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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 superantigen while still retaining full enterotoxicity. 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* and dsbA− 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+ and dsbA− 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 prosesequences are dispensable for STa secretion (8). Mature STa without the prosesequence 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− and dsbA+ 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 enterotoxin activity (see Fig. 1). Plasmid pHPC0838 was constructed from pDEV14155 (20) by in vitro site-directed mutagenesis (25) using mutagenic and selection primers. The former primer (5'-CGCGCAGTCTAGTATTAACTA-ATTGGCGTTA-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'-GGAGCAAAAGGCGCCTG-3') and plasmid pHProSTC28 were engineered from pHPC0838 as follows. A synthetic double-stranded DNA (the 5'–3' single coding strand was CGCGCAGTCTAGTATTAACTA-ATTGGCGTTA and the 3'–5' single coding strand was TGTCGATGAATCAAGTGCTGATTACCCGTGCA-TGTGAACTTTGTTGTAATCCTGCCTGTACAGGATGTTACTA) encoding the last 15 amino acids of mature STh and containing Smal-flanked ends was inserted in frame in the correct orientation into the 

\[
\text{Sph}^{+} \text{HpaI site and added a valine between the penultimate tyrosine and the last C-terminal residue (asparagine) of ClpG. The latter primer (5'-GGAGCAAAAGGCGCCTG-3')}\]

containing exponentially growing cells was poured onto LB agar plates, 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 method was as follows: the hybrid protein was determined using STa-specific monoclonal horseradish peroxidase-conjugated antibodies and ELISA plates coated with synthetic STa peptides. 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 H2O2 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 M H2SO4, 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 antibodies, as detected by optical density in sample solutions at 490 nm with a cutoff of 0.1. The positive samples were defined as giving A490 < 0.2 (as this is a competitive assay), and the negative samples were defined as giving A490 ≥ 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 STa (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) 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 nM H2O2 and 10 mg of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt were added to each well and washed twice with PBS and 0.05% Tween 20 and once with PBS. The negative samples were defined as giving at least 0.200 absorbance, as detected by optical density in sample solutions at 490 nm with a cutoff of 0.1. The positive samples were defined as giving A490 < 0.2 (as this is a competitive assay), and the negative samples were defined as giving A490 ≥ 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 STa (Calbiochem) ranging from 0 to 1.25 μg/ml.

**Nonreducing SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting**—Samples containing the hybrids were mixed with an equal volume of 2× Laemml 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 antisera 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 H2O2/e-chloranaphthol as a substrate. For probing with anti-STa antisera, filters were developed with H2O2/e-chloronaphthol (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) diammonium salt to produce a purple color in the presence of nitric acid and 125 mM Na2HPO4, 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 (pDP5H24) served as a negative control. In terms of molecular mass, ClpG represents 93% of the hybrid protein. Therefore, the concentration of ClpG in the fusion protein mixture is equivalent to 1.1 μg of ClpG-STh hybrid.

**Determination of Protein Concentration**—The concentration of ClpG was determined by using the BCA protein assay (Pierce Chemical). The absorbance at 490 nm was measured using a microplate reader (MTP-1100, Dynatech). The standard curve was constructed using purified ClpG in PBS, and the concentration of ClpG in the fusion protein mixture was 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 antisera 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 H2O2/e-chloranaphthol as a substrate. For probing with anti-STa antisera, filters were developed with H2O2/e-chloronaphthol (2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)) diammonium salt to produce a purple color in the presence of nitric acid and 125 mM Na2HPO4, 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 (pDP5H24) served as a negative control. In terms of molecular mass, ClpG represents 93% of the hybrid protein. Therefore, the concentration of ClpG in the fusion protein mixture is equivalent to 1.1 μg of ClpG-STh hybrid.
First, we genetically deleted ClpG from ClpG-STh fusions (Fig. 1). In all cases, the \( \text{dsb}^+ \) and \( \text{dsb}^- \) 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 \( \text{dsb} \) background. Therefore, the dithiothreitol (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 \( \text{dsb}^- \) and \( \text{dsb}^+ \) cells producing pProSTC28-encoded STh
Table I  
Strains and plasmids used in this study

| Strain/plasmid | Relevant genotype or phenotype | Ref. or source |
|----------------|--------------------------------|----------------|
| E. coli B41    | Bovine ETEC<sup>©</sup> isolate (0101.K·H·K99,F41) producing ST<sub>pa</sub> | 18 |
| pH50          | F<sup>−</sup>supE44, ΔargF/lanZTAU169 (Δ804 lanZAM15), hsdR17 (F<sup>−</sup> mk<sup>−</sup> recA1, endA1, gyrA296 (Nal<sup>+</sup>), thi-1, relA1 | Life Technologies, Inc. |
| JCB570        | araD139 ΔaraABC-leu) 7679 galU galK Δ(lac X74 rpmL thi phoR zih12 :Tn10 | 14 |
| JCB571        | JCB570 dsa<sup>−</sup> | 19 |
| JCB819        | JCB570 (malF-lacZ129) dsa<sup>−</sup> kan<sup>1</sup> | 19 |
| JCB818        | JCB570 (malF-lacZ129) dsa<sup>−</sup> dsaB<sup>−</sup> kan<sup>1</sup> | 19 |
| Plasmid       |                                 |                |
| pSK           | High-copy number cloning vector pBluescript SK<sup>+</sup>, pColE1 ori; Ap<sup>R</sup> | Stratagene |
| pDEV41155     | pSK<sup>−</sup> carrying the clpG gene | 20 |
| pHPCO838      | pDEV41155 with an additional GGT triplet just before the final codon AAC of clpG | This study |
| pSTN24        | pHPCO838 with a synthetic STh gene fused to the 5′-end of clpG | This study |
| pSTC22        | pHPCO838 with a synthetic STh gene fused to the 3′-end of clpG | This study |
| pProSTC28     | pHPCO838 with a synthetic STh gene fused to the 3′-end of clpG | This study |
| pSTC17        | pHPCO838 with a synthetic STh gene fused to the 3′-end of clpG | This study |
| pPGST71       | pHPCO838 with clpG deleted and STh directly fused to the ClpG leader peptide | This study |
| pSTD126       | pSTN24 with clpG deleted | This study |
| pGMSTC71      | pSTC22 with clpG deleted | This study |
| pGMSTC710     | pSTC17 with clpG deleted | This study |
| pGMSTC13/19   | pProSTC28 with clpG deleted | This study |
| pH50ST75      | Low-copy number cloning vector, pSC101 ori; Cm<sup>R</sup> | 21 |
| pH524        | pHSG575 carrying the CS31A fibronectin-encoding clp operon | 22 |
| pDPSH245     | pEH524 with clpG deleted | 20 |
| pH50STN24      | pEH524 in which clpG was replaced by the clpG<sup>−</sup> STh fusion gene from pSTN24 | This study |
| pH50STC22     | pEH524 in which clpG was replaced by the clpG<sup>−</sup> STh fusion gene from pSTC22 | This study |
| pEHProSTC28   | pEH524 in which clpG was replaced by the clpG<sup>−</sup> STh fusion gene of pProSTC28 | This study |
| pTrec99A      | Expression vector with IPTG-inducible P<sub>lac</sub> promoter, lacP<sup>−</sup>, rnsB; pColE1 ori; Ap<sup>R</sup> | Amersham Pharmacia Biotech |
| pTrecProSTC28 | pTrec99A carrying the clpG<sup>−</sup> STh fusion gene of pProSTC28 | This study |

a ETEC, enterotoxigenic E. coli; Ap, ampicillin; Cm, chloramphenicol.

Extracellular STh from dsa<sup>+</sup> and dsa<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 dsa<sup>+</sup> and dsa<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.

To determine the redox state of intracellular STh, the JCB570 and JCB818 (dsa<sup>−</sup> dsaB<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-un-treated 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-monomeric 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 (dsa<sup>−</sup>) and JCB571 (dsa<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>lac</sub> promoter. The resulting plasmid, pTrecProSTC28 (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 unduced 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...
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. 3b) and then as two bands reacting to anti-STα antibodies (Fig. 3c), and then as two bands reacting to anti-STα antibodies (Fig. 3c), 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-STα antibodies (Fig. 3c, 65–115 min), the upper appearing last. Emergence of the lower

| Strain/plasmid | Production<sup>a</sup> | Enterotoxicity | Liquid medium |
|---------------|--------------------------|----------------|---------------|
|               | ng STh/10<sup>10</sup> cfu | G/C           | MU/10<sup>10</sup> cfu | MU/μg STh<sup>b</sup> |
| B41           | 123                      | 0.120 ± 0.015  | 7.5           | 63            | 0.134 ± 0.020 | 329 |
| pSTN24        | ND                       | 0.062 ± 0.002  | NT            | NT            | NT            | NT |
| pSTC22        | 30                       | 0.149 ± 0.008  | 3.6           | 120           | 0.150 ± 0.015 | 1136 |
| pSTC17        | 39                       | 0.161 ± 0.009  | 5.2           | 133           | 0.161 ± 0.019 | 909 |
| pEHProSTC28   | 33                       | 0.154 ± 0.012  | 14.4          | 254           | 0.153 ± 0.023 | 1074 |
| pEHProSTC28   | NT                       | 0.142 ± 0.010  | 3.6           | NT            | NT            | NT |
| pSTN24        | NM                       | 0.143 ± 0.012  | 5.3           | NM            | 0.150 ± 0.018 | 909 |
| pSTC22        | 132                      | 0.126 ± 0.008  | 18.2          | 138           | 0.148 ± 0.035 | 1173 |
| pSTC17        | 57                       | 0.133 ± 0.020  | 12.5          | 219           | 0.151 ± 0.020 | 1430 |
| pEHProSTC28   | 112                      | 0.141 ± 0.011  | 14.0          | 125           | 0.136 ± 0.025 | 1950 |
| JCB571 dsbA<sup>b</sup> dsbB<sup>b</sup> | NT                       | 0.135 ± 0.033  | 8.2           | NT            | NT            | NT |
| pSTN24        | NM                       | 0.150 ± 0.025  | 9.9           | NM            | 0.131 ± 0.031 | 1140 |
| pSTC22        | 61                       | 0.141 ± 0.017  | 10.9          | 178           | 0.170 ± 0.010 | 1444 |
| pSTC17        | 51                       | 0.137 ± 0.024  | 16.1          | 315           | 0.151 ± 0.020 | 699 |
| pEHProSTC28   | 55                       | 0.134 ± 0.023  | 16.4          | 298           | 0.140 ± 0.022 | 1894 |
| JCB571 dsbA<sup>b</sup> dsbB<sup>b</sup> | NT                       | 0.134 ± 0.020  | 2.1           | NT            | NT            | NT |
| pSTN24        | NM                       | 0.167 ± 0.006  | 5.8           | NM            | 0.157 ± 0.007 | 708 |
| pSTC22        | 98                       | 0.143 ± 0.026  | 9.3           | 95            | 0.161 ± 0.007 | 727 |
| pSTC17        | 122                      | 0.160 ± 0.008  | 17.1          | 140           | 0.145 ± 0.025 | 1140 |
| pEHProSTC28   | 110                      | 0.166 ± 0.014  | 17.0          | 155           | 0.157 ± 0.039 | 1172 |

<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 0.990); ND, not detectable in undiluted sample following ELISA; NT, not tested; NM, not measurable in ELISA because of a lack of affinity for anti-STα monoclonal antibodies.

<sup>c</sup> Each value represents the mean G/C ratio ± S.E. of a group of five suckling mice. Values ≥0.990 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.
oxidized protein species coincided with that of STh enterotox-icity (Fig. 3, a and c, 15 and 40 min), suggesting that it was correctly folded. Both AMS-treated and AMS-derivatized overnight supernatant samples (Fig. 3, b and c, Cox and O.N., respectively) migrated indistinctly, 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” and dab Cells Is Properly Folded, in Contrast to Intracellular STh” under “Results”), the most probable explanation for these conflicting results is that the upper oxidized isofrom 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. 3c) were detected in the absence of inducer, demonstrating that we are 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 superna-ntants 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 treat-ment 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 dab mutants than in the dsb wild-type strain (Table II). In conclusion, chimeric STh translocates across the outer membrane as an unfolded inter-mEDIATE 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

### Table IV

| Strain/plasmid | Enterotoxicity<sup>a</sup> |
|---------------|--------------------------|
|               | Periplasm | Lysate |
| JCB570 dabA<sup>b</sup> dabB<sup>b</sup>, pProSTC28 | 0.067 ± 0.017 | 0.059 ± 0.005 |
| JCB571 dabA<sup>b</sup> dabB<sup>b</sup>, pProSTC28 | 0.067 ± 0.016 | NT |
| JCB819 dabA<sup>b</sup> dabB<sup>b</sup>, pProSTC28 | 0.063 ± 0.008 | NT |
| JCB818 dabA<sup>b</sup> dabB<sup>b</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 into 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.

STh peptides to the N or C terminus of the major protein subunit of E. coli CS31A fimbriae (ClpO). 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 flex-ible since the STh moiety in the different hybrids is certainly not always the same. This is consistent with an x-ray crystal-lography study of mature STa peptide indicating inherent flex-ibility at the junctions of the three segments and in the central
Fig. 3. Time course of extracellular folding. The JCB571 (dsbA−) strain was grown in 500 ml of LB broth to A600 = 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 sucking 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 μg/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 chimera. Redox state of extracellular STa (Ref. 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− 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−) 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−dsbB− 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− and dsbA− 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− and dsbA− cells by two indirect procedures consisting of examination of the effect of β-mercaptoethanol on its electrophoretic mobility in SDS-polyacrylamide gel electrophoresis and evaluation of the toxicity of STp-containing polyacrylamide gel pieces in the sucking mouse assay (13). However, on one hand, β-mercaptoethanol is less effective than DTT in completely reducing fully folded STa2 and is thus capable of generating different degrees of unfolding, and on the other hand, spontaneous oxi-

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2 I. Batission 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 produced the secretion, 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 a favorable redox environment (12), explaining why folding in this system 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) STH is transported through the outer membrane in an unfolded state in both dsbA+ and dsbA− 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 man-designated, peptides of medical, biochemical, and pharmacological interest. As an ideal example, recent studies demonstrated that guanylyl cyclase C, the intestinal receptor for 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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