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Thermoactivation of a Periplasmic Heat-Stable Enterotoxin of *Escherichia coli*

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Strains of *Escherichia coli* that host a plasmid that codes for the heat-stable (ST) enterotoxin showed 160 times more extracellular enterotoxin than intracellular activity. However, when washed bacteria were sonicated and incubated at between 50 and 85°C, an activity similar to that of the ST enterotoxin was detected. No such effect was present in strains lacking the plasmid, in a plasmid ST^- mutant, or in chromosomal mutants that lack a cyclic AMP-linked positive regulatory system which previously were shown to yield an ST^- phenotype. The thermoactivation was inhibited by iodoacetamide and *N*-ethylmaleimide; chloramphenicol did not affect the phenomenon. The heat-activated ST-like enterotoxin was localized in the periplasmic space. The results are discussed in relation to the export of the toxin from the periplasm to the outside of the cell.

Enterotoxigenic *Escherichia coli* strains are well-known etiological agents of diarrheal disease in humans and other animals (12, 26). At least two types of enterotoxins have been described: the heat-labile toxin and the heat-stable toxin (ST). In addition, two types of ST toxins (STa and STb) are known to be produced by an *E. coli* strain isolated from swine (6). All three enterotoxins, which are plasmid coded, are exported from the cell to the culture medium and thus have to cross the complex cell envelope of *E. coli*.

Other plasmid-coded gene products found in the growth medium of *E. coli* cultures are hemolysin (30) and colicins (14, 19, 24); in the latter case, there is either a lethal synthesis or a general increase in the permeability of the producing cell. Colicins E1 and E3 leave the cell by a process of lysis or quasilysis (14). The export (understood as release of a product to the medium) of hemolysin (30) is independent of energy and de novo protein synthesis and probably requires one plasmid-specified product (22). In the case of heat-labile enterotoxin, a polymyxin B-released product with heat-labile toxin activity was obtained (13) and periplasmic activity was associated with outer membrane fragments (33). Mug-Opstelten and Witholt (20) have suggested that this association could be an active event in the export of proteins from the periplasm to the outside of the cell.

Little is known regarding the export of the ST enterotoxin. In this report we present evidence for the periplasmic location of a heat-activated ST-like toxin which is biologically indistinguishable from the ST enterotoxin; ST toxin was measured as fluid accumulation in the intestine of neonatal mice (9). The activation process is inhibited by compounds that bind to —SH groups. This thermoactivation is discussed in relation to the export of the ST enterotoxin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and the plasmids used and their relevant properties are listed in Table 1. pYK007 is an 80-megadalton Ap^r ST^+ Tra^+ plasmid isolated from a human strain of *E. coli* (32); pYK008 is an Ap^r ST^- Tra^+ derivative of pYK007 (17). The medium used throughout this work (11, 17) was of the following composition: Casamino Acids, 20 g; yeast extract, 1.5 g; NaCl, 2.5 g; K_2HPO_4, 8.71 g; trace salts, 1 ml; in 1 liter of water adjusted to pH 8.5. The trace salts solution consisted of 5% $MgSO_4$, 0.5% $MnCl_2$, and 0.5% $FeCl_3$ dissolved in 0.001 N H_2SO_4. We refer to this medium as T broth.

Preparation of cell extracts. Sonic extracts were prepared as previously described (17). A 100-ml amount of 37°C overnight-grown cultures in T broth was washed two times by centrifugation with cold phosphate-buffered saline (0.137 M NaCl, 0.003 M KCl, 0.016 M Na_2HPO_4, 0.002 M KH_2PO_4). The pellet was suspended in the same buffer, submerged in an ice bath, and sonicated (MSE 150-W ultrasonic MK2 disintegrator equipped with a 9.5-mm-diameter titanium probe) by pulses of 10 s with 40-s intervals at a setting of 13μ. Osmotic shock treatment of cells was adapted from Neu and Heppel (21). Cultures (100 ml) grown overnight at 37°C in T broth were centrifuged, and the pellet was washed three times with 0.01 M

TABLE 1. Bacterial strains and plasmids[a]

Strain/plasmid	Relevant genotype/ phenotype	Source
Strain		
CA8000	HfrH *thi*	4
CA8306	HfrH *thi* Δ*cya*	4
CA8445	HfrH *thi* Δ*cya* Δ*crp rpsL*	25
J54	*pro-22 met-63* Nal^r	6
Plasmid		
pYK007	EntST$^+$ Ap^r Tra	16
pYK008	EntST$^-$ Ap^r Tra	16

[a] Bacterial nomenclature follows the recommendations of Demerec et al. (10) and Bachmann et al. (2); plasmid nomenclature complies with that proposed by Novick et al. (23).

Tris, pH 8.0. The pellet was suspended to a density of 10^{10} cells per ml in 20% sucrose in 0.03 M Tris, pH 8, and transferred to a 1-liter flask. EDTA was added to a final concentration of 10^{-3} M, and the flask was agitated in a rotary shaker (180 rpm) at room temperature for 10 min. The suspension was centrifuged at 13,000 × *g* for 10 min at 4°C. The pellet was rapidly resuspended in 20 ml of 4°C distilled water and agitated at 100 rpm for 10 min at 4°C. The sample was centrifuged: the supernatant is referred to as osmotic shock fluid (periplasm), and the pellet, resuspended in an equal volume of water, is referred to as the shocked cells (intracellular protein).

Determination of ST activity. The ST assay was done as described by Dean et al. (9), using five newborn Swiss albino mice per sample (17). Samples, 0.1 ml, of filtered (0.45 μm; Schleicher & Schuell) cell cultures containing 2 drops of a 2% solution of Evans blue per ml were injected intragastrically; 4 h after inoculation, the mice were killed by exposure to chloroform vapors. From each group of five mice the intestines were removed, pooled, and weighed. The remainder of the bodies was weighed, and the ratio of intestine weight to body weight minus intestine was determined. (Animals with no dye in the intestine or with dye within the peritoneal cavity were discarded, a rare ocurrence of <0.1%.) In each experiment a positive [J54(pYK007)] and a negative (J54) control were included. The mean values and their standard error for strains J54(pYK007) and J54 from different experiments were 0.11952 ± 0.00154 and 0.05443 ± 0.0081, respectively. Toxin activity is reported as the reciprocal of the dilution that gave an intestine/body weight ratio of ≥0.090.

Enzyme assays. Alkaline phosphatase was determined as described by Brickman and Beckwith (4), using *p*-nitrophenyl-phosphate. β-Galactosidase was assayed with *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (18). The method of Lowry et al. (16) was used to quantify protein concentration. Trypsin (Worthington Diagnostics) was used as indicated in the *Worthington Enzyme Manual* (35); pronase (Calbiochem) and proteinase K (E. Merck AG) were used as described in reference 3.

Chemicals. The proteinase inhibitors pepstatin, *N*,α-*p*-tosyl-L-lysine chloromethyl ketone, leupeptin, diazoacetyl-DL-norleucine methyl ester, phenylmethylsulfonyl fluoride, iodoacetamide, and *N*-ethylmaleimide were purchased from Sigma Chemical Co. All but the last two were dissolved separately in methanol (100-fold concentrated); iodoacetamide and *N*-ethylmaleimide were dissolved directly in phosphate-buffered saline. Sodium bisulfite was obtained from Merck.

RESULTS

Heat activation of an ST-like enterotoxin. When strains of *E. coli* (i.e., CA8000, J54) harboring plasmid pYK007 (Ap^r ST$^+$) were sonically disrupted, the level of ST activity obtained was variable from experiment to experiment. Even though the sonication was carried out in an ice water bath (see above), a potential temperature increase in the microenvironment during sonication was hypothesized to explain the variable increase in ST-like activity. For this reason the effect of temperature on the yield of ST activity from cellular extracts was examined. Figure 1 shows the time-dependent activation of the ST-like toxin upon incubation at 65°C of the sonic extracts of strain CA8000(pYK007). After incubation for 20 min, there is an increase from 0 to 32 in the cellular ST activity. The heat activation of the ST-like enterotoxin incubated for 30 min had a maximum at 70°C and decreased to nonobservable levels above 85°C. Table 2 indicates that no ST-like activity could be detected in strains lacking the EntST plasmid or in chromosomal mutants deficient in catabolite repression (CA8306 and CA8445) with pYK007. It is

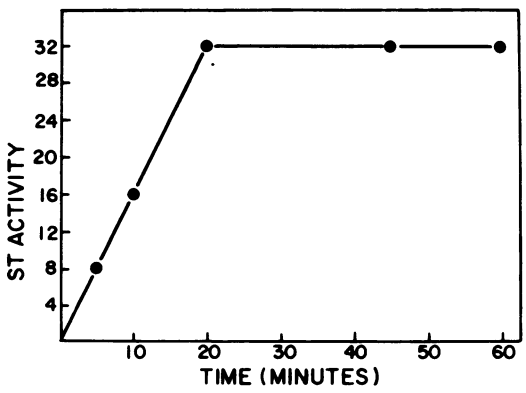

FIG. 1. Thermoactivation of ST-like enterotoxin activity. Strain CA8000(pYK007) was grown and sonicated as described in the text. The preparation was incubated at 65°C for the times indicated, and samples were assayed for ST-like enterotoxin as described in the text. Toxin activity is the reciprocal of the dilution that gave an intestine/body weight ratio of >0.090.

TABLE 2. Extracellular and intracellular ST-like activity[a]

Strain	ST activity		
	Supernatant (extracellular)	Cells (intracellular)	
		Sonicated	Sonicated and activated
CA8000	<1	<0.2	<0.2
CA8445	<1	<0.2	<0.2
CA8000(pYK007)	32	0.2	12.8
CA8306(pYK007)	<1	<0.2	<0.2
CA8445(pYK007)	<1	<0.2	<0.2
J54(pYK007)	32	0.2	12.8
J54(pYK008)	<1	<0.2	<0.2

[a] Cultures (100 ml), of the strains studied were incubated at 37°C for 18 h in T broth; the cultures were centrifuged, and the supernatant was assayed for ST activity as described in the text. The cell pellet was washed once with phosphate-buffered saline, resuspended in 20 ml of the same buffer, sonicated, and assayed as described (see text). The sonicated extracts were activated by incubation at 65°C for 30 min. In all cases, between 70 and 85% of the total cellular protein was solubilized by sonication. ST activity refers to the original 100-ml cultures.

shown also that a plasmid carrying a mutation in the ST gene (pYK008) did not specify the heat-activated ST-like toxin (Table 2). Sonic extracts of phenotypic ST^- strains did not have an affect on the suckling mouse model (Table 2). When these extracts were mixed with extracellular ST enterotoxin, they did not inhibit or enhance the activity shown by the ST^+ supernatant.

Inhibition of heat activation. No effect on heat activation of the ST-like activity was seen when a cell suspension of J54(pYK007) was sonicated in the presence of chloramphenicol (200 μg/ml) followed by incubation at 65°C for 30 min (Table 3). This rules out the possibility that the temperature effect was due to de novo protein synthesis. Also, when the overnight culture was resuspended in T broth containing *N*-ethylmaleimide or iodoacetamide, there was an eight- or fourfold reduction, respectively, in the level of heat-activated intracellular ST-like activity (Table 3). Neither *N*-ethylmaleimide, iodoacetamide, nor chloramphenicol affected the suckling mouse model (Table 3). We also tested pepstatin (4 to 40 μg/ml), *N*,α-tosyl-L-lysine chloromethyl ketone (7.4 to 74 μg/ml), diazoacetyl-DL-norleucine methyl ester (2.66 to 26.6 μg/ml), and phenylmethylsulfonyl fluoride (2×10^{-3} and 2×10^{-4} M); they did not affect the mouse model or the thermal activation of the ST-like enterotoxin. Sodium bisulfite (50 mM) gave false-positive results in the assay system and thus could not be tested as an inhibitor of thermoactivation. These data suggest the participation of free —SH groups in the thermoactivation of the ST-like toxin. No enhancement or inhibition of the activation induced by heat was observed when the cultures were sonicated in the presence of cysteine (100 μg/ml), EDTA (1×10^{-3} to 5×10^{-3} M), or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid (1×10^{-3} to 5×10^{-3} M).

Cellular localization of the heat-activated ST-like toxin. The cell pellet of an overnight culture of strain J54(pYK007) was osmotically shocked. The osmotic fluid and the cells were separated and assayed for ST activity, heat-activated ST-like toxin, alkaline phosphatase (a periplasmic protein), β-galactosidase (an intracellular protein), and total protein. It can be seen in Table 4 that, under our conditions, the marker enzymes were recovered in the proper fraction. It can also be seen (Table 4) that no ST activity was detected in any of the sonicated preparations that were not subjected to heat activation. However, 50% of the total ST-like activity was found in the heat-activated shock fluid and 6% was found in the shocked cells treated in the same way. Similar results (data not shown) were obtained with spheroplasts formed by the method of Witholt et al. (34) and fractionated into spheroplast supernatant and pellet. From these results we conclude that the heat activation phenomenon is located in the osmotic shock fluid, i.e., in the periplasmic space.

To explore the existence of a heat-labile inhibitor, thermoactivated periplasm of strain

TABLE 3. Inhibition of thermoactivation of the ST-like activity

Toxin source[a]	ST activity			
	Control	*N*-ethylmaleimide (10^{-3} M)	Iodoacetamide (5×10^{-4} M)	Chloramphenicol (200 μg/ml)
ST culture supernatant	32	32	32	32
ST cell pellet	16	2	4	16
T broth	0	0	0	0

[a] Overnight T-broth cultures of strain J54(pYK007) were centrifuged at 5,000 rpm for 10 min. Potential inhibitors were added to the supernatant and to the cell pellet, which had been suspended in fresh T broth. Both fractions were then sonicated and incubated at 65°C for 30 min.

TABLE 4. Cellular location of the heat-activated ST-like enterotoxin

Prepn	ST activity[a]		% of total activity		
	Untreated	Heated at 65°C, 30 min	Alkaline phosphatase	β-Galacto-sidase	Protein
Intact cells	0	32	100	100	100
Osmotic shock fluid	0	16	70.8	8	8.4
Shocked cells	0	2	22.6	90	90.7
Remixed osmotic shock fluid and cells	0	16	93.4	99.5	99.1

[a] Overnight T-broth cultures of strain J54(pYK007) were pelleted by centrifugation, and the cells were resuspended in 0.01 M Tris, pH 8, and washed three times. A sample was removed (intact cells) and the suspension was then osmotically shocked as described in the text. After the osmotic shock all fractions were sonicated and assayed for ST, alkaline phosphatase, β-galactosidase, and protein (see text).

J54(pYK007) was mixed with J54 and J54(pYK008) sonic extracts and periplasm. In no case did we observe inhibition of the thermoactivated ST-like activity; furthermore, there was no difference if these same extracts were incubated at 65°C for 30 min before mixing with the thermoactivated ST$^+$ periplasm.

Comparison of heat-activated ST-like toxin and ST enterotoxin. The ST enterotoxin has been shown to be resistant to proteolytic action and to heat (28). The heat-activated ST-like toxin is as resistant to pronase, proteinase K, trypsin, and heat as the ST enterotoxin (Table 5).

When the supernatants of ST$^+$ cultures were dialyzed (Spectrapor 4; Spectrum Medical Industries) against phosphate-buffered saline for 18 h at 4°C, the ST activity dropped from 32 to 0. When the heat-induced ST-like toxin from sonicated cells was dialyzed under the same conditions, the activity fell from 32 to 8. However, if an ST culture supernatant was used to resuspend a cell pellet from a nontoxigenic *E. coli* strain, dialysis reduced the ST activity from 32 to 4. This suggests that the heat-activated ST-like toxin and the ST enterotoxin are under 14 kilodaltons but that they bind to a larger protein in cell sonicates that delays their permeability through a dialysis membrane with a molecular weight cutoff of 14,000.

Table 6 shows the ultrafiltration behavior of the heat-activated ST-like toxin and the ST enterotoxin. When the periplasm of strain J54(pYK007) was ultrafiltered through a PM10 membrane (Amicon Corp.; nominal cut-off level of 10,000 daltons) and then incubated at 65°C for 30 min, the ST-like activity was found in the retained solution; however, if the incubation at 65°C preceded the ultrafiltration, the ST activity was found in the filtrate. Like the extracellular ST, the periplasmic heat-activated ST enterotoxin was retained by UM05 membranes (Amicon Corp.; nominal cutoff level of 500 daltons). No ST-like activity was found if the strain used did not harbor an ST$^+$ plasmid.

DISCUSSION

ST is an extracellular toxin (17, 28) and thus has to be transported through the cell envelope of *E. coli*. We found 160 times more toxin in the supernatant of cell cultures than in the sonicated cell pellet (Table 2), and we did not detect ST activity in untreated osmotic shock fluid. Thus, we can say that no active toxin can be found either within the cells or in the periplasmic space; unlike other proteins localized in the periplasmic space of gram-negative bacteria (8), the active ST is found in the medium.

TABLE 5. Resistance of the enterotoxin and the thermoactivated ST-like toxin to heat and proteolytic action

Treatment	ST enterotoxin[a]		Thermoactivated ST-like toxin[b]	
	TCA-precipitable protein (%)	Activity	TCA-precipitable protein (%)	Activity
None	100[c]	32	100[d]	16
Trypsin (1:10)[e]			42	16
Proteinase K (1:2,000)[e]	50	32	40	16
Pronase (1:10)[e]	15	32	22	16
Heat, 80°C, 10 min	100	32	100	16

[a] ST$^+$ culture supernatant. TCA, Trichloroacetic acid.
[b] Osmotic shock fluid incubated for 30 min at 65°C.
[c] 150 μg of protein per ml.
[d] 320 μg of protein per ml.
[e] Ratio of protease to protein (both in micrograms per milliliter) in the untreated extract.

TABLE 6. Ultrafiltration behavior of periplasmic and extracellular ST activities

Treatment	ST activity			
	Periplasmic		Extracellular	
	Filtered	Retained	Filtered	Retained
PM10	2	0	128	0
65°C for 30 min, then PM10	64	8	128	0
PM10, then 65°C for 30 min	2	64	128	0
UM05	0	2	0	128
65°C for 30 min, then UM05	0	64	0	128
UM05, then 65°C for 30 min	0	64	0	128

When sonicates of J54(pYK007) or CA-8000(pYK007) were incubated at 65°C, an activity like that of the ST enterotoxin was detected (Fig. 1). No such activity was found in strains without the ST plasmid or in strains with deletions in the *cya* and *crp* genes (Table 2). When strain J54 harbors plasmid pYK008, an Ap^r ST^- derivative of pYK007, it does not exhibit the heat activation phenomenon. pYK007 DNA is 81.5 megadaltons as judged by *Eco*RI restriction analysis (32), and all but one of the *Eco*RI fragments of pYK008 comigrate with the *Eco*RI digestion products of pYK007 (32; H. Stieglitz, R. Fonseca, J. Olarte, and Y. M. Kupersztoch-Portnoy, unpublished data). The heat-activated ST-like toxin exhibits the same resistance to heat and to proteolytic action as the ST enterotoxin (Table 5), and both toxins have a low molecular weight as suggested by their behavior upon dialysis. The heat-activated ST-like activity behaves upon ultrafiltration like the extracellular ST (Table 6). However, if it is ultrafiltrated before being heat activated, it behaves as a large molecule, thus suggesting a change in molecular weight or a dissociation into active subunits as a consequence of the thermoactivation. In summary, strains that do not produce ST enterotoxin (Δ*cya* Δ*crp* $EntST^-$) lack the heat-activated toxin. Both activities are biologically indistinguishable, and based on the above-mentioned observations we suggest that the ST enterotoxin is generated from a larger or aggregated form.

When cells harboring pYK007 were resuspended in buffer that contained iodoacetamide or *N*-ethylmaleimide and subsequently tested for the heat-activated ST-like toxin, a reduction in the level of toxin was observed (Table 3). These results suggest that free —SH groups are involved in the activation of the pretoxin. Alderete and Robertson (1) have shown that an ST enterotoxin of porcine origin has a high content of half-cystines (6 of 47 amino acids). Staples et al. (31) have shown that one-third of the 18 amino acids of a human ST toxin are half-cystines and that upon elimination of the disulfide bridges the activity is lost. It is possible that the heat treatment favors the formation of disulfide bridges, thus rendering the precursor active. However, since cysteine did not inhibit the thermoactivation, this possibility is unlikely. Alternatively, the effect of iodoacetamide and *N*-ethylmaleimide could be on an enzyme responsible for the heat activation, and that enzyme may have free —SH groups in the active site. Silva and co-workers (27) isolated chromosomal mutants that yield an ST^- phenotype; they suggested that the mutation could be in a processing enzyme. We found an optimum temperature of 70°C for the activation, and no activation took place above 85°C. These observations favor the second hypothesis, that an enzyme is responsible for the heat activation of the ST-like activity. We were not able to detect a heat-labile inhibitor when either ST^+ or ST^- periplasm was mixed with either thermoactivated ST-like activity or extracellular ST. Thus, if the inhibitor is present, it is very diluted or its affinity for ST is very low.

The in vivo activation of ST could be analogous to the in vitro heat activation and takes place as the pretoxin travels across the cytoplasmic membrane, as is the case for the proteolytic cleavage at the leader or signal sequence (8) of many periplasmic and outer membrane proteins. Alternatively, the activation could take place as the toxin is exported from the periplasmic space through the outer membrane to the exterior of the cell. We found (Table 4) that the heat activation phenomenon is located in the periplasmic space, suggestive of the latter case. Although we have not demonstrated that in vitro activation reflects the mechanism by which the toxin is exported to the exterior of the cell, conceivably it is a phenomenon shared by the few fully exported proteins of *E. coli*.

The EntST toxin has been cloned into a multicopy cloning vehicle (15, 29) and into pBR328, pBR327, and pBR329-2 (Stieglitz et al., unpublished data). Cells harboring cloned ST in these multicopy plasmids show the same level of activity after heat activation as does the parental EntST (pYK007). This suggests that upon an increase in the copy number of the EntST gene, the rate-limiting factor for a parallel increase in enterotoxin is either the hypothetical exportase or a regulatory mechanism by which, regardless of the increase in gene copies, the ST gene product regulates its own synthesis. Lathe et al. (15) have shown evidence for the processing of the ST gene product in a cell-free transcription-translation system capable of the synthesis of a biologically active ST product. It is likely that

the protein fraction of such a system carries the exportase as it also has the ability to generate two discrete bands coded by a single 700-base pair cloned fragment. Experiments are in progress to analyze the biochemical changes that our cloned ST gene product undergoes in vivo as it travels from the interior of the cell through the periplasmic space to the exterior of the cell. We previously reported the effect of *cya* and *crp* genes on ST enterotoxin activity (17); experiments also are in progress to assess whether the effect of catabolite repression is on the hypothetical exportase or on the transcription of the EntST mRNA.

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