicillin, 50 µg/ml; chloramphenicol, 25 µg/ml; kanamycin, 50 µg/ml; and tetracycline, 12.5 µg/ml.

**ClpG::Sth Fusion Genes: Cloning Strategies, Constructions, and Features**—We made a variety of constructs to increase our chances of success in producing chimeric proteins with enterotoxigenic activity (see Fig. 1). Plasmid pHPC0838 was constructed from pDEV41155 (20) by *in vitro* site-directed mutagenesis (23) using mutagenic and selection primers. The former primer (5'-CGTGGCAGTAACCTATGTTAACTAATTGGCTTGA-3') created a unique *HpaI* site and added a valine between the penultimate tyrosine and the last C-terminal residue (asparagine) of ClpG. The latter primer (5'-CGCAGGAAAGAAGATCTGAGCAAAAGGCG-3') mutated the single *AflIII* restriction site of the pSK<sup>+</sup> plasmid vector into a single *BglIII* site. Plasmid pSTN24 was engineered from pHPC0838 as follows. A synthetic double-stranded DNA (the 5' → 3' single coding strand was CGGCAGATCTGTACTGCTGTGAACCTTTGTTGTAATCTGCCTGTACAGGATGTTACCTGCA-GATCCTCATG) encoding the last 15 amino acids of mature STh and containing *SphI*-flanked ends was inserted in frame in the correct orientation into the *SphI* site of pHPC0838; the initial signal peptidase cleavage sequence (-<sup>3</sup>AHA-<sup>1</sup>↓<sup>+1</sup>WT<sup>+2</sup>) was changed to an improved processing site (-<sup>3</sup>AHA-<sup>1</sup>↓<sup>+1</sup>AD<sup>+2</sup>) based on the predictive accuracy estimation of von Heijne (24). Plasmids pSTDG126 and pSPGST1, expressing the last 15 amino acids of mature STh and the entire 19-amino acid sequence of mature STh, respectively, were constructed after insertion of paired oligonucleotides (the two 5' → 3' single coding strands were CGGCAGATCTGTACTGCTGTGAACCTTTGTTGTAATCTGCCTGTACAGGATGTTACTAAGTT and CGAATTCCTTCTAACTA-CTGCTGTGAACCTTTGTTGTAATCTGCCTGTACAGGATGTTACTAAGTT, respectively) between the *SphI* and *HpaI* sites of pHPC0838. Plasmid pSTC22 was constructed by cloning a synthetic DNA duplex (the 5' → 3' single coding strand was AACCTGATAACCCCGGGAACTACTGCTGTGAACCTTTGTTGTAATCTGCCTGTACAGGATGTTACTA-CTAA) encoding the last 16 amino acids of mature STh and containing *HpaI* and *SmaI* sites between the *HpaI* and *XbaI* sites of pHPC0838. Plasmid pSTC17 was pSTC22 with the *HpaI*-*SmaI* fragment deleted, thus placing a glycine precisely at the ClpG-STh junction. Plasmid pProSTC28 was made by inserting a blunt-ended DNA fragment (the 5' → 3' single coding strand was AACAAAAGTGGTCTGAATCGATGAATTCTAGC) encoding amino acid residues 46–53 (NKSGPESM) of the pro-STh peptide plus the first three residues (NSS) of mature STh between the *HpaI* and *SmaI* sites of pSTC22. We hypothesized that this natural linker might favor folding of the STh moiety in the hybrid in a more effective way. To obtain plasmid pEHProSTC28, the *MunI*-*Ecl136II* fragment of pProSTC28 containing the *clpG*::*Sth* fusion gene was subcloned in the low-copy number plasmid pEH524 within the *clp* operon in place of the *MunI*-*HpaI* fragment carrying native *clpG*. This same fusion gene was IPTG<sup>1</sup>-overexpressed by cloning the *KpnI*-*BglIII* fragment of pProSTC28 downstream of the *P<sub>trc</sub>* promoter between the *KpnI* and *BamHI* sites of pTrc99A, resulting in pTrcProSTC28. A synthetic gene (the 5' → 3' single coding strand was CGGTTAACATGAAT-TCTGCAGTT) encoding the last 16 amino acids of mature STh and containing an *HpaI* site was placed in frame between the *SphI* and *HpaI* sites of pSTC22, and the resulting construct was either *HpaI*/*HpaI*-deleted, leading to pGMSTC71, or *HpaI*/*SmaI*-deleted, leading to pGMSTC710. The same DNA fragment was also introduced between the *SphI* and *HpaI* sites of pProSTC28, and the resulting plasmid was *HpaI*/*HpaI*-deleted, leading to pGMSTC13/19.

All other standard techniques were performed essentially as described previously (25). The oligonucleotides used in this study were supplied by Eurogentec (Belgium). Fusion genes were checked by sequencing (26).

**Production of Fusion Proteins**—LB broth preculture (1 or 2 ml) containing exponentially growing cells was poured onto LB agar plates, which were incubated overnight at 37 °C in a humid atmosphere with the agar surface facing up. Bacteria were carefully harvested by scrap-

ing from the agar surface. After centrifugation at 12,000 × *g* for 10 min, the resulting supernatant was separated from the cell pellet and used as the solid culture supernatant fraction. Supernatants from LB broth cultures were obtained after centrifugation and used as the liquid culture supernatant fraction. The bacterial enumeration expressed in colony-forming units/ml was done by spreading out dilutions of PBS-suspended cells on MacConkey lactose agar medium containing the appropriate antibiotic.

**Competitive ELISA**—Competitive ELISA using a commercially available assay kit for *E. coli* STa (COLI ST EIA, Denka Seiken Co., Ltd., Tokyo, Japan) (27) was performed to detect and quantify fusion proteins in culture supernatants. The reactivity of the STa moiety of hybrids was determined using STa-monospecific horseradish peroxidase-conjugated antibodies and ELISA plates coated with synthetic STa peptide. After the wells were washed once with the supplied buffer, 200 µl of serially diluted samples and 10 µl of conjugated monoclonal antibody were added. After incubation for 90 min at room temperature, the contents were removed, and the wells were washed five times with buffer. Enzyme substrate solution (100 µl), prepared by adding H<sub>2</sub>O<sub>2</sub> solution to *o*-phenylenediamine, was added, and the plate was left in the dark at room temperature for 30 min. After the reaction was stopped by addition of 100 µl of 1.5 N H<sub>2</sub>SO<sub>4</sub>, the absorbance at 490 nm was measured using a Dynatech MR5000 microplate reader. A positive sample was identified by inhibition of binding of horseradish peroxidase-conjugated monoclonal antibodies to the well, as demonstrated by a decrease in A<sub>490</sub>. The positive samples were defined as giving A<sub>490</sub> < 0.2 (as this is a competitive assay), and the negative samples were defined as giving A<sub>490</sub> ≥ 1.2. The concentration of STa in culture supernatants was determined by reference to a standard curve, which was constructed using known concentrations of purified native STp (Calbiochem) ranging from 0 to 1.25 µg/ml.

**Double Antibody Sandwich ELISA**—Double antibody sandwich ELISA was performed for determination of the amount of ClpG in supernatant samples. Microtiter plates (Immulon 2, Dynatech Laboratories Inc.) were coated by overnight incubation at 4 °C with 100 µl of rabbit anti-ClpG antiserum (28) diluted (1:200) in 50 mM carbonate buffer (pH 9.6). After removal of the buffer, the plates were incubated overnight at 4 °C with blocking buffer (PBS, 2% dry milk, and 1% fetal calf serum) and washed twice with PBS and 0.05% Tween 20 and once with PBS. The supernatant samples were serially plated in 2-fold dilutions (100 µl/well) in antibody buffer (PBS, 0.2% dry milk, and 0.5% fetal calf serum) and incubated for 2 h at 37 °C. For the standard curve, known concentrations of purified ClpG in PBS were serially plated in 2-fold dilutions in antibody buffer. Purification of native ClpG has been described elsewhere (28). After washing twice with PBS and 0.05% Tween 20 and once with PBS, 100 µl of a 1:500 dilution of mouse anti-ClpG antiserum were added to each well, and the plates were incubated for 1.5 h at 37 °C. After washing, 100 µl of a 1:1000 dilution of goat anti-mouse IgG were added, and the plates were incubated for 2 h at 37 °C. After washing, 100 µl of 2 mM H<sub>2</sub>O<sub>2</sub> and 10 mg of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (Sigma) substrate in 100 ml of citrate/phosphate buffer (80 mM citric acid and 125 mM Na<sub>2</sub>HPO<sub>4</sub>) were added, and the plates were incubated for 20 min in the dark at room temperature. The absorbance at 405 nm was read on a Dynatech MR5000 reader. An overnight supernatant of *E. coli* DH5α (pDSPH524) served as a negative control. In terms of molecular mass, ClpG represents 93% of the hybrid protein. Therefore, 1 µg of ClpG in the fusion protein mixture is equivalent to ~1.1 µg of ClpG-STh hybrid.

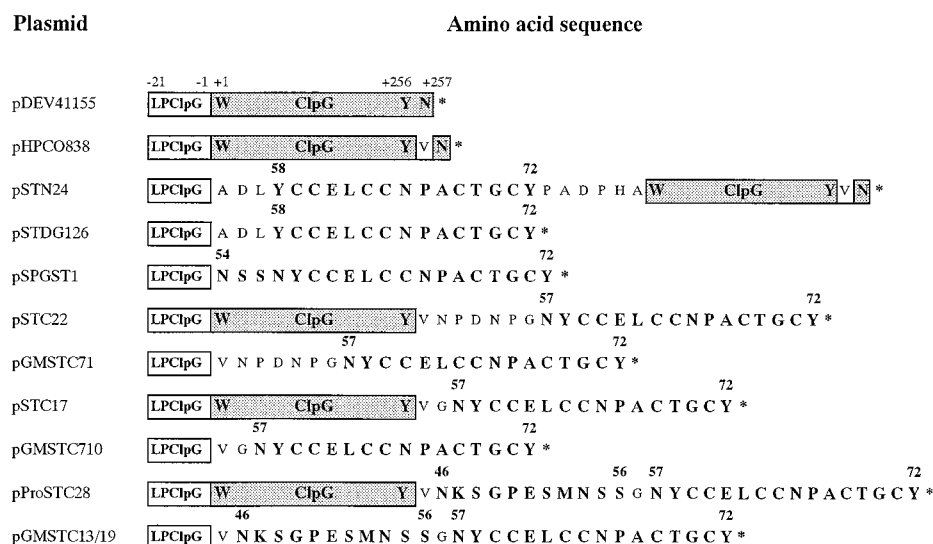
**Nonreducing SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—Samples containing the hybrids were mixed with an equal volume of 2× Laemmli buffer without reducing agent, applied without boiling to a 12% SDS-polyacrylamide gel, and semidry-electrotransferred onto 0.2-µm nitrocellulose paper (Bio-Rad) as described by Towbin *et al.* (29). Blots were blocked and washed with 1% bovine serum albumin and 0.1% Tween 20 in Tris-buffered saline and incubated overnight with rabbit ClpG-specific antiserum or with a mixture of the STa-specific monoclonal antibodies 11C (30) and 20C1 (31). For probing with anti-ClpG antibodies, filters were developed with horseradish peroxidase-labeled goat anti-rabbit secondary antibodies and with H<sub>2</sub>O<sub>2</sub>/α-chloronaphthol as a substrate. For probing with anti-STa antibodies, a 1:1000 dilution of biotinylated goat anti-mouse IgG (Sigma) in Tris-buffered saline and 0.05% Tween 20 was used as a secondary antibody. After incubation for 1 h and washing, membranes were again incubated for 30 min with a 1:100 dilution of horseradish peroxidase-conjugated streptavidin (250 µg/ml; Sigma) in Tris-buffered saline and 0.05% Tween 20 and developed with H<sub>2</sub>O<sub>2</sub>/α-chloronaphthol.

**Assay for Enterotoxin Activity**—The enterotoxin activity of superna-

<sup>1</sup> The abbreviations used are: IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; G/C, gut weight/carcass weight ratio; DTT, dithiothreitol; AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid.



**FIG. 1. Structures of the fusion proteins.** In pSPGST1, STh is directly fused to the leader peptide of ClpG (LPClpG), whereas pSTDG126, pGMSTC71, pGMSTC13/19 are pSTN24, pSTC22, pSTC17, and pProSTC28 deleted of *clpG*, respectively. The shaded boxes represent the native mature ClpG protein, and the lightface numbers above the boxes are the positions of the indicated amino acid residues relative to signal peptide cleavage site -1/+1 of the ClpG precursor sequence. Residues from the original STh (STa3) precursor (prepro-STa3) are shown in **boldface**, and additional residues are in *lightface*. The boldface numbers refer to the positions of the indicated amino acid residues relative to the prepro-STa3 precursor sequence. Asterisks indicate stop codons (TAA) after the *STh* or *clpG* gene.



tant fractions was examined in the suckling mouse assay as described previously (32). Three-day-old Swiss OF1 suckling mice were separated from their mothers immediately before use and randomly divided into groups. A 0.1-ml aliquot of each sample was directly delivered to the stomachs of infant mice using flexible plastic tubing. Three hours later, the entire intestine from each mouse was removed and weighed, and the ratio of the gut weight to remaining carcass weight (G/C) was calculated. The mean G/C ratio and the S.E. of several separated assays were determined for each mouse batch. A G/C ratio of  $\geq 0.090$  corresponded to the unambiguous accumulation of fluid in the gut lumen. One mouse unit was defined as the enterotoxin activity corresponding to a minimum effective dose that gave a positive response (G/C  $\geq 0.090$ ), and the enterotoxin titer was expressed as the highest dilution that gave 1 mouse unit of enterotoxin activity. The G/C value was plotted semilogarithmically versus dilution of the sample fraction, and the point at which the curve crossed the line equal to a G/C ratio of 0.090 was defined as the dilution that corresponds to 1 mouse unit of STh. In our study, the minimum effective dose of STh necessary to produce an activity of 1 mouse unit was 8 ng, as determined using a standard curve, which was developed following intragastric inoculation of suckling mice with 0.1 ml of sterile PBS containing amounts of pure STp ranging from 0.2 to 100 ng (data not shown).

**Redox State of STh in the Fusion Protein**—The redox state of fusion proteins was assessed as described previously (33). To determine the redox state of intracellular fusion proteins, bacteria were cultured in LB broth at 37 °C with or without 5 mM dithiothreitol (DTT). Cultures (25 ml) were grown to  $A_{550} \sim 0.5$  and centrifuged. Cell pellets were suspended in PBS and mixed with an equal volume of 10% trichloroacetic acid in PBS, followed by incubation on ice for 1 h. For determination of the redox state of extracellular fusion proteins, supernatants were treated or not with 100 mM DTT for 10 min at 37 °C before trichloroacetic acid treatment as described above. In all cases, precipitates of trichloroacetic acid-denatured proteins were pelleted by centrifugation and washed with cold acetone to remove trace acid. The samples were centrifuged, and the acetone supernatants were discarded. The resulting pellets were air-dried and dissolved in a solution (freshly made) containing 50 mM Tris-HCl (pH 8.0), 1% SDS, 1 mM EDTA, and 10 mM 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS; Molecular Probes, Inc.). Proteins were separated by 12% SDS-polyacrylamide gel electrophoresis without using any reducing agent. Reduced and oxidized forms of proteins were visualized on Western blots as described above. AMS reacts specifically and irreversibly with sulfhydryl groups, conjugating a large 490-Da moiety to the free cysteines (34). This major change in molecular mass is used to clearly separate oxidized from reduced proteins on SDS-polyacrylamide gels (33). Indeed, unlike oxidized forms, reduced forms, which are sensitive to reaction with AMS, shift to a high molecular mass protein position.

## RESULTS

**DsbA and DsbB Are Not Required for Secretion and Enterotoxicity of STh Chimeras**—Plasmids carrying various *clpG::STh* fusion genes (Fig. 1) were introduced into *E. coli* strains JCB570 (*dsbA*<sup>+</sup>*dsbB*<sup>+</sup>), JCB571 (*dsbA*<sup>-</sup>), JCB819

(*dsbB*<sup>-</sup>), and JCB818 (*dsbA*<sup>-</sup>*dsbB*<sup>-</sup>) (Table I). The transformants were grown overnight in LB broth or LB agar, and the supernatant fractions were analyzed for STh production by competitive ELISA and for STh activity by the suckling mouse assay. All STh chimeras were secreted with high biological activity in solid and liquid media by both the *dsb*<sup>+</sup> wild-type and *dsb*<sup>-</sup> mutant cells (Table II). The percentage of positive mice reached 100% for each of the recombinant plasmids tested in all mutant strains and in both solid and liquid media. Activity was 80–170-fold higher in liquid medium than in solid medium (data expressed in mouse units/10<sup>10</sup> colony-forming units in Table II), probably because of a better air oxygenation. In comparison to the wild-type strain, the production and enterotoxicity levels of the *dsb*<sup>-</sup> mutants were mostly improved. Nevertheless, STh-specific activities (data expressed in mouse units/ $\mu$ g of STh in Table II) remained for the most part unchanged, indicating a similar DsbA/DsbB-independent toxicity against these various fusion proteins. This is in contrast with other reports showing that the *dsbA* mutation caused a marked reduction in the secretion (13) and activity (12) of the native toxin. Therefore, questions remained regarding whether our results might be accounted for by the presence of ClpG or by a periplasmic leakage due to the high-copy number of the recombinant plasmids.

First, we genetically deleted ClpG from ClpG-STh fusions (Fig. 1). In all cases, the *dsb*<sup>+</sup> and *dsb*<sup>-</sup> strains secreted active STh (Table III), indicating that STh free of ClpG reached the extracellular milieu simply upon its entry into the periplasm, once guided by the leader peptide of ClpG. However, the production and activity of STh were mostly slightly higher in the presence (Table II) than in the absence (Table III) of ClpG.

Second, we subcloned most of the *clpG::STh* fusion genes in a pSC101-related plasmid (Table I). Only results relative to the fusion protein carried by pProSTC28 (Fig. 1) are preferentially reported in the rest of our study, especially since all the ClpG-STh chimeras acted similarly. As shown in Table II, the STh chimera expressed by the corresponding low-copy number recombinant plasmid pEHProSTC28 was released as an active fusion protein whatever the *dsb* background. Therefore, the discrepancy (as discussed above) between our findings and those of Yamanaka *et al.* (12, 13) cannot merely be the result of the hybrid state of STh or a periplasmic leakage in our experiments, although pEHProSTC28 displayed a 1.7–7.8-fold reduction in activity compared with pProSTC28 (Table II). Furthermore, none of the test periplasmic and cell lysate fractions from *dsb*<sup>+</sup> and *dsb*<sup>-</sup> cells producing pProSTC28-encoded STh

TABLE I  
Strains and plasmids used in this study

Strain/plasmid	Relevant genotype or phenotype	Ref. or source
<i>E. coli</i>		
B41	Bovine ETEC <sup>a</sup> isolate (0101:K <sup>-</sup> :H <sup>-</sup> :K99,F41) producing STp	18
DH5α	F <sup>-</sup> <i>supE44</i> , Δ( <i>argF-lacZYA</i> ) <i>U169</i> (Δ80d <i>lacZΔM15</i> ), <i>hsdR17</i> (rk <sup>-</sup> mk <sup>+</sup> ), <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> (Nal <sup>r</sup> ), <i>thi-1</i> , <i>relA1</i>	Life Technologies, Inc.
JCB570	<i>araD139</i> Δ( <i>araABC-leu</i> ) 7679 <i>galU galK</i> Δ( <i>lac</i> ) <i>X74 rpsL thi phoR zih12::Tn10</i>	14
JCB571	JCB570 <i>dsbA::kan1</i>	14
JCB819	JCB570 ( <i>malF-lacZ102</i> ) <i>dsbB::kan1</i>	19
JCB818	JCB570 ( <i>malF-lacZ102</i> ) <i>dsbA::kan1 dsbB::kan1</i>	Bardwell collection
Plasmid		
pSK <sup>+</sup>	High-copy number cloning vector pBluescript SK <sup>+</sup> , pColE1 <i>ori</i> ; Ap <sup>R</sup>	Stratagene
pDEV41155	pSK <sup>+</sup> carrying the <i>clpG</i> gene	20
pHPCO838	pDEV41155 with an additional <i>GTT</i> triplet just before the final codon AAC of <i>clpG</i>	This study
pSTN24	pHPCO838 with a synthetic <i>STh</i> gene fused to the 5'-end of <i>clpG</i>	This study
pSTC22	pHPCO838 with a synthetic <i>STh</i> gene fused to the 3'-end of <i>clpG</i>	This study
pProSTC28	pHPCO838 with a synthetic <i>STh</i> gene fused to the 3'-end of <i>clpG</i>	This study
pSTC17	pHPCO838 with a synthetic <i>STh</i> gene fused to the 3'-end of <i>clpG</i>	This study
pSPGST1	pHPCO838 with <i>clpG</i> deleted and <i>STh</i> directly fused to the ClpG leader peptide	This study
pSTDG126	pSTN24 with <i>clpG</i> deleted	This study
pGMSTC71	pSTC22 with <i>clpG</i> deleted	This study
pGMSTC710	pSTC17 with <i>clpG</i> deleted	This study
pGMSTC13/19	pProSTC28 with <i>clpG</i> deleted	This study
pHSG575	Low-copy number cloning vector, pSC101 <i>ori</i> ; Cm <sup>R</sup>	21
pEH524	pHSG575 carrying the CS31A fimbria-encoding <i>clp</i> operon	22
pDSPH524	pEH524 with <i>clpG</i> deleted	20
pEHSTN24	pEH524 in which <i>clpG</i> was replaced by the <i>clpG::Sth</i> fusion gene from pSTN24	This study
pEHSTC22	pEH524 in which <i>clpG</i> was replaced by the <i>clpG::Sth</i> fusion gene from pSTC22	This study
pEHProSTC28	pEH524 in which <i>clpG</i> was replaced by the <i>clpG::Sth</i> fusion gene of pProSTC28	This study
pTrc99A	Expression vector with IPTG-inducible P <sub>trc</sub> promoter, <i>lacI<sup>P</sup></i> , <i>rrnB</i> ; pColE1 <i>ori</i> ; Ap <sup>R</sup>	Amersham Pharmacia Biotech
pTrcProSTC28	pTrc99A carrying the <i>clpG::Sth</i> fusion gene of pProSTC28	This study

<sup>a</sup> ETEC, enterotoxigenic *E. coli*; Ap, ampicillin; Cm, chloramphenicol.

exhibited enterotoxicity (Table IV), strengthening the idea that active STh was absent in these cells.

**Extracellular STh from *dsb*<sup>+</sup> and *dsb*<sup>-</sup> Cells Is Properly Folded, in Contrast to Intracellular STh**—The three correct intramolecular disulfide bonds of STa are needed for enterotoxicity (5). Therefore, the above findings strongly suggest that, in contrast to intracellular STh, extracellular STh from the *dsb*<sup>+</sup> and *dsb*<sup>-</sup> strains was correctly folded in an active disulfide-bonded form. We verified this hypothesis by determining the redox state of STh outside (Fig. 2A) and inside (Fig. 2B) these bacteria.

Extracellular fusion protein from the JCB570 (*dsbA*<sup>+</sup>) and JCB571 (*dsbA*<sup>-</sup>) strains appeared as two clearly distinguishable protein species (Fig. 2A, -AMS, -DTT). These two species do not represent singly reduced and oxidized forms since they tandemly migrated identically with or without AMS when not reduced by DTT. They consist of oxidized species since they were tandemly subjected to an upward change in position on immunoblots when treated with both AMS and DTT, but not with AMS alone. The shift of the band position signifies the chemical binding of AMS to sulfhydryl groups generated by the conversion of oxidized cysteines by DTT (see "Experimental Procedures"). The two oxidized species do not correspond to disulfide-bonded isoforms since they did not migrate as one band once reduced by DTT prior to AMS derivatization. Taken together with the finding that they reacted with STh-specific antibodies (not shown in Fig. 2A since they are indicated in Fig. 3c, samples marked Cox and O.N.), these results identify the upper and lower species as the folded full-length chimera and presumably a truncated disulfide-bonded fusion protein derivative, respectively. Thus, STh in the fusion protein was totally oxidized in the *dsbA*<sup>+</sup> and *dsbA*<sup>-</sup> supernatants.

To determine the redox state of intracellular STh, the JCB570 and JCB818 (*dsbA*<sup>-</sup>*dsbB*<sup>-</sup>) strains carrying pProSTC28 were directly cultured with or without DTT prior to AMS treatment. As shown in Fig. 2B, AMS-conjugated fusion proteins reduced or not with DTT had similar electrophoretic

profiles, in which they appeared as two distinguishable protein species. They represent reduced forms since they tandemly migrated identically at the "red" position on immunoblots when treated with only AMS or additionally with DTT and since they migrated more slowly than the DTT/AMS-untreated oxidized control. In addition, given that they did not react with antibodies to the ClpG portion as one band once treated with DTT, they do not consist of reduced isoforms, but most certainly of unoxidized precursor and mature forms. Moreover, no sample reacted with STa-monospecific antibodies (data not shown), which are known to react less or not at all with reduced toxin (30), thus confirming that the STh chimera is unoxidized in JCB570 and JCB818 cells. Together with the fact that, in agreement with earlier studies (12, 13), no intracellular enterotoxin activity was found in the JCB570 strain (Table IV), these findings support the conclusion that disulfide formation does not happen in the periplasmic space through the DsbA pathway, in clear contrast to a previous report (12).

**STh Is Secreted as an Unfolded Intermediate before Being Extracellularly Disulfide-bonded**—The above data suggest that STh from the JCB570 (*dsbA*<sup>+</sup>) and JCB571 (*dsbA*<sup>-</sup>) strains folded after transit through the periplasm. To confirm this hypothesis, the redox state of STh was determined as soon as it translocated across the outer membrane during secretion by JCB570 and JCB571. For this purpose, we cloned the fusion gene of pProSTC28 under the control of the IPTG-inducible P<sub>trc</sub> promoter. The resulting plasmid, pTrcProSTC28 (Table I), was transferred into the JCB570 and JCB571 strains, and an IPTG-induced time course of folding (increased oxidized form) was performed (Fig. 3). A supernatant sample from the induced and uninduced cultures was taken at different time points and processed for determination of both the redox state and enterotoxin activity of the STh chimera.

Approximately equal amounts of the oxidized form (resistant to AMS (Fig. 3b) and reacting with anti-STh antibodies (Fig. 3c)) and the reduced form (sensitive to AMS and lacking affinity for anti-STh antibodies) were found early in the culture

TABLE II  
Secretion and activity of extracellular STh chimeras

Strain/plasmid	Solid medium				Liquid medium	
	Production <sup>a</sup> ng STh/10 <sup>10</sup> cfu <sup>b</sup>	Enterotoxigenicity			Enterotoxigenicity	
		G/C <sup>c</sup>	MU/10 <sup>10</sup> cfu	MU/ $\mu$ g STh <sup>d</sup>	G/C	MU/10 <sup>10</sup> cfu
B41 <sup>e</sup>	123	0.120 $\pm$ 0.015	7.5	63	0.134 $\pm$ 0.020	329
JCB570 dsbA <sup>+</sup> dsbB <sup>+</sup>						
pHPC0838 <sup>f</sup>	ND	0.062 $\pm$ 0.002	NT	NT	NT	NT
pSTN24	NM	0.131 $\pm$ 0.031	3.5	NM	0.116 $\pm$ 0.033	236
pSTC22	30	0.149 $\pm$ 0.008	3.6	120	0.150 $\pm$ 0.015	1136
pSTC17	39	0.161 $\pm$ 0.009	5.2	133	0.161 $\pm$ 0.019	909
pProSTC28	33	0.154 $\pm$ 0.012	8.4	254	0.153 $\pm$ 0.023	1072
pEHProSTC28	NT	0.142 $\pm$ 0.010	3.6	NT	NT	NT
JCB571 dsbA <sup>-</sup> dsbB <sup>+</sup>						
pSTN24	NM	0.143 $\pm$ 0.012	5.3	NM	0.150 $\pm$ 0.018	909
pSTC22	132	0.126 $\pm$ 0.008	18.2	138	0.148 $\pm$ 0.035	1173
pSTC17	57	0.133 $\pm$ 0.020	12.5	219	0.151 $\pm$ 0.020	1430
pProSTC28	112	0.141 $\pm$ 0.011	14.0	125	0.136 $\pm$ 0.025	1950
pEHProSTC28	NT	0.135 $\pm$ 0.033	8.2	NT	NT	NT
JCB819 dsbA <sup>+</sup> dsbB <sup>-</sup>						
pSTN24	NM	0.150 $\pm$ 0.025	9.9	NM	0.131 $\pm$ 0.031	1140
pSTC22	61	0.141 $\pm$ 0.017	10.9	178	0.170 $\pm$ 0.010	1444
pSTC17	51	0.137 $\pm$ 0.024	16.1	315	0.151 $\pm$ 0.020	609
pProSTC28	55	0.134 $\pm$ 0.023	16.4	298	0.140 $\pm$ 0.022	1894
pEHProSTC28	NT	0.134 $\pm$ 0.020	2.1	NT	NT	NT
JCB818 dsbA <sup>-</sup> dsbB <sup>-</sup>						
pSTN24	NM	0.167 $\pm$ 0.006	5.8	NM	0.157 $\pm$ 0.007	708
pSTC22	98	0.143 $\pm$ 0.026	9.3	95	0.161 $\pm$ 0.007	727
pSTC17	122	0.160 $\pm$ 0.008	17.1	140	0.145 $\pm$ 0.025	1140
pProSTC28	110	0.166 $\pm$ 0.014	17.0	155	0.157 $\pm$ 0.039	1172
pEHProSTC28	NT	0.115 $\pm$ 0.021	2.4	NT	NT	NT

<sup>a</sup> The amount of STh hybrids in supernatants was estimated by a competitive ELISA from a standard curve as described under "Experimental Procedures."

<sup>b</sup> cfu, colony-forming units; MU, mouse units (the activity corresponding to a minimum effective dose that gives a G/C ratio of 0.090); ND, not detectable in undiluted sample following ELISA; NT, not tested; NM, not measurable in ELISA because of a lack of affinity for anti-STa monoclonal antibodies.

<sup>c</sup> Each value represents the mean G/C ratio  $\pm$  S.E. of a group of five suckling mice. Values  $\geq$  0.090 were considered positive.

<sup>d</sup> Specific activity.

<sup>e</sup> Strain used as a positive reference control.

<sup>f</sup> Plasmid used as a negative control.

TABLE III  
Effect of ClpG deletion on secretion and activity of STh

Strain/plasmid	Solid medium			Liquid medium		
	Production <sup>a</sup> ng STh/10 <sup>10</sup> cfu <sup>b</sup>	Enterotoxigenicity		Production ng STh/10 <sup>10</sup> cfu	Enterotoxigenicity	
		G/C <sup>c</sup>	Score <sup>d</sup>		G/C	Score
JCB570 dsbA <sup>+</sup>			%			%
pSPGST1	23	0.130 $\pm$ 0.035	100	11	0.085 $\pm$ 0.012	66
pSTDG126	23	0.115 $\pm$ 0.036	66	20	0.087 $\pm$ 0.003	50
pGMSTC71	9	0.092 $\pm$ 0.017	42	16	NT	NT
pGMSTC710	14	0.113 $\pm$ 0.030	66	13	NT	NT
pGMSTC13/19	31	0.124 $\pm$ 0.016	100	25	NT	NT
JCB571 dsbA <sup>-</sup>						
pSPGST1	10	0.120 $\pm$ 0.024	100	14	0.082 $\pm$ 0.020	50
pSTDG126	12	0.099 $\pm$ 0.016	83	21	0.090 $\pm$ 0.004	75
pGMSTC71	9	0.099 $\pm$ 0.021	75	22	0.106 $\pm$ 0.034	50
pGMSTC710	9	0.095 $\pm$ 0.025	57	23	0.091 $\pm$ 0.008	50
pGMSTC13/19	30	0.132 $\pm$ 0.027	100	25	0.124 $\pm$ 0.033	100

<sup>a</sup> As described in Table II, Footnote a.

<sup>b</sup> cfu, colony-forming units; NT, not tested.

<sup>c</sup> Each value represents the mean G/C ratio  $\pm$  S.E. of a group of three to seven suckling mice.

<sup>d</sup> Number of positive mice/total number of mice.

supernatants of JCB571 (<15 min after induction). Apparently, the level of the reduced form decreased with time (Fig. 3b), whereas that of the oxidized form increased (Fig. 3c) so that a completely oxidized state was observed at the last time point (Fig. 3, b and c, O.N.). Likewise, both the enterotoxin activity (proper folding) (Fig. 3a) and concentration of hybrids in the supernatants increased with time (see legend to Fig. 3). Taken together, these observations support the idea that an increase in the oxidized form parallels a decrease in the reduced form, although, in view of Fig. 3b, it is not clear whether

there is really more protein in the area of the oxidized form. Fig. 3b suggests that the quantity of the hybrid protein detected at 15 and 40 min was more than that at 65, 90, and 115 min, although its concentration in the supernatant increased with time. The most likely explanation is that variations in protein band thickness reflect variations in the yield of proteins recovered after trichloroacetic acid precipitation. The oxidized form first migrated as one band (Fig. 3c, 15 min and 40 min) and then as two bands reacting to anti-STa antibodies (Fig. 3c, 65–115 min), the upper appearing last. Emergence of the lower



TABLE IV  
Intracellular activity of STh chimeras

Strain/plasmid	Enterotoxigenicity <sup>a</sup>	
	G/C <sup>b</sup>	
	Periplasm	Lysate
JCB570 <i>dsbA</i> <sup>+</sup> <i>dsbB</i> <sup>+</sup> , pProSTC28	0.067 ± 0.017	0.059 ± 0.005
JCB571 <i>dsbA</i> <sup>+</sup> <i>dsbB</i> <sup>+</sup> , pProSTC28	0.067 ± 0.016	NT <sup>c</sup>
JCB819 <i>dsbA</i> <sup>+</sup> <i>dsbB</i> <sup>+</sup> , pProSTC28	0.063 ± 0.008	NT
JCB818 <i>dsbA</i> <sup>+</sup> <i>dsbB</i> <sup>+</sup> , pProSTC28	NT	0.077 ± 0.009

<sup>a</sup> After the cells were cultured in solid medium and harvested by centrifugation, the pellets were suspended in 10 mM Tris-HCl buffer (pH 7.5). The suspension was then divided in two equal parts. One part was sonicated and centrifuged, and the resulting supernatant is referred to as the cell lysate fraction. The other part was treated with polymyxin B to prepare the periplasmic fraction as described by Yamanaka *et al.* (13).

<sup>b</sup> Each value represents the mean G/C ratio ± S.E. of a group of 5–12 suckling mice.

<sup>c</sup> Not tested.

oxidized protein species coincided with that of STh enterotoxigenicity (Fig. 3, *a* and *c*, 15 and 40 min), suggesting that it was correctly folded. Both AMS-untreated and AMS-derivatized overnight supernatant samples (Fig. 3, *b* and *c*, *Cox* and *O.N.*, respectively) migrated identically, indicating that neither of the two protein species included any free thiol groups. In addition, simultaneous treatment with DTT and AMS generated only one band, confirming that supernatants sampled between 65 and 115 min after induction contained two disulfide-bonded isoforms. Consequently, there is a contradiction between these data and those presented in Fig. 2A showing that an overnight extracellular STh chimera appeared as two oxidized protein bands instead of only one band when treated with both AMS and DTT. In agreement with our previous hypothesis about the redox state of extracellular STh (see “Extracellular STh from *dsb*<sup>+</sup> and *dsb*<sup>−</sup> Cells Is Properly Folded, in Contrast to Intracellular STh” under “Results”), the most probable explanation for these conflicting results is that the upper oxidized isoform seen in Fig. 3c (65–115 min after induction) is subsequently subjected to proteolytic cleavage, perhaps due to a protease-sensitive misfolded structure (as native toxin is resistant to proteases (35)), thus generating the overnight lower oxidized species seen in Figs. 2A (−AMS, −DTT) and 3 (*Cox*). No protein material (Fig. 3, *b* and *c*, 115 min, −IPTG, +AMS, −DTT) and no activity (Fig. 3a) were detected in the absence of inducer, demonstrating that we were analyzing the STh hybrid.

Overall, the experiments with JCB570 led to the same conclusions as those with JCB571. Indeed, both oxidized and reduced forms emerged in the IPTG-induced culture supernatants of JCB570 and were eventually completely converted overnight into an active oxidized form (data not shown). The latter form migrated as two distinguishable bands after treatment with AMS alone and as one band after treatment with both AMS and DTT, demonstrating that the upper and lower oxidized species corresponded to two disulfide-bonded isoforms. A slight difference with respect to JCB571 is that the lower isoform from JCB570, which probably represents an active chimera since it correlated with STh activity, appeared later (90 min after induction) than the active lower isoform from JCB571 (Fig. 3c), thus probably explaining why production and toxicity levels were higher in the *dsb*<sup>−</sup> mutants than in the *dsb*<sup>+</sup> wild-type strain (Table II). In conclusion, chimeric STh translocates across the outer membrane as an unfolded intermediate and folds correctly in the extracellular environment through a *DsbA*-independent pathway.

#### DISCUSSION

To overcome the difficulties in the analysis of processing of *E. coli* heat-stable enterotoxin STa, we genetically fused different

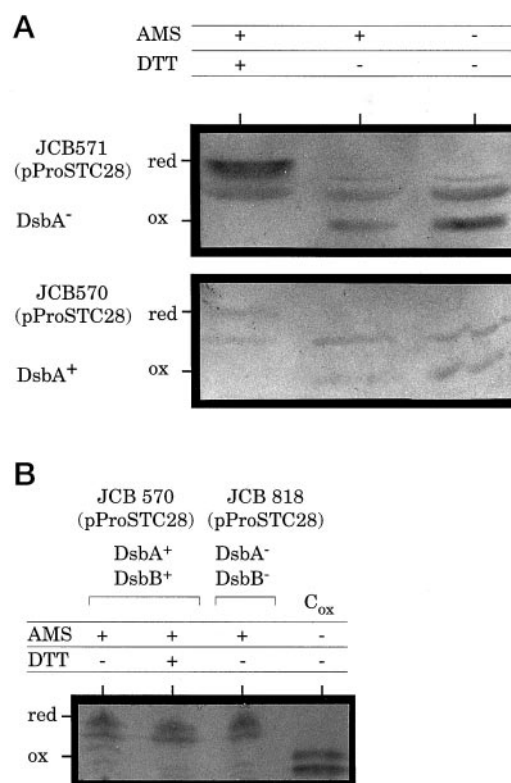
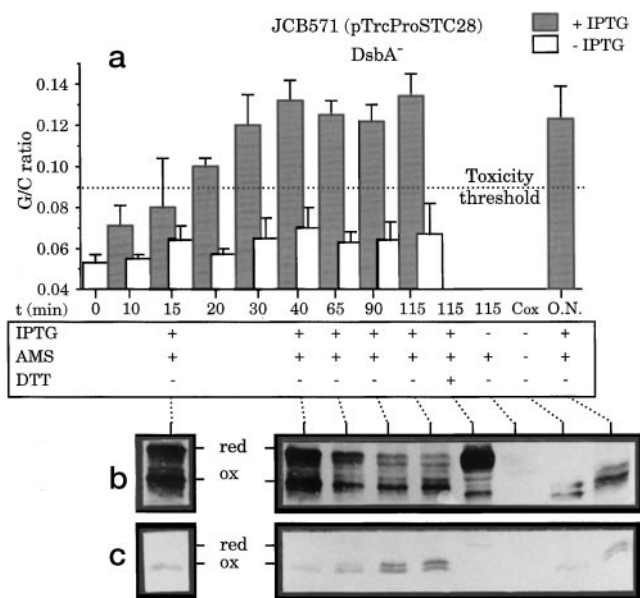


FIG. 2. Redox states of chimeric STh. A, redox state of extracellular STh. The indicated recombinant strains were cultured overnight in LB broth (50 ml) to  $\sim 1.1 \times 10^9$  cells/ml. Supernatants (25 ml) were (+) or were not (−) first reduced with 100 mM DTT at 37 °C for 10 min before being precipitated on ice for 1 h with 5% trichloroacetic acid (final concentration). The concentration of hybrid proteins in the supernatants was determined through a double antibody sandwich ELISA, which enabled the quantification of ClpG as described under “Experimental Procedures.” It was estimated at  $\sim 3.6$   $\mu$ g/ml for JCB570 (pProSTC28) and 4.4  $\mu$ g/ml for JCB571 (pProSTC28). B, redox state of intracellular STh. The indicated strains were grown to  $A_{550} \sim 0.5$  in LB broth containing (+) or not (−) a nonlethal concentration of DTT (5 mM). After cell density monitoring,  $2.5 \times 10^9$  cells were suspended in PBS. Cell lysis and precipitation of whole cell proteins were performed by direct treatment of cell suspensions with an equal volume of 10% trichloroacetic acid, followed by incubation on ice for 1 h. In all cases (A and B), protein precipitates were collected by centrifugation, washed with acetone, air-dried, and dissolved in 200  $\mu$ l of freshly prepared solution containing 1% SDS, 50 mM Tris-HCl (pH 8.0), and 1 mM EDTA with (+) or without (−) 10 mM AMS. Samples (40  $\mu$ l) were added to 40  $\mu$ l of 2 $\times$  SDS sample buffer without reducing agent, and the mixture was loaded on each lane of a 12% SDS-polyacrylamide gel. After electrophoresis followed by electrotransfer onto nitrocellulose paper, the separated oxidized (ox) and reduced (red) forms of the fusion protein were immunolabeled with anti-ClpG antibodies. Reaction of the STh chimera with AMS meant that we were analyzing the redox state of the cysteines in the STh part of the hybrid since ClpG protein contains no cysteine residues (17). The position labeled red refers to the AMS-conjugated unoxidized ClpG-STh chimera, and the position labeled ox designates either the oxidized ClpG-STh hybrid treated or not with AMS or the unoxidized ClpG-STh fusion protein not conjugated to AMS. *C<sub>ox</sub>* designates the oxidized form control corresponding to the extracellular STh hybrid from *E. coli* DH5 $\alpha$  (pEHProSTC28).

STh peptides to the N or C terminus of the major protein subunit of *E. coli* CS31A fimbriae (ClpG). Four fusion proteins were obtained. In all cases, STh was able to mobilize ClpG to the extracellular environment while still retaining its native biological properties. Therefore, the conformation of the native toxin compatible with secretion and activity must be very flexible since the STh moiety in the different hybrids is certainly not always the same. This is consistent with an x-ray crystallography study of mature STa peptide indicating inherent flexibility at the junctions of the three segments and in the central



**FIG. 3. Time course of extracellular folding.** The JCB571 (*dsbA*<sup>-</sup>) strain was grown in 500 ml of LB broth to  $A_{600} \sim 0.6$ , and expression of fusion proteins was (+) or was not (-) induced by addition of 2.5 mM IPTG, followed by continued shaking at 37 °C. Culture fractions (25 ml) were taken at the indicated times and immediately centrifuged to discard the cell pellets. At each time point, a volume (3 ml) of supernatant was taken and stored at -20 °C until it was used to evaluate enterotoxin activity (G/C ratio) in the suckling mouse assay (a) and to determine the levels of hybrid protein by a double antibody sandwich ELISA as described in the legend to Fig. 2. The concentrations of ClpG at 10, 15, 20, 30, 40, 65, 90, and 115 min and overnight (O.N.) were ~0.7, 1.2, 1.4, 1.9, 2.2, 2.8, 3.5, 4.0, and 4.5  $\mu$ g/ml, respectively. The rest of the supernatant was subjected to DTT, trichloroacetic acid, AMS, and nonreducing SDS-polyacrylamide gel electrophoresis treatments as described in the legend to Fig. 2. The mobility of hybrid proteins was visualized by Western blotting using ClpG antisera (b) and anti-STa antibodies consisting of a mixture of monoclonal antibodies 11C and 20C1 (c). The electrophoretic mobility of proteins from overnight supernatants (b and c), which appeared as two distorted bands in the far right end of the gel, was unexpectedly delayed due to an unusual migration. Cox, the oxidized form control corresponding to the extracellular STh hybrid from *E. coli* DH5 $\alpha$  (pEHProSTC28); ox and red, the oxidized and reduced forms of the fusion protein, respectively. The dotted line equal to a G/C ratio of 0.090 represents the toxicity threshold above which the samples are considered positive.

segment composing the molecule (36). In addition, a strict specific three-dimensional structure of STh compatible with release is unlikely as there is secretion of unfolded STa fused (this study) or not (13) to a foreign protein. Furthermore, as discussed below, the STh part of the secreted chimera folded in a biologically active disulfide-bonded form, dismissing the idea that eventual folding took place after release of the STh moiety from the fusion protein by some proteolytic events. There have been a number of efforts directed at developing genetic fusions between STa and a heterologous protein other than ClpG (11). However, in most cases, the resulting hybrids were defective in secretion and enterotoxin activity. Removal of ClpG from the ClpG-STh fusions negatively influenced (but did not completely abolish) production and thus activity. The explanation for the slight effect of ClpG may be that it improved secretion by acting on the three-dimensional structure of its cognate leader peptide for correct inner membrane presentation, allowing optimal signal peptidase recognition. Conversely, ClpG alone was previously shown to be extracellularly undetectable in the absence of the CS31A fimbrial helper proteins (20). Altogether, these data suggest that mature STh might be able to gain access to the extracellular milieu upon its entry into the *E. coli* periplasm, once guided into this compartment by the ClpG leader peptide. Together with the fact that the natural pre- and pro-STa neigh-

boring sequences are not essential for full secretion of mature STa (Refs. 8 and 15 and this work), these findings led us to conclude that STh in the fusion protein folds in a similar manner to the free native toxin and to hypothesize that STh governs the translocation of ClpG-STh chimeras across the outer membrane.

We have found several discrepancies between our results and those of Yamanaka *et al.* (12, 13), who support the notion that disulfide bonds are formed before translocation of STa across the outer membrane by the catalysis of DsbA in the periplasm. First, in agreement with our study, they observed no STa activity in cell lysate fractions from *E. coli* JCB570 (12), thus favoring the idea that STa with correct disulfide bonds is absent in *dsbA*<sup>+</sup> cells. Second, they reported that the *dsbA* mutation caused a marked reduction in STa activity (12), thus concluding that DsbA takes part in disulfide bond formation. However, the amount of STa produced by JCB571 (*dsbA*<sup>-</sup>) might be diminished in relation to that produced by JCB570, thus having repercussions on the activity level. In addition, in more recent studies, the authors showed that production of toxin by JCB571 was actually lower (13). Therefore, quantification of the STa peptide in supernatants of strains JCB570 and JCB571 allowing the measure of toxicity in terms of specific activity is essential. In our hands, the use of these same *E. coli* recipient strains revealed no negative effect of *dsbA* and *dsbB* mutations on secretion and enterotoxicity. Third, Yamanaka *et al.* (12) also concluded that DsbA participated in the disulfide bond formation of STa based on two additional indirect approaches: the induction of conversion of an inactive synthetic STa peptide into an active form and an increase in the STa activity of the culture supernatant derived from JCB571 merely by incubation with purified DsbA protein. The same results should remain unchanged with another thiol-disulfide oxidoreductase or any strong oxidizing agent. Last, we showed that at steady state, the intracellular STh chimera was present exclusively in a reduced form in wild-type cells and in *dsbA*<sup>-</sup>*dsbB*<sup>-</sup> cells. On the other hand, a mixture of the oxidized and reduced forms was detected early in the culture supernatants of JCB570 and JCB571 and eventually shifted to a completely oxidized form. Considering that the ratio of reduced to oxidized proteins in the culture supernatant apparently decreased with time, whereas the biological activity (proper folding) increased, these results suggest that the extracellular oxidized form derived from the extracellular reduced forms and did not come directly from the cell. There are several possible reasons for the discrepancies between our findings and those of Yamanaka *et al.* (12, 13). First, we used the STh gene and analyzed the STh-ClpG chimera, whereas Yamanaka *et al.* used the STp gene and studied native STp; the route by which STh chimera and STp are delivered to the exterior may differ although mature STh is highly homologous to mature STp. On the other hand, there was no significant difference between *dsbA*<sup>+</sup> and *dsbA*<sup>-</sup> cells in the secretion and toxicity of STh free of ClpG, thus minimizing the accountability of ClpG for responses with the *dsbA* mutant. Second, the authors estimated the redox state of extracellular STa from *dsbA*<sup>+</sup> and *dsbA*<sup>-</sup> cells by two indirect procedures consisting of examination of the effect of  $\beta$ -mercaptoethanol on its electrophoretic mobility in SDS-polyacrylamide gel electrophoresis and evaluation of the toxicity of STp-containing polyacrylamide gel pieces in the suckling mouse assay (13). However, on one hand,  $\beta$ -mercaptoethanol is less effective than DTT in completely reducing fully folded STa<sup>2</sup> and is thus capable of generating different degrees of unfolding, and on the other hand, spontaneous oxi-

<sup>2</sup> I. Batisson and M. Der Vartanian, unpublished observations.



dation might occur during purification of STp from polyacrylamide gel. Moreover, native STa does not react in conventional protein analysis procedures and, under certain conditions, displays unexpected electrophoretic mobilities due to its high cysteine content (9, 16), making its detection during secretion difficult. Taking advantage of the easy probing of the poorly antigenic STa using the highly antigenic ClpG as a reporter protein, we followed the production, secretion, and folding of chimeric STh with antibodies to the ClpG portion. In addition, we determined the redox state according to a recently improved methodology based on the use of AMS as a potent irreversibly thiol-trapping agent allowing unequivocal separation of the reduced and oxidized forms of STh (37).

Finally, our observations suggest that chimeric STh translocates across the outer membrane as a reduced intermediate and folds outside the cell as a biologically active structure. Oxidation of cysteine residues in STh may occur spontaneously because of a favorable redox environment (12), perhaps due to the presence of oxidized compounds (38) in the extracellular milieu. Molecular oxygen as a main source of oxidizing power is difficult to imagine because air oxidation is a slow, mechanistically complex reaction (39), explaining why folding in this case can take hours or even days (40). In this work, the culture supernatant samples taken at close intervals (5–25 min) during the time course of extracellular folding were immediately treated with trichloroacetic acid and exposed to AMS, which irreversibly prevents spontaneous disulfide bond formation. Therefore, folding of ClpG-STh chimeras exclusively through air oxidation is unlikely.

This study constitutes the first demonstration that, *in vitro*, (i) STa is transported through the outer membrane in an unfolded state in both *dsbA*<sup>+</sup> and *dsbA*<sup>−</sup> cells; (ii) STa disulfide bond formation occurs outside the *E. coli* cell, independently of the *dsbA* background; and (iii) STa, when joined covalently to a heterologous protein, is capable of mobilizing it to the extracellular environment while still retaining full native biological activities and is therefore capable of efficiently recognizing and activating its receptor on intestinal cells. For these reasons, using STa as a carrier peptide could be of interest in different fields of basic and applied research for delivering natural, or even man-designed, peptides of medical, biochemical, and pharmacological interest. As an ideal example, recent studies demonstrated that guanylyl cyclase C, the intestinal receptor of STa, represents a specific marker for human metastatic colorectal cancer that may have use as a target in directing therapeutic agents for colorectal tumors using STa as a targeting vector (41–43). After coupling STa to a cancer drug and cell internalization of the resulting conjugate via guanylyl cyclase C, one could hope to stop metastases. One could also foresee the use of the STa-ClpG fusion protein as a biomarker in the development of diagnostic tests to probe guanylyl cyclase C in metastatic cells with the help of specific antibodies directed against the large, highly antigenic ClpG marker protein rather than the small, poorly antigenic STa peptide. However, in view of this, it would first be necessary to demonstrate that the entire hybrid binds to guanylyl cyclase C and penetrates *in vitro* into human intestinal cells such as Caco-2 and T84 cell lines and *in vivo* into mouse enterocytes.

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