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GENETIC LABELING OF AN ENT PLASMID THAT ENCODES HEAT-STABLE ENTEROTOXIN OF ENTEROTOXIGENIC *ESCHERICHIA COLI* ISOLATED FROM PATIENTS

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SUMMARY Ent plasmids of enterotoxigenic *Escherichia coli* that encode heat-stable enterotoxin (ST) and both ST and heat-labile enterotoxin (LT) were labeled with a kanamycin- and an ampicilin-resistance transposon, respectively. These transposons were kept stably in the respective Ent plasmids. By monitoring antibiotic resistance we could easily select strains carrying Ent plasmids. Strains carrying the labeled plasmids produced enterotoxins that were immunologically indistinguishable from those produced by the parent strains. Moreover the strain carrying a labeled ST-Ent plasmid produced 16 times more ST than the parent strain.

INTRODUCTION

During investigations on heat-stable (ST) and heat-labile (LT) enterotoxins of enterotoxigenic *Excherichia coli*, a sudden loss of toxin production or marked decrease in its production is often encountered. This is assumed to be due to loss of an Ent plasmid that encodes ST and/or LT. Thus, it is necessary to ensure that the Ent plasmid remains in the cell used for experiments. One method to do this is to label the Ent plasmid genetically with an antibiotic resistance transposon.

Attempts to do this have been made by several workers. (So, Heffron and Falkow, 1978; Yokota et al., 1979; Yokota, Yamamoto and Kuwahara, 1980). We also achieved genetic labeling of the Ent plasmid with a kanamycin resistance transposon (Soedarmono et al., 1980; Takeda et al., 1981b). In these studies, however, the Ent plasmid encoding only ST was not labeled. Yokota et al. (1979) labeled an Ent plasmid encoding ST-LT with an ampicilin-resistance transposon, but the isolated plasmid was not carried stably by the cells. In this paper, we report

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genetic labeling with a kanamycin resistance transposon of an Ent plasmid of a human isolate that encodes ST and labeling with an ampicilin resistance transposon of one that encodes both ST and LT, using transformation and conjugation techniques.

MATERIALS AND METHODS

1. *Bacterial strains*

The bacterial strains used in this work were *E. coli* 53402 A-1 (ST^+, LT^-, $ColE_1^-$, Str^s, Rif^s, Nal^s, Kan^s) isolated from a patient and provided by Dr. M. Merson, World Health Organization, *E. coli* 269–2 (ST^+, LT^+, $ColE_1^-$, Str^s, Rif^s, Nal^s, Amp^s) isolated from a patient with traveller's diarrhea at Osaka Airport Quarantine Station (Miwatani et al., 1981), *E. coli* 20SO (ST^-, LT^-, Str^r, Sa^r, Azi^r, Rif^r, Nal^s, Amp^s, Kan^s, Lac^-, λ^r) (Terawaki et al., 1967) provided by Dr. T. Yokota, Juntendo University and an Str^r mutant (ST^-, LT^-, Str^r, F^-, Thr^-, Leu^-) of *E. coli* C600.

A naturally occurring nalidixic acid resistant mutant of *E. coli* 20SO was selected on nutrient agar containing 30 μg/ml of nalidixic acid.

2. *Enterotoxin assays*

CAYE medium described previously (Takeda et al., 1979) was used for production of ST and LT. LT was assayed with Chinese hamster ovary (CHO) cells as described by Takeda et al. (1981a) and by the Biken test (modified Elek test) described by Honda et al. (1981). ST was assayed by intragastric administration of the toxin to suckling mice of 2—3 days old as described previously (Takeda et al., 1979).

3. *Preparation of plasmid DNA*

Cleared lysates of *E. coli* cells were prepared by the sodium dodecyl sulfate (SDS)-salt precipitation method described by Guerry, LeBlanc and Falkow (1973). Then, DNA was precipitated with cold ethanol as described by Meyers et al. (1976). The precipitated DNA was suspended in 3.3 ml of 0.4% sodium sarcosinate (Nakarai Chemical Co., Kyoto) in 0.01 *M* Tris-HCl buffer (pH 7.4) containing 1 m*M* EDTA, and then mixed with 3.5 g of cesium chloride (Sigma Chemical Co.) and 0.35 ml of 5 mg/ml of ethidium bromide (Aldrich Chemical Co.) in distilled water and the refractive index was adjusted to 1.387—1.389 n_D^{25} by adding distilled water. The preparation was centrifuged at 37,000 rpm for 40 h in 4 C in a Beckman SW50.1 rotor. DNA was measured by monitoring the absorbance at 260 nm, assuming that $0.02A_{260}$ corresponds to 1 μg DNA/ml.

4. *Transformation of enterotoxigenic E. coli*

E. coli 53402 A-1 and *E. coli* 269–2 were transformed with pMKl [$ColE_1$ with Tn5 (Kan^r)] (Yokota, Yamamoto and Kuwahara, 1980) and pYSl $ColE_1$ with Tn3 (Amp^r)] (Sugino et al., 1980), respectively, by the method of Lederberg and Cohen 1974). Chilled samples of 20 ng of DNA in 5 μl of 10 m*M* Tris-HCl buffer (pH 8.0) containing 1 m*M* EDTA were added to 0.1 ml of each cell suspension. The mixture were incubated for 60 min in an ice-bath and then for 2 min at 42 C. Five ml of L broth was added to each mixture and incubation with mild rotation was continued at 37 C for 60 min. Finally, the cells of *E. coli* 53402 A-1 and *E. coli* 260–2 were plated on nutrient agar containing 30 μg/ml of kanamycin and 20 μg/ml of ampicilin, respectively.

5. *Conjugation of transformed enterotoxigenic E. coli with nonenterotoxigenic E. coli*

About 1×10^8 cells/ml of transformed enterotoxigenic *E. coli* were mixed with about the same number of recipient cells in 2 ml of tryptic soy broth, and cultured at 37 C overnight with rotation at 60 rpm. The cells were plated on nutrient agar containing appropriate antibiotics to select transconjugants.

6. *Electron micrographs of plasmid DNA*

The plasmid DNA preparation was diluted with 50% formamide containing 0.1 *M* Tris-HCl buffer (pH 8.5) and 0.01 *M* EDTA. The DNA solution (1.5 μg/ml) was mixed with cytochrome c and marker DNA of fd-RFII phage, and spread on a hypophase which contained 0.01 *M* Tris-HCl buffer (pH 8.5) and 1 m*M* EDTA in 18% formamide (Yamagishi, Inokuchi and Ozeki, 1976). The DNAs were adsorbed to Parlodion-covered grids, stained with 0.05 m*M* uranyl acetate in 90% ethanol and shadowed with platinum-palladium. Electron micrographs were taken in a Hitachi electron microscope HU-11B at an instrumental magnification of 12,500-fold.

7. *Colicin immunity*

Colicinogenicity of the cells was tested as described by Fredericq (1957).

8. *Preparation of anti-ST antiserum*

Anti-ST antiserum was prepared by immunizing rabbits with purified ST produced by *E. coli* 53402 A-1 (Takeda et al., 1979). Purified ST (700 μg) in 1 ml of phosphate buffered saline (pH 7.4) was polymerized by adding 25 μl of tolylene-2,4diisocyanate (Wako Chemical Industries, Osaka), with vigorous shaking for 1 min at room temperature. The polymerized ST was then emulsified with an equal volume of Freund complete adjuvant (Difco), and the emulsion was inoculated intramuscularly into young rabbits weighing about 2 kg. Three booster injections of 500 μg of polymerized purified ST emulsified with Freund incomplete adjuvant were given at one-month intervals. The highest dilution of antiserum that neutralized 2 mouse units (5 ng) of purified ST was 1 : 32.

9. *Preparation of anti-LT antiserum*

Anti-LT antiserum was prepared by immunizing rabbits with purified LT (Takeda et al., 1981a) as described previously (Honda, Takeda and Miwatani, 1981).

10. *Ouchterlony double gel diffusion test*

The double gel diffusion test was performed as described by Ouchterlony (1949) in 0.05 *M* Tris-HCl buffer (pH 7.5) containing 1 m*M* EDTA disodium salt, 3 m*M* NaN_3 and 0.2 *M* NaCl. After the samples had been applied, the plate was placed in a humidified incubator at room temperature for about 16—24 h. Then the plate was washed extensively with a solution of 0.4% NaCl and 0.4% $Na_2B_4O_7$ and dried and stained with 0.5% Coomassie brilliant blue in a solution of 50% methanol and 10% acetic acid, and destained with a solution of 50% methanol and 10% acetic acid.

RESULTS

1. *Transformation of enterotoxigenic E. coli with antibiotic-resistant derivatives of $ColE_1$ plasmid DNA*

E. coli 53402 A-1 (ST^+ LT^-) was transformed with pMK1 plasmid DNA, which is a $ColE_1$ carrying Kan^r transposon (Tn5). When 10^7 cells were plated on nutrient agar containing 30 μg/ml of kanamycin, 20 colonies were formed on the agar, indicating that these 20 strains had acquired kanamycin-resistance. Ten of these 20 strains were examined for colicin immunity and 4 strains were found to be immune. These 4 strains were found to produce ST also, indicating that they had acquired pMK1 plasmid DNA without losing the Ent plasmid. We designated these transformants SCK-1, 2, 3 and 4.

E. coli 269-2 (ST^+, LT^+) was similarly transformed with pYS1 plasmid DNA, which is a $ColE_1$ carrying Amp^r transposon (Tn3). Of 10^7 cells, 130 transformants grew on nutrient agar containing 20 μg/ml of ampicilin. Two of 10 strains examined were immune to colicin and also produced both ST and LT, indicating that they had acquired pYS1 plasmid DNA without losing the Ent plasmid. We designated these transformants LSCA-1 and 2.

2. *Conjugation of transformed enterotoxigenic E. coli with nonenterotoxigenic E. coli*

About 1×10^8 cells/ml of *E. coli* SCK-1 (ST^+, LT^-, Kan^r, Str^s, $ColE_1^+$) were mixed with about the same number of recipient cells, the Str^r mutant of *E. coli* C600, in 2 ml of tryptic soy broth and cultured overnight at 27 C with rotation (60 rpm). When about 9×10^7 cells were plated on nutrient agar containing 100 μg/ml of streptomycin and 30 μg/ml of kanamycin, about 3.3×10^2 colonies formed on the plate. Three of 100 strains examined were found to be ST positive. However, these 3 strains were also immune to colicin, indicating that both the pMK1 plasmid and the Ent plasmid may be transferred to the recipient strain, the Str^r mutant of *E. coli* C600. Thus, one of these transconjugants was subjected to additional conjugation with *E. coli* 20SO (ST^-, LT^-, Kan^s, str^r, Rif^r, Lac^-, $ColE_1^-$). Of the 9×10^7 cells plated about 72 grew on nutrient agar containing 100 μg/ml of streptomycin, 30

TABLE 1. *Neutralization of ST activity of culture supernatants of E. coli SK-1, LSA-44 and LSA-45 by anti-ST antiserum against purified ST from E. coli 53402 A-1*

Strain	ST activity (FA ratio[a])	
	+PBS (control)	+anti-ST antiserum
E. coli 53402 A-1	0.116±0.007	0.051±0.001
E. coli SK-1	0.112±0.006	0.056±0.001
E. coli 269-2	0.103±0.015	0.051±0.003
E. coli LSA-44	0.141±0.008	0.056±0.003
E. coli LSA-45	0.135±0.025	0.052±0.001
Purified ST (3MU)	0.155±0.008	0.054±0.002

[a] Culture supernatant of each strain was diluted to 30 MU/ml with phosphate buffered saline (PBS), an equal volume of either PBS of anti-ST serum was added and the mixture was incubated at 37 C for 15 min. One ml of anti-ST serum neutralized 40 MU of purified ST. Suckling mice (3–5 day old) were given 0.1 ml of each of these mixtures. The FA ratio was calculated as described previously (Takeda et al. 1979). An FA value of more than 0.090 is positive and one of less than 0.065 is negative. Values are means for 5 determinations ± standard deviations.

μg/ml of kanamycin and 100 μg/ml of rifampicin. Twenty of these transconjugant strains were examined for ST production and colicin sensitivity. Nineteen were ST positive and 6 of these 19 were sensitive to colicin, indicating that these 6 strains carried an Ent plasmid with a kanamycin transposon, but did not carry pMK1 plasmid DNA. To confirm that kanamycin resistance is always associated with ST producibility, we subjected one of these 6 strains, named SK-1, to reconjugation with an Nal^r mutant of *E. coli* 20SO. Reconjugation occurred at a frequence of 1.4×10^{-3}. Fifty colonies grown on nutrient agar containing 100 μg/ml of streptomycin, 30 μg/ml of kanamycin, 100 μg/ml of rifampicin and 30 μg/ml of nalidixic acid were examined for ST production and 47 were found to be ST positive. These data suggest that the Ent plasmid of *E. coli* SK-1 was labeled with a Tn5 (Kan^r) transposon. We designated this plasmid as pBT1.

E. coli LSCA-1 (ST^+, LT^+, Amp^r, Str^s, Rif^s, $ColE_1^+$) was also conjugated with *E. coli* 20SO (ST^-, LT^-, Amp^s, Str^r, Rif^r, Lac^-, $ColE_1^-$). *E. coli* LSCA-1 and 20SO were mixed and cultured, and then 1×10^7 cells were plated on nutrient agar containing 100 μg/ml of streptomycin, 20 μg/ml of ampicilin and 100 μg/ml of rifampicin to select 20SO that acquired Amp^r. A total of 135 colonies were formed on the plate and 9 of 100 transconjugants were found to produce both LT and ST, and to be sensitive to colicin. These data suggest that these 9 strains of *E. coli* 20SO had acquired Ent plasmid labeled with the Tn3 (Amp^r) transposon. To confirm this, we subjected 2 of the 9 strains to conjugation with the Nal^r mutant of *E. coli* 20SO. Fifty colonies grown on nutrient agar containing 100 μg/ml of streptomycin, 20 μg/of ampicilin, 100 μg of rifampicin and 30 μg of nalidixic acid were examined for enterotoxin production. It was found that all of them produced both ST and LT. Thus, it was concluded that the Ent plasmid of these 2 strains was labeled with the Tn3 (Amp^r) transposon. These strains were named *E. coli* LSA-44 and LSA-45, respectively, and the plasmid was named pBT2.

Figure 1 shows schematically the procedure for labeling the Ent plasmid (ST^+) of *E. coli* 53402 A-1 with the Tn5 (Kan^r) transposon.

3. *Stability of labeled Ent plasmids in E. coli SK-1, LSA-44 and LSA-45*

The stability of the antibiotic resistance transposon on the Ent plasmid was tested by subculturing *E. coli* SK-1, LSA-44 and LSA-45 separately in tryptic soy broth without antibiotics. Each culture was incubated overnight at 37 C and transferred serially 5 times to the same medium. Then the cultures were finally plated on nutrient agar without antibiotics. Fifty colonies from each strain were picked up at random and grown in CAYE medium without antibiotics and

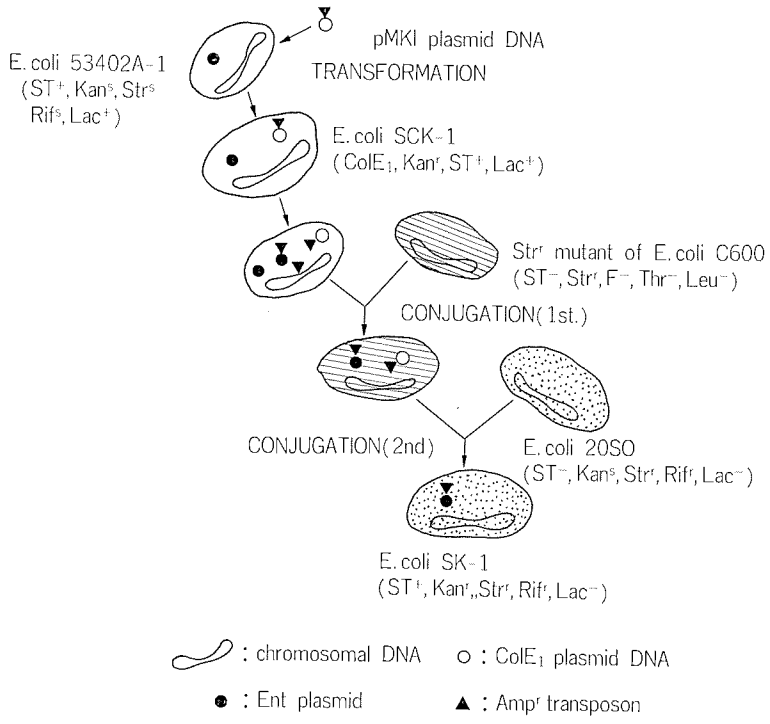


FIGURE 1. Scheme of genetic labeling of the ST-Ent plasmid of *E. coli* 53402 A-1 with the Tn5 (Kan^r) transposon.

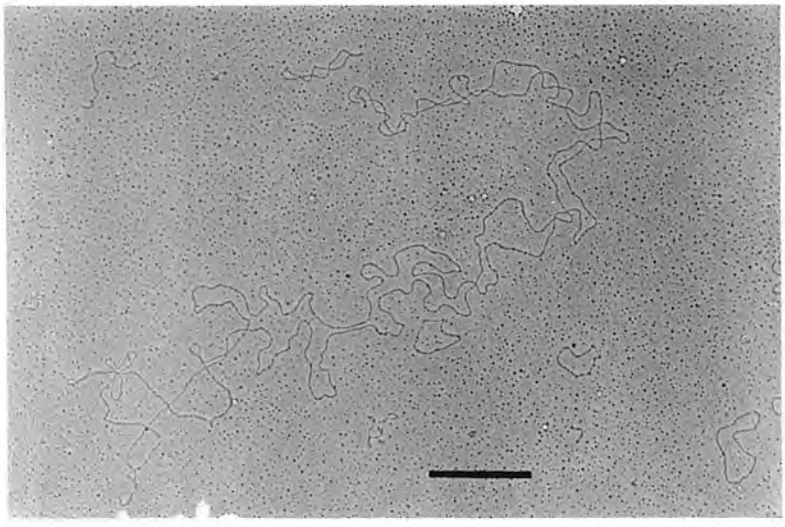
using the culture supernatants were examined for enterotoxin production. Forty-nine colonies of *E. coli* SK-1, 46 of *E. coli* LSA-44 and 35 of *E. coli* LSA-45 were enterotoxin-positive. All these enterotoxin-positive strains grew on nutrient agar containing the respective antibiotic.

4. *Electron micrographs of plasmid DNA of E. coli SK-1 and LSA-44*

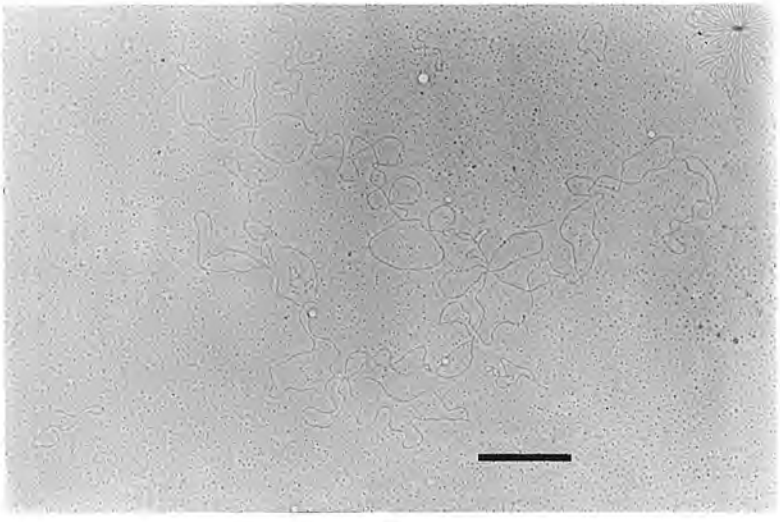
Plasmid DNAs of *E. coli* SK-1 and LSA-44 were prepared and electron micrographs were taken. Typical pictures are shown in Fig. 2. The lengths of the plasmid DNAs of *E. coli* SK-1 and LSA-44 were measured from similar pictures and are shown in Table 2. An fd-RFII DNA that has been reported to have 6,408 base pairs (Beck et al., 1978) was used as a standard. Assuming that the molecular weight of one base pair of DNA is 660 daltons, the molecular weights of the plasmid DNAs of SK-1 and LSA-44 were estimated to be 86 ± 3.3 and 101 ± 3.5 megadaltons, respectively.

5. *Characterization of enterotoxins produced by E. coli SK-1, LSA-44 and LSA-45*

The enterotoxins produced by the strains carrying these genetically labeled Ent plasmids pBT1 and pBT2 were characterized. For this, *E. coli* SK-1, and *E. coli* LSA-44 and LSA-45 were cultured in CAYE medium and the culture supernatants were used for experiments. *E. coli* SK-1 was found to produce about 16 times more ST than the parent strain *E. coli* 53402 A-1. *E. coli* LSA-44 and LSA-45 produced about the same amount of ST as the parent strain *E. coli* 269-2. The ST activities of these culture supernatants were all neutralized equally well by anti-ST



a



b

FIGURE 2. Electron micrographs of plasmid DNAs isolated from *E. coli* SK-1(a), and *E. coli* LSA-44(b). fd-RFII phage DNA (6,408 base pairs) was used as a standard marker. Scale: 1 μm.

TABLE 2. *Lengths of plasmid DNAs of E. coli SK-1 and LSA-44*

Strain	Number of molecules examined	Average length (95% confidence interval)	
E. coli SK-1	24	130±5.0 kilobase	86±3.3 megadaltons
E. coli LSA-44	26	153±5.3	101±3.5

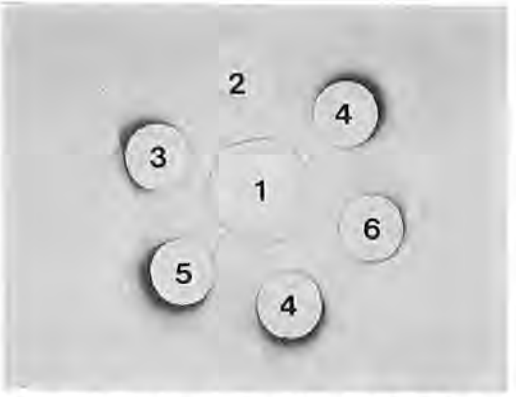


FIGURE 3. Ouchterlony double gel diffusion test of crude LT from *E. coli* LSA-44 and LSA-45. 1, Anti-LT antiserum; 2, purified LT; 3, crude LT from *E. coli* LSA-44; 4, crude LT from *E. coli* LSA-45; 5, crude LT from *E. coli* 269–2; 6, cell extract from *E. coli* 20SO. Five micrograms of purified LT in 10 μl was used. Ten μl of anti-LT serum was used, which was twice the minimum dose for a clear precipitin line with 5 μg of purified LT. Crude LT preparations or cell extracts were prepared as described in the text and 50 μl of each sample was used for the gel diffusion test.

antiserum prepared against purified ST from *E. coli* 53402 A-1, the parent strain of *E. coli* SK-1 (Table 1).

For characterization of LT, cells of *E. coli* LSA-44 and LSA-45 were cultivated overnight in 500 ml of CAYE medium at 37 C and then harvested and suspended in 5 ml of 50 m*M* Tris-HCl buffer (pH 7.5). They were then sonicated and centrifuged at 12,000 rpm for 40 min, and the resulting cell lysates were used as crude preparations or cell extracts. Crude LT preparations were subjected to an Ouchterlony double gel diffusion test with anti-LT antiserum prepared against purified LT. As shown in Fig. 3, crude LT preparations from *E. coli* LSA-44, LSA-45 and 269-2 and purified LT gave a line of identity on the gel.

These data suggest that the ST and LT produced by strains carrying the pBT1 or pBT2 plasmid are immunologically indistinguishable from those produced by parent strains.

DISCUSSION

Genetic labeling of the LT-Ent plasmid has been reported by several workers (So, Heffron and Falkow, 1978; Yokota et al., 1979; Yokota, Yamamoto and Kuwahara, 1980; Soedarmono et al., 1980; Takeda et al., 1981b). In this paper we report labeling of Ent plasmids encoding ST and both ST and LT from human isolates with Tn5 (Kan^r) and Tn3 (Amp^r) transposons, respectively. It was found that the labeled plasmids were carried stably by the cells. Moreover, *E. coli* SK-1, synthesizes 16 times more ST than the parent strain *E. coli* 53402 A-1. Thus, *E. coli* SK-1 will be useful for obtaining a large quantity of ST.

The molecular weights of pBT1 and pBT2 were estimated to be 86 and 101 megadaltons, respectively. Gyles, So and Falkow (1974) reported that ST-Ent plasmids were heterologous in size and varied from 21 to 80 megadaltons, whereas ST-LT-Ent plasmids were homogeneous in size, being 60 megadaltons. In their study molecular weights were measured by sucrose density gradient centrifugation. Myers et al. (1976) reported that the ST-Ent plasmid from a human isolate was 91 megadaltons in size as determined both from its contour length and by agarose gel electrophresis. Wachsmuth, Falkow and Ryder (1976) reported that the ST-Ent plasmid from a human isolate was 30 megadaltons in size as measured by agarose gel electrophoresis. pBT1 is comparable in size to the above preparations but pBT2 is larger.

The Ent plasmid has been found only in *E. coli*, but enterotoxin or enterotoxin-like materials have been isolated from *Salmonella* (Sanderfur and Peterson, 1976), *Shigella* (Takeda, Okamoto and Miwatani, 1977) and *Yersinia enterocolitica* (Okamoto et al. 1980 and 1981). The possible transfer of the Ent plasmid from *E. coli* to other enteric bacteria is interesting problem which we are now ex-

amining using *E. coli* SK-1 and LSA-44.

REFERENCES

Beck, E., Sommer, R., Auerswald, E. A., Kurz, C., Zink, B., Osterburg, G., Schaller, H., Sugimoto, K., Sugisaki, H., Okamoto, T., Takanami, M. 1978. Nucleotide sequence of bacteriophage fdDNA. Nucleic Acids Res. 5: 4495–4503.

Fredericq, P. 1957. Colicins. Annu. Rev. Microbiol. 11: 7–22.

Guerry, P., LeBlanc, D. J., Falkow, S. 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. 116: 1064–1066.

Gyles, C., So, M., Falkow, S. 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. 130: 40–49.

Honda, T., Takeda, Y., Miwatani, T. 1981. Isolation of special antibodies which react only with homologous enterotoxins from *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. Infect. Immun. 34: 2, in press.

Honda, T., Taga, S., Takeda, Y., Miwatani, T. 1981. A modified Elek test for detection of heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. 13: 1–5.

Lederberg, E. M., Cohen, S. N. 1974 Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. J. Bacteriol. 119: 1072–1074.

Miwatani, T., Takeda, Y., Takeda, T., Arita, M., Abe, H., Kanda, T., Tsukamoto, T., Kinoshita, Y. 1981. Epidemiology of enterotoxigenic *Escherichia coli* in the Osaka area. in press. *In* Kuwahara, S., Zinnaka, Y. [ed.] Proceedings 15th Joint Conference US-Japan Cooperative Medical Science Program, Cholera Panel, Toho University, Tokyo.

Myers, J. A., Sanchez, D., Elwell, L. P., Falkow, S. 1976. Simple agarose gel electrophoretic method for the identification and characterization of plasmid deoxyribonucleic acid. J. Bacteriol. 127: 1529–1537.

Okamoto, K., Ichikawa, H., Kawamoto, Y., Miyama, A., Yoshii, S. 1980. Heat-stable enterotoxin produced by *Yersinia enterocolitica* isolated from patients. Microbiol. Immunol. 24: 401–408.

Okamoto, K., Inoue, T., Ichikawa, H., Kawamoto, Y., Miyama, A. 1981. Partial purification and characterization of heat-stable enterotoxin produced by *Yersinia enterocolitica*. Infect. Immun. 31: 554–559.

Ouchterlony, Ö. 1949. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26: 507–515.

Sandefur, P. D., Peterson, J. W. 1976. Isolation of skin permeability factors from culture filtrates of *Salmonella typhimurium*. Infect. Immun. 14: 671–679.

So, M., Heffron, F., Falkow, S. 1978. Method for the genetic labeling of cryptic plasmids. J. Bacteriol. 133: 1520–1523.

Soedarmono, P., Takeda, T., Matsushiro, A., Yamamoto, K., Takeda, Y., Miwatani, T., Yoshikawa, M. 1980. Genetic labeling of an Ent plasmid that encodes heat-labile enterotoxin of enterotoxigenic *Escherichia coli* with kanamycin resistance transposon. FEMS Microbiol. Letters 9: 307–310.

Sugino, Y., Tsunasawa, S., Yutani, K., Ogasahara, K. 1980. Amino-terminally formylated tryptophan synthase α-subunit produced by the trp operon cloned in a plasmid vector. J. Biochem. 87: 351–354.

Takeda, Y., Okamoto, K., Miwatani, T. 1977. Toxin from the culture filtrate of *Shigella dysenteriae* that causes morphological changes in Chinese hamster ovary cells and is distinct from the neurotoxin. Infect. Immun. 18: 546–548.

Takeda, Y., Takeda, T., Yano, T., Yamamoto, K., Miwatani, T. 1979. Purification and partial characterization of heat-stable enterotoxin of enterotoxigenic *Escherichia coli*. Infect. Immun. 25: 978–985.

Takeda, Y., Honda, T., Taga, S., Miwatani, T. 1981a. In vitro formation of hybrid toxins between subunits of *Escherichia coli* heat-labile enterotoxin and those of cholera enterotoxin. Infect. Immun. 34: 341–346.

Takeda, Y., Takeda, T., Soedarmono, P., Morita, T., Matsushiro, A., Yamamoto, K., Miwatani, T., Yoshikawa, M. 1981b. Development of a novel method to label an Ent plasmid of enterotoxigenic *Escherichia coli* with kanamycin resistance transposon. in press. *In* Kuwahara, S., Zinnaka, Y. [ed.] Proceedings 16th Joint Con-

ference US-Japan Cooperative Medical Science Program, Cholela Panel, Oct. 6–8, 1980, Toho University, Tokyo.

Terawaki, Y., Takayasu, H., Akiba, T. 1967. Thermosensitive replication of a kanamycin resistance factor. J. Bacteriol. 94: 687–690.

Wachsmuth, I. K., Falkow, S., Ryder, R. W. 1976. Plasmidmediated properties of a heat-stable enterotoxin-producing *Escherichia coli* associated with infantile diarrhea. Infect. Immun. 14: 403–407.

Yamagishi, H., Inokuchi, H., Ozeki, H. 1976. Excision and duplication of su3$^+$-transducing fragments carried by bacteriophage ϕ80. I. Novel structure of ϕ80 sus 2 psu 3$^+$ DNA molecule. J. Virol. 18: 1016–1023.

Yokota, T., Sekiguchi, R., Yamamoto, T., Ito, T., Kano, T., Kuwahara, S. 1979. Enterotoxin plasmids genetically labeled with a drug-resistance gene (transposon) and its application to adenylate cyclase-less mutants of *Escherichia coli*, p. 207–216, *In* Takeya, K., Zinnaka, Y. [ed.], Proceedings 14th Joint Conference US-Japan Cooperative Medical Science Program, Cholera Panel, Sept. 27–29, 1978, Toho University, Tokyo.

Yokota, T., Yamamoto, T., Kuwahara, S. 1980. DNA-cloning of the ent (LT) and ent (ST) genes from an ent plasmid derived from *Escherichia coli* H10407, p. 476–482, Proceedings 15th Joint Conference US-Japan Cooperative Medical Science Program, Cholera Panel, July 23–25, 1979, National Institute of Health, Bethesda, Md.