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NOTES

Intracellular Distribution of Heat-Labile Enterotoxin in a Clinical Isolate of *Escherichia coli*

JOHN D. CLEMENTS,* KARIN L. LOWE, LYNN BONHAM, AND SAWSAN EL-MORSHIDY

Department of Microbiology and Immunology, Tulane University Medical Center, New Orleans, Louisiana 70112

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The intracellular distribution of heat-labile enterotoxin in a human isolate of enterotoxigenic *Escherichia coli* varied significantly as a result of changing incubation time, media, and degree of aeration. Direct comparison with a K-12 plasmid recipient revealed a similar but less dramatic response to environmental factors.

Enterotoxigenic strains of *Escherichia coli* cause diarrhea by production of one or more enterotoxins. One of these enterotoxins, the heat-labile enterotoxin (LT), has been purified to homogeneity and has been shown to be immunologically and structurally similar to the enterotoxin of *Vibrio cholerae* (choleragen) (2, 3, 6, 8, 13). Unlike cholera toxin, which is primarily secreted into the culture medium, LT is predominantly an intracellular or cell-associated protein (12, 16, 17, 19, 20). The intracellular distribution of LT within *E. coli*, however, is not clearly defined. Recent attempts to determine the distribution have been performed with recombinant plasmids containing LT genes inserted into a variety of laboratory strains of *E. coli* (11, 18). Many of these studies have found that LT is located principally in the periplasm. The study reported here was designed to determine the effect of various environmental influences on the intracellular distribution of LT in a clinical isolate of enterotoxigenic *E. coli*.

The intracellular distribution of LT in human enterotoxigenic *E. coli* Throop D (9) was studied with three media representing both enriched (ML medium [1] and brain heart infusion broth [BHI;Difco Laboratories, Detroit, Mich.]) and minimal nutritional environments (minimal medium [7]). Periplasmic, cytoplasmic, and membrane fractions were prepared as described by Witholt et al. (21). Alkaline phosphatase was assayed according to the procedure of Malamy and Horecker (14). Glucose 6-phosphate dehydrogenase was assayed as follows: 1 ml of each cell fraction was mixed with 1.1 ml of 0.05 M Tris (pH 8.0) and 0.3 ml each of 4 μM NAD, 0.1 M $MgCl_2$, and 10 μM glucose 6-phosphate. The reaction mixture was incubated at room temperature, and glucose 6-phosphate dehydrogenase activity was measured spectrophotometrically at A_{340}. The amount of LT in each cellular fraction was determined by an enzyme-linked immunosorbent assay (ELISA), as previously described (4, 5).

Table 1 shows the relative distribution of both A and B subunits of LT (LT-A and LT-B, respectively) in either periplasm or spheroplasts after incubation with and without aeration at 37°C. The percentage of LT-B in the periplasmic fraction at 6 h ranged from a high of 96% in BHI to a low of 0% in minimal medium. In 18-h aerated and nonaerated cultures the relative distribution remained the same, with more LT-B associated with the spheroplasts under minimal and nonaerated conditions than in enriched aerated media. This difference could not be attributed to differences in efficiency of spheroplast formation since marker enzymes (alkaline phosphatase for periplasm and glucose 6-phosphate dehydrogenase for spheroplasts) consistently partitioned with the appropriate fraction. A higher percentage of LT-A than LT-B remained associated with the spheroplasts, probably reflecting the excess LT-A resulting from the A1B5 configuration of the holotoxin (10).

Overall production of LT was also quantitated and compared with the number of organisms as a function of incubation time, aeration, and nutrition. The maximum amount of LT was produced in ML medium, with 86% of the final

TABLE 1. Distribution of two marker enzymes[a] and subunits of LT[b] after growth in three media as a function of time and degree of aeration

Medium	% distribution in fraction:							
	Periplasm				Spheroplast			
	AP	G-6-P	LT-B	LT-A	AP	G-6-P	LT-B	LT-A
Aerated for 6 h								
BHI	96	2	96	100	4	98	4	0
ML	92	12	78	72	8	88	22	28
Minimal	ND[c]	ND	0	0	ND	ND	100	ND
Aerated for 18 h								
BHI	91	7	91	83	9	93	9	17
ML	89	20	83	70	11	80	17	30
Minimal	58	6	34	15	42	93	66	85
Stationary for 18 h								
BHI	82	3	85	ND	18	97	15	ND
ML	85	3	76	ND	16	97	24	ND
Minimal	84	4	36	35	16	96	64	65

[a] Alkaline phosphatase (AP) and glucose 6-phosphate dehydrogenase (G-6-P). One unit of alkaline phosphatase is defined as the amount required to hydrolyze 1.0 μmol of *p*-nitrophenyl phosphate to *p*-nitrophenol and inorganic phosphate per min at pH 9.6 at room temperature. One unit of glucose 6-phosphate dehydrogenase is defined as the amount of enzyme in a 1-ml sample that at 25°C in a 3-ml assay mixture increases the optical density of NADH at A_{340} by 0.001 in 1 min.

[b] LT in each cellular fraction was determined by ELISA as previously described (4,5). Amounts of LT or subunits of LT in each sample were determined by extrapolation from a standard curve of a known quantity of purified *E. coli* LT, LT-A, or LT-B (6).

[c] ND, Not done.

* Corresponding author.

TABLE 2. Production of LT after growth in three media as a function of time and degree of aeration

Medium	CFU/ml	Amt of LT (μg)[a]		Molecules/ CFU
		Periplasm	Spheroplast	
Aerated for 6 h				
BHI	3.0×10^9	0.91	0.07	93
ML	4.0×10^9	2.56	1.13	264
Minimal	1.8×10^8	0.00	0.06	100
Aerated for 18 h				
BHI	5.6×10^9	3.33	0.70	205
ML	6.1×10^9	3.16	1.13	200
Minimal	2.9×10^9	0.24	1.47	168
Stationary for 18 h				
BHI	1.0×10^9	0.18	0.03	61
ML	1.0×10^9	0.17	0.10	79
Minimal	1.1×10^9	0.11	0.43	140

[a] Determined by ELISA.

yield produced in 6 h (Table 2). This may be compared with production in minimal medium, where the LT production per CFU was lower (100 molecules per CFU for minimal medium versus 264 molecules per CFU for ML medium), and the overall yield was lower because of the greatly reduced growth. After 18 h, the differences among the media were not so striking in the aerated group, with the total yield being somewhat higher in the enriched media. In the nonaerated cultures, growth, molecules per CFU, and total yield were greatly reduced compared with aerated cultures, with minimal medium showing the highest total and per-cell yields.

The intracellular distribution of LT was further investigated by subfractionating the cells. Fractions were designated as periplasm, cytoplasm, outer membrane, or inner membrane. The outer membrane fraction was principally outer membranes obtained by high-speed centrifugation of the spheroplast supernatant and wash. The inner membrane fraction was a mixture of cytoplasmic and outer membranes remaining after lysis of the spheroplasts. Efficiency of fractionation was confirmed by monitoring marker enzymes, as noted above. Table 3 shows the distribution of LT in each fraction after overnight growth at 37°C with aeration. Once again, the relative distribution of LT within each fraction varied according to the type of medium in which the cells were grown. After growth in minimal medium, the majority of LT (56%) was in the cytoplasm, with 26% in the periplasm, and the remainder was associated with outer or inner membranes. Growth in the more enriched media produced a markedly different distribution, with the majority of LT being found in the periplasm. Statistical analysis of these data by the Student *t* test for two unpaired means indicated a highly significant degree of difference between values for periplasm and cytoplasm between each medium ($P \leq 0.01$). As previously noted, ML medium produced the highest total yield of LT.

The inner and outer membranes from cells grown overnight with aeration in minimal medium were subsequently purified, separated by isopycnic sucrose gradient centrifugation (15), and analyzed for the presence of LT by ELISA. All of the detectable LT in the membrane fraction was found to be associated with the cytoplasmic membrane. NADH oxidase and 2-keto-3-deoxyoctonate were used as markers for the cytoplasmic and outer membranes, respectively.

The intracellular distribution of LT was compared in the wild-type enterotoxigenic isolate strain Throop D and in a laboratory-derived strain designated 711 (10407)—an LT^+ ST^+ transconjugant derived by phenotypically tagging the enterotoxin plasmid of *E. coli* H10407 by transposition from an F'ts *lac*::Tn*5* plasmid and conjugally transferring the Tn*5*-tagged plasmid to *E. coli* K-12 711 (4, 5). Both strains were grown in ML medium and in minimal medium overnight at 37°C with aeration. As with strain Throop D, LT was found predominantly in the periplasm (75%) when the K-12 strain was grown in ML medium, but was more evenly distributed between periplasm (43%) and spheroplasts (57%) when the K-12 was grown in minimal medium. Data for Throop D are presented in Table 1.

We have previously examined the intracellular distribution of LT in two strains of *E. coli* containing the same genes for production of LT: H10407, an enterotoxigenic human isolate that produces both LT and heat-stable enterotoxin and 711 (10407). The distribution of LT in both strains, as assayed by ELISA, was compared with that of alkaline phosphatase. In that study (L. Bonham and J. D. Clements, Abstr. Annu. Meet. Am. Soc. Microbiol. 1984, B138, p. 40), LT clearly partitioned with alkaline phosphatase in the periplasm of the K-12 strain and was found principally in spheroplasts of the clinical isolate. It should be noted that the laboratory strain contained the entire LT–heat-stable enterotoxin plasmid from an enterotoxigenic human isolate, and not a high-copy-number, overly efficient recombinant plasmid, as has been the case with other such studies.

The current study examined the intracellular distribution of LT in another clinical isolate, *E. coli* Throop D, and investigated the effect of a number of environmental influences upon that distribution. The relative distribution of LT within each of the fractions varied according to type of medium and degree of aeration; the more highly enriched media and higher aeration resulted in a periplasmic location for LT, whereas growth in minimal medium and reduced

TABLE 3. Subcellular distribution of LT after growth in three media at 37°C for 18 h[a]

Fraction	Amt of LT					
	Minimal medium		BHI		ML medium	
	μg	%	μg	%	μg	%
Periplasm	8.3 ± 2.4	26 ± 9	14.0 ± 2.9	57 ± 3	34.1 ± 9.9	78 ± 3
Cytoplasm	21.9 ± 9.7	56 ± 11	5.4 ± 1.4	21 ± 3	2.9 ± 1.1	6 ± 2
Inner membrane[b]	2.7 ± 0.7	10 ± 4	3.9 ± 1.1	16 ± 3	4.1 ± 1.4	10 ± 4
Outer membrane[b]	2.7 ± 0.4	8 ± 1	1.8 ± 1.3	6 ± 3	2.3 ± 1.3	5 ± 2

[a] Mean ± the standard error of the mean for three independent determinations. Differences between media for periplasmic and cytoplasmic LTs are significantly different ($P \leq 0.01$).

[b] Cytoplasmic and outer membranes were separated according to the procedure of Mizushima and Yamada (15).

aeration caused LT to remain principally in the cytoplasm. Only a minor portion was found to be associated with either outer or cytoplasmic membranes. The reason for the differences in our observations on processing of LT by clinical isolates and that in laboratory strains, as reported by others, is not known. Certainly, most K-12 strains have been multiply mutagenized and possess undetected genetic alterations other than the auxotrophic markers scored for during mutagenesis. It would therefore seem unwise to predict the pathogenic behavior of wild-type strains based on a limited number of observations in laboratory strains carrying recombinant plasmids capable of overproducing certain virulence determinants by orders of magnitude.

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LITERATURE CITED

1. **Clements, J. D., and S. El-Morshidy.** 1984. Construction of a potential live oral bivalent vaccine for typhoid fever and cholera-*Escherichia coli*-related diarrheas. Infect. Immun. **46**:564–569.
2. **Clements, J. D., and R. A. Finkelstein.** 1978. Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **22**:709–713.
3. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24**:760–769.
4. **Clements, J. D., D. C. Flint, R. F. Engert, and F. A. Klipstein.** 1983. Cloning and molecular characterization of the B subunit of *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **40**:653–658.
5. **Clements, J. D., D. C. Flint, and F. A. Klipstein.** 1982. Immunological and physicochemical characterization of heat-labile enterotoxins isolated from two strains of *Escherichia coli*. Infect. Immun. **38**:806–809.
6. **Clements, J. D., R. J. Yancey, and R. A. Finkelstein.** 1980. Properties of homogeneous heat-labile enterotoxin from *Escherichia coli*. Infect. Immun. **29**:91–97.
7. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17–28.
8. **Finkelstein, R. A.** 1976. Progress in the study of cholera and related enterotoxins, p. 54–84. *In* A. W. Bernheimer (ed.), Mechanisms of bacterial toxinology. John Wiley & Sons, Inc., New York.
9. **Finkelstein, R. A., M. K. LaRue, D. W. Johnston, M. L. Vasil, G. J. Cho, and J. R. Jones.** 1976. Isolation and properties of heat-labile enterotoxin(s) from enterotoxigenic *Escherichia coli*. J. Infect. Dis. **133**(Suppl.):S120–S137.
10. **Gill, D. M., J. D. Clements, D. C. Robertson, and R. A. Finkelstein.** 1981. Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **33**:677–682.
11. **Hirst, T. R., L. L. Randall, and S. J. S.Hardy.** 1984. Cellular location of heat-labile enterotoxin in *Escherichia coli*. J. Bacteriol. **157**:637–642.
12. **Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. S. Hardy, and J. Holmgren.** 1984. Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**:7752–7756.
13. **Kunkel, S. L., and D. C. Robertson.** 1979. Purification and chemical characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. Infect. Immun. **25**:586–596.
14. **Malamy, M., and B. L. Horecker.** 1961. The location of alkaline phosphatase in *E. coli* K-12. Biochem. Biophys. Res. Commun. **5**:104–108.
15. **Mizushima, S., and H. Yamada.** 1975. Isolation and characterization of two outer membrane preparations from *Escherichia coli*. Biochim. Biophys. Acta **375**:44–53.
16. **Neill, R. J., B. E. Ivins, and R. K. Holmes.** 1983. Synthesis and secretion of the plasmid-coded heat-labile enterotoxin of *Escherichia coli* in *Vibrio cholerae*. Science **221**:289–291.
17. **Neill, R. J., E. M. Twiddy, and R. K. Holmes.** 1983. Synthesis of plasmid-coded heat-labile enterotoxin in wild-type and hypertoxinogenic strains of *Escherichia coli* and in other genera of *Enterobacteriaceae*. Infect. Immun. **41**:1056–1061.
18. **Palva, E. T., T. R. Hirst, S. J. S. Hardy, J. Holmgren, and L. Randall.** 1981. Synthesis of a precursor to the B subunit of heat-labile enterotoxin in *Escherichia coli*. J. Bacteriol. **146**:325–330.
19. **Pearson, G. D. N., and J. J. Mekalanos.** 1982. Molecular cloning of *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K-12. Proc. Natl. Acad. Sci. USA **79**:2976–2980.
20. **Wensink, J., H. Gankema, W. H. Jansen, P. A. M. Guinee, and B. Witholt.** 1978. Isolation of the membranes of an enterotoxigenic strain of *Escherichia coli* and distribution of enterotoxin activity in different subcellular fractions. Biochim. Biophys. Acta **514**:128–136.
21. **Witholt, B., M. Boekhout, M. Brock, J. Kingma, H. van Heerikhuizen, and L. de Leij.** 1976. An efficient and reproducible procedure for the formation of spheroplasts from variously grown *Escherichia coli*. Anal. Biochem. **74**:160–170.