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Release of Heat-Labile Enterotoxin Subunits by *Escherichia coli*

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Most of the heat-labile enterotoxin (LT) synthesized by *Escherichia coli* is cell associated; however, a small portion of LT (approximately 10%) is released by bacterial cells into the culture supernatant. The LT subunit B (LT-B) produced by a cloned LT-B gene (*toxB*) was released in amounts equal to the parent LT release. In contrast, no release of LT subunit A (LT-A) or its smaller derivatives was observed in strains containing cloned *toxA* genes. The data suggest that LT-B is necessary for the release of LT-A across the bacterial membrane.

Enterotoxigenic *Escherichia coli* strains produce two distinct types of enterotoxins (7): a heat-labile toxin (LT) and a heat-stable toxin (ST). LT and *Vibrio cholerae* enterotoxin (cholera toxin) share several common properties. (i) Both holotoxins consist of one molecule of subunit A and five molecules of subunit B (4); (ii) subunit B binds to target cells, and subunit A stimulates the cellular adenylate cyclase-cyclic AMP system (3, 5); (iii) antibodies to isolated subunits precipitate the corresponding subunit from either toxin (1, 12).

In previous studies, we cloned and characterized the LT region of an LT- and ST-coding plasmid, pJY11, which was present in a clinically isolated enterotoxigenic *E. coli* strain, H10407 (11–13). In those studies, we identified two LT gene (*toxA* and *toxB*) products: LT subunit A (LT-A), with a molecular weight of 26,800 to 27,000, and LT subunit B (LT-B), with a molecular weight of 11,800 to 12,000 (12). The latter is apparently larger than cholera toxin subunit B (molecular weight, 11,590). In contrast, recent studies have shown that the LT-B produced by the LT region from a porcine *E. coli* strain has a molecular weight indistinguishable from that of cholera toxin subunit B (6). We also succeeded in constructing recombinant plasmids which produce only LT-B by using a promoter resident on the vector and others which produce a derivative of LT-A (LT-A*) by using the LT promoter (12, 13). LT-A* is biologically inactive because it lacks the carboxy-terminal third of subunit A; however, it does possess the immunological determinants of LT-A. For *E. coli* strains carrying such toxoid-producing recombinant plasmids to be used as a live vaccine or as a source of toxoids, it is important to know whether LT subunits can be released from bacterial cells into the culture supernatant.

The recombinant plasmids used in this study are summarized in Fig. 1 and Table 1. Cells harboring recombinant plasmids were grown in liquid medium, and the levels of LT subunit antigen in bacterial cells and in the culture supernatants were determined. Antigen levels were measured by agglutination tests by using latex particles adsorbed with antiserum to cholera toxin subunit A or B; our previous experiments indicated that each LT subunit antigen (A or B) could be identified by using antiserum to cholera toxin subunit A or B (12).

E. coli cells harboring pJY11 or pJYL2299, both of which carry the entire LT region, released approximately 10% or more of the total LT subunit antigen (A or B) into the culture supernatant (Table 2); this agreed well with results obtained with intact LT (data not shown). Cells harboring pJYL2299 produced more LT antigen than did cells harboring pJY11, probably because the copy number of the former plasmid is higher. As purified LT subunits A and B were not available for this study, we could not accurately determine the molar ratios of the LT-A and LT-B antigens. However, our previous studies on LT gene products in minicells suggested that the amount of LT-B is greater than that of LT-A or its related peptide (12). If that is the case even in *E. coli* 20SO, LT-B would be predominant.

Significant amounts of LT-B antigen (ca. 20% of the total) were observed in the culture supernatant of *E. coli* cells harboring LT-B-coding recombinant plasmids (pJY24, pJY26, pJY35, or pJY37a). In contrast, cells harboring recombinant plasmids which code for LT-A or its derivatives (LT-A′ or LT-A*) released no LT-A antigen into the culture supernatant, although these cells did produce significant amounts of LT-A antigen. LT-B′, produced by cells harboring

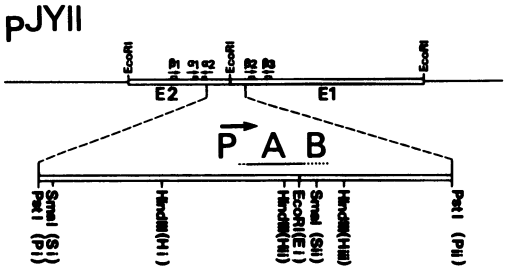

FIG. 1. LT region of the LT- and ST-coding plasmid pJY11. Of the four *Eco*RI sites present on pJY11, only three sites are indicated; the two *Eco*RI fragments are referred to as E1 and E2. α and β represent repeated sequences. The restriction endonuclease cleavage sites indicated here are only those used for cloning of the LT region and are abbreviated as shown in the parentheses. P, A, and B are the LT promoter, LT-A gene (*toxA*), and LT-B gene (*toxB*), respectively. The large arrow indicates the transcriptional direction of the LT operon.

pJY31, was not detected in these experiments; as previous results indicated significant antigenic heterogeneity between this peptide and cholera toxin subunit B (13), this result was not

TABLE 1. List of recombinant plasmids

Recombinant plasmid	Cloned region[a]	Cloning vehicle	Major products[b] of the LT region cloned	Reference
pJYL2299	Pi–Pii	pBR322	LT-A, LT-B	11, 12
pJY31	Si–Sii	pACYC177	LT-A, LT-B′	13
pJY23	Pi–Ei	pBR322	LT-A′	12
pJY34b	Hi–Hii	pACYC177	LT-A*	13
pJY24	Ei–Pii	pBR322	LT-B	12
pJY26	E1	pGA24	LT-B	12, this study
pJY35	Ei–Hiii	pGA24	LT-B	13
pJY37a	Hii–Hiii	pACYC177	LT-B	13, this study

[a] Refer to Fig. 1 for restriction endonuclease cleavage site abbreviations.

[b] Products of the LT regions were identified in minicells and partially characterized. LT-A is a peptide of molecular weight 26,800 to 27,000, and LT-B is a peptide of molecular weight 11,800 to 12,000. LT-A′ is a peptide of molecular weight 24,500 which is produced from a deleted *toxA* lacking a small carboxy-terminal region (ca. 50 base pairs) and shows LT activity in the presence of LT-B. LT-A* is a peptide of molecular weight 17,000, produced by cells harboring a more truncated *toxA* which lacks the last 250 base pairs, and does not show LT activity even in the presence of LT-B. LT-B′ is a smaller LT-B derivative produced by cells harboring a deleted *toxB*; the deletion apparently includes essential antigenic determinants, and the peptide does not show LT activity even in the presence of LT-A. pJY24, pJY26, pJY35, and pJY37a code for LT-B by a plasmid promoter.

TABLE 2. Relative production of LT subunits[a]

Plasmid carried by *E. coli* 20SO (LT gene products)	LT-A antigens detected in:		LT-B antigens detected in:		LT activity in culture supernatant[c]
	Culture supernatant	Sonicated cells[b]	Culture supernatant	Sonicated cells[b]	
pJY11 (LT-A, LT-B)	1	1	1	1	1
pJYL2299 (LT-A, LT-B)	6	4	8	6	7.6
pJY31 (LT-A, LT-B′)	<0.2	2	<0.2	<0.02	—[d]
pJY23 (LT-A′)	<0.2	4	<0.2	<0.02	—
pJY34b (LT-A*)	<0.2	2	<0.2	<0.02	—
pJY24 (LT-B)	<0.2	<0.02	12	6	—
pJY26 (LT-B)	<0.2	<0.02	12	6	—
pJY35 (LT-B)	<0.2	<0.02	12	6	—
pJY37a (LT-B)	<0.2	<0.02	6	6	—
pJY31 (LT-A, LT-B′), pJY24 (LT-B)	3	NT[e]	12	NT	2.0
pJY23 (LT-A′), pJY26 (LT-B)	6	NT	12	NT	2.2
pJY34b (LT-A*), pJY24 (LT-B)	<0.2	2	12	6	—

[a] *E. coli* 20SO cells carrying recombinant plasmids were grown in L broth at 37°C. After 18 to 24 h of incubation, bacterial cultures were centrifuged, and the culture supernatant and cell pellets were obtained. The pellets were suspended in an equal volume of L broth and then disrupted by sonic oscillation. Supernatants obtained by centrifugation of the sonicated cells and the original culture supernatants were tested for LT-A and LT-B antigens by latex immunoassay (T. Itoh and T. Yokota, manuscript in preparation). For the latex assay, twofold serial dilutions of samples were made with 0.5 M glycine buffer (pH 8.2)–0.1% bovine serum albumin, and the dilutions (25 μl of each) were mixed with equal volumes of latex suspension; before the experiments, latex particles were adsorbed with antiserum to cholera toxin subunit A or B. The mixtures were kept at room temperature (20°C) for 18 h, and the endpoints of latex agglutination reaction were determined. The data shown are from the last dilutions in which agglutination was observed; the relative increase in antigen titer or LT activity as compared with cells harboring pJY11 is shown in each case. The mean of three or four trials is shown; hence, the figures do not correspond to the actual dilution.

[b] As indicated in the text, the intracellular antigen titer was approximately 10-fold higher than the extracellular titer.

[c] The LT activity was assayed by using Chinese hamster ovary cells as previously described (11, 12).

[d] —, None detected.

[e] NT, Not tested.

surprising. The introduction of LT-B-coding plasmids into cells producing LT-A or LT-A′ resulted in a marked release of LT-A antigens and intact LT into the culture supernatant; the data also suggested that LT-A′ may have less biological activity than intact LT-A. However, pJY34b, which codes for LT-A*, released no LT-A antigen into the culture supernatant even when LT-B was present.

Recent studies of the LT region and its products in a porcine-colonizing *E. coli* strain suggested the presence of signal peptides for both subunits A and B (2, 6, 8); these signal peptides should be lost during release of the peptides across the bacterial inner membrane (9). It was also reported that the majority of LT synthesized by *E. coli* cells is accumulated in the bacterial outer membrane (10). We have previously shown that cells can produce LT even if the LT-A and LT-B genes (*toxA* and *toxB*) are separately placed on two compatible cloning vehicles (12). This suggests that clustering of these genes is not essential for the synthesis of LT; perhaps subunits A and B are independently released across the inner membrane. The present experiments clearly showed that LT-A is released into the culture supernatant only if LT-B is present. We speculate that LT-A, released across the inner membrane presumably owing to a signal peptide, requires LT-B to pass through the outer membrane into the culture supernatant. If this is so, we speculate that LT-A* cannot interact with LT-B and therefore remains inside the cells.

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