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Molecular Cloning of an *Escherichia coli* Plasmid Determinant That Encodes for the Production of Heat-Stable Enterotoxin

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A conjugative plasmid, ESF0041, was isolated from an enterotoxigenic strain of *Escherichia coli* from calves. ESF0041 was found to be 65 × 10^6 daltons in mass and a member of the F incompatibility complex. Acquisition of ESF0041 by *E. coli* K-12 was invariably associated with the capacity to produce heat-stable (ST) enterotoxin. ESF0041 and pSC101 deoxyribonucleic acids were cleaved with *Eco*RI, and the fragments were ligated with polynucleotide ligase. Transformation of *E. coli* K-12 with the ligation mixture led to the isolation of an ST^+ clone. Further analysis of the plasmid deoxyribonucleic acid from this clone showed that the structural gene(s) associated with ST biosynthesis had been isolated as a 5.7 × 10^6-dalton ESF0041 fragment in pSC101. In turn, the 5.7 × 10^6-dalton fragment was ligated to a multicopy ColE1 derivative, RSF2124, so that toxin synthesis was amplified about threefold.

Enterotoxin production by enteropathogenic *Escherichia coli* isolated from calves, piglets, and humans has been shown, in many instances, to be plasmid mediated (19, 29-32). *E. coli* enterotoxins have been divided into two general classes. The heat-labile class, called LT, is an immunogenic protein, the size of which has been variously estimated to be from 24,000 to 100,000 daltons (8, 17, 18, 28). The LT toxin shares partial antigenic identity with the well-characterized enterotoxin of *Vibrio cholera* (17, 33). Cholera toxin has been shown to exert its effect on small-bowel epithelial cells via stimulation of adenyl cyclase activity, and it is now apparent that the effects of *E. coli* enterotoxin are also mediated through the same system (7, 15).

The other class of *E. coli* enterotoxin, called ST, is a heat-stable substance that is nonantigenic and of unknown molecular weight (16, 21, 28; M. H. Merson, G. K. Morris, D. A. Sack, J. G. Wells, D. M. Bessudo, and E. J. Gangarosa, N. Engl. J. Med., in press). The response of a sensitive small bowel to ST is characterized by an immediate accumulation of fluid in the bowel lumen, but the duration of its action, unlike that observed with LT, is short lived and not mediated through the adenyl cyclase system.

The *E. coli* ST enterotoxin remains a perplexing problem. The observed differences between ST and LT with respect to their immunogenicity, mode of action, and apparent differences in molecular size support the concept that these two enterotoxins are distinctly different. Yet, whereas some *E. coli* plasmids encode for only ST or only LT, a significant proportion encodes for both ST and LT (19, 29). It is not clear whether the ST enterotoxins are homogeneous, as seems to be the case for LT toxins (17), or whether there is some variation in their structure or mode of action. Certainly there does not appear to be a direct relationship between plasmids encoding for ST only and those encoding for both ST and LT enterotoxins on the basis of deoxyribonucleic acid (DNA)-DNA homology experiments (26). Such DNA homology data do not, of course, provide the necessary information concerning the relationship between the actual structural genes for ST, which are expected to comprise only a small proportion of an Ent plasmid genome. In large part, the enigma of the ST enterotoxin is compounded by the fact that it may be reliably assayed only in the ligated intestinal loop of animals (16, 28) or by intragastric injection of suckling mice (6). Moreover, one finds considerable differences in the degree of toxicity and animal specificity when dealing with ST toxin preparations (28).

One approach to the study of the *E. coli* ST toxin is to focus attention more precisely on the genetic determinant(s) of ST toxin biosynthesis. To isolate the ST genes in a form that would be more useful for genetic analysis, as

well as potentially simplify the isolation of ST toxin and the determination of its mode of action, we decided to exploit recent advances in the use of restriction endonucleases, which permit the cloning and amplification of specific sequences of DNA as part of a recombinant plasmid structure. This methodology of molecular cloning has proved to be a powerful tool for the isolation of specific genetic determinants and has been successfully employed in the isolation of the *E. coli* tryptophan operon (20) and R plasmid antibiotic resistance genes (3), as well as the ribosomal DNA of *Xenopus laevis* (24), fragments of *Drosophila* DNA (13), and the histone genes of sea urchins (22). In the present paper, we describe the molecular cloning of a DNA fragment containing genes determining ST toxin biosynthesis, as well as the amplification of ST toxin production.

MATERIALS AND METHODS

Bacterial strains. All strains used, except for HB101, are derivatives of *E. coli* K-12. Strain 711 (ESF0041) is *trp*$^-$ *phe*$^-$ *pro*$^-$ *his*$^-$ *lac*$^-$ *nal*^{r} and harbors the 65 × 10^6-dalton (Mdal) ST toxin plasmid ESF0041. Strains C600 (pSC101) and C600 (RSF2124) are *thr*$^-$ *leu*$^-$ *thi*$^-$ *lac*$^-$ and harbor plasmids pSC101 and RSF2124, respectively. Both pSC101 (3) and RSF2124 (27) were described in detail previously. HB101 is an *E. coli* B strain that is *leu*$^-$ *pro*$^-$ *lac*$^-$ *gal*$^-$ *strA*^{r} *thi*$^-$ *recA*, r$^-$ m$^-$.

In vitro generation of recombinant DNA molecules. ESF0041, pSC101, and RSF2124 plasmid DNA was isolated as described previously (27). The DNAs were mixed in a ratio of 1 ESF0041 molecule to 10 pSC101 molecules, at a final concentration of 4 μg. The DNA mixture was digested to completion with *Eco*RI endonuclease in TMN buffer [100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, 5 mM $MgCl_2$, 50 mM NaCl; pH 7.5] and dialyzed overnight in 10 mM Tris-hydrochloride (pH 7.5). The DNA mixture was concentrated to a final volume of 100 μl and adjusted to a final concentration of 50 mM Tris-hydrochloride (pH 8.0), 5 mM $MgCl_2$, 10 mM $(NH_4)SO_4$, 25 μM nicotinamide adenine dinucleotide, and 50 μg of bovine serum albumin per ml. Ligation of the DNA was performed as described by Dugaiczyk et al. (9). The ligated DNA was sedimented in an SW65 rotor at 46,000 rpm and 15°C for 145 min in a Beckman L2-65B ultracentrifuge. The gradient tube was punctured from the bottom and 5-drop fractions were collected. After dialysis overnight in TEN buffer (20 mM Tris-hydrochloride, 1 mM ethylenediaminetetraacetate, 20 mM NaCl; pH 8.0), each fraction was used as the source of DNA for transformation of *E. coli* HB101.

For cloning the ST determinant from ESF3000 (a pSC101-ST$^+$ hybrid) to RSF2124, 4 μg of each DNA was mixed and digested to completion with *Eco*RI and ligated as described above. After ligation, the DNA was dialyzed overnight in TEN buffer and used to transform *E. coli* C600. Transformants were selected for resistance to ampicillin (Ap) by being streaked onto nutrient agar (Difco) plates containing 100 μg of ampicillin (Calbiochem) per ml. Twenty Ap^r clones were purified for screening for colicinogeny and for resistance to tetracycline (Tc). Colicin assays were performed as described by Ozeki (25).

Agarose gel electrophoresis was performed by the method of Green et al. (14).

DNA-DNA-hybridization. ^{3}H-labeled ESF0041 plasmid DNA was hybridized to representative plasmids of different incompatibility groups by the S1 endonuclease method described by Crosa et al. (5).

Assay for the production of ST toxin. Organisms were grown in 5 ml of PF medium (10) for 18 to 22 h with shaking at 37°C. The culture was filtered through a 0.22-μm membrane filter (type HA, Millipore Corp.). A 0.05-ml volume of Evan blue dye (2% in physiological saline) was added to 1 ml of filtered supernatant, and 0.1 ml of the solution was injected intragastrically into 1- to 3-day-old mice. After 4 h the mice were sacrificed, and the ratio of gut to body weight was determined as described by Dean et al. (6).

RESULTS

Characterization of the Ent plasmid ESF0041. Smith (28) reported that 7 of 127 *E. coli* strains isolated from the alimentary tract of calves were toxigenic in a ligated loop model. One of these strains, *E. coli* B41, was chosen for analysis of the genetic nature of enterotoxin biosynthesis. *E. coli* B41 causes severe diarrhea when given orally to calves less than 48 h old but caused minor symptoms or was innocuous in older calves (28; L. P. Williams and S. Falkow, unpublished data). *E. coli* B41 possessed the serotype O101:KA(?):K99, and culture supernatant fluid (both unheated and heated at 65°C for 1 h) gave positive suckling mouse tests but was negative in the Chinese hamster ovary cell assay of Guerrant et al. (15), which is specific for LT toxin. Additionally, strain B41 was resistant to tetracycline and streptomycin (Sm). Strain B41 was mated with the nalidixic acid-resistant (*nal*) *E. coli* recipient 711, and the transconjugants were analyzed for inheritance of the ability to produce enterotoxin. Approximately 1% of the *E. coli* 711 cells grown in mixed culture with strain B41 were found to give a strongly positive suckling mouse test, indicating the inheritance of a genetic element encoding for ST toxin biosynthesis. Cleared lysates were prepared from a number of Ent$^+$ transconjugants, and several revealed the presence of a single plasmid species, 65 Mdal in size. Retransfer of this plasmid species to various other nontoxigenic *E. coli* K-12 sublines was always associated with the acquisition of heat-stable enterotoxin biosyn-

thesis, and it was concluded that this plasmid, ESF0041, called hereafter Ent 41 for simplicity, encoded for the ST enterotoxin. (It should be noted that a 52-Mdal plasmid encoding for the K99 antigen, as well as for tetracycline and streptomycin resistance, was also transferred successfully from strain B41 to recipient *E. coli* K-12 strains. This plasmid was transmitted independently of Ent 41 and is currently under investigation.)

Purified, [^{3}H]thymine-labeled Ent 41 DNA employed in DNA-DNA hybridization studies was found to share 41% of its polynucleotide sequences with the FII incompatibility group plasmid R1*drd-19* and 33% of its sequences with the classical F-factor (incompatibility group FI). It appears, therefore, that Ent 41 belongs to one of the F incompatibility complexes. Since Ent 41 can coexist stably with both F-*lac*$^+$ and R1*drd-19* (unpublished data), we presume that it does not fall into the common FI and FII incompatibility groups. Although the Ent ST+LT plasmids of porcine origin are all closely related (more than 85% polynucleotide sequences in common; 26), we have found that Ent 41 and the Ent ST+LT plasmid P307 have only 40% of their sequences in common, and Ent 41 shared less than 5% of its sequences with Ent plasmids of porcine origin which encode for only ST (Table 1).

TABLE 1. *Polynucleotide sequence relationships between Ent plasmid ESF0041 and representatives of various other plasmid groups*

Plasmid	Phenotype	Mol wt (Mdal)	Relatedness with ESF0041 (%)[a]
ESF0041	ST (bovine) Inc?	65	100
P307	ST + LT (porcine) IncFI	60	36
SF119	ST + LT (human) IncFI	60	36
P95	ST (porcine) Inc?	20	3
P16	ST (porcine) Inc?	25	1
F$^+$	IncFI sex factor	62	33
R1*drd-19*	IncFII Ap^r Cm^r Sm^r Su^r Km^r	65	41
N3	IncN Tc^r Sm^r Su^r	32	16
R144	IncI Tc^r Km^r	63	3
RP4	IncP Ap^r Tc^r Km^r	34	0
Sa	IncW Cm^r Sm^r Su^r Km^r	25	0

[a] The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (3). In every case the degree of duplex formation was calculated relative to the homologous reaction. The actual extent of binding of (^{3}H) ESF0041 purified plasmid DNA with whole cell *E. coli* K-12 (ESF0041) DNA was 95%, which was corrected for an 8% reaction between (^{3}H) ESF0041 DNA and whole-cell DNA extracted from an *E. coli* F$^-$ strain. Each value shown is an average of three to six reactions, and the range was within 2% of the given value.

Because Ent 41 was the best-characterized plasmid available to us which encoded for a potent ST toxin "easily" assayed in suckling mice, it appeared to be the logical choice from which to clone the gene(s) encoding for the ST enterotoxin.

Cleavage of ESF0041 with *Eco*RI and the cloning of ST determinant with pSC101. Purified, covalently closed Ent 41 DNA was cleaved with the restriction enzyme *Eco*RI. Figure 1 shows that migration in 0.7% agarose of cleaved Ent 41 DNA fragments relative to similarly cleaved pSC101 and λ bacteriophage DNA. Ent 41 possesses 11 sites susceptible to *Eco*RI cleavage. With the λ DNA fragments as standards, the extrapolated molecular sizes of the Ent 41 fragments were calculated to be 15.5, 13.2, 7.3, 6.2, 6.2, 5.7, 5.1, 4.3, 3.4, 2.4, and 0.4 Mdal for an estimated molecular size of 69.4 Mdal. Previous contour length measurements of open circular DNA molecules placed the molecular size of Ent 41 at 65.5 Mdal, which was in approximate agreement with the value given above.

Ent 41 and pSC101 DNAs were digested and the fragments were ligated with *E. coli* polynucleotide ligase. The ligation products were sedimented through a linear 5 to 20% neutral sucrose gradient. Fractions from the gradient were used to transform *E. coli* HB101 with selection for resistance to tetracycline. In the absence of a direct selection for ST or, alternatively, a means to distinguish clones harboring pSC101 and one or more ligated Ent 41 fragments, random Tc^r clones from each fraction were tested for the production of ST by using the suckling mouse assay. Of 72 clones examined, 1 was positive for ST.

Plasmid DNA was prepared from this Tc^r ST$^+$ clone, examined in an electron microscope and, after *Eco*RI cleavage, subjected to agarose gel electrophoresis. Figure 2 (bottom) shows an open circular molecule of the single plasmid species, designated ESF3000 (hereafter called Ent 3000), isolated from the Tc^r ST$^+$ clone. Contour length measurements of Ent 3000 relative to pSC101 (Fig. 2, top) places its molecular size at 11.5 Mdal. These data were consistent with the view that Ent 3000 is a hybrid molecule consisting of the pSC101 genome (5.8 Mdal) and a fragment of Ent 41 of approximately 5.7 Mdal. Agarose gel electrophoresis of Ent 3000 confirmed the presence of two fragments of DNA, one migrating at the position of pSC101 and one at the position of the 5.7-Mdal fragment of Ent41 (Fig. 3D and E).

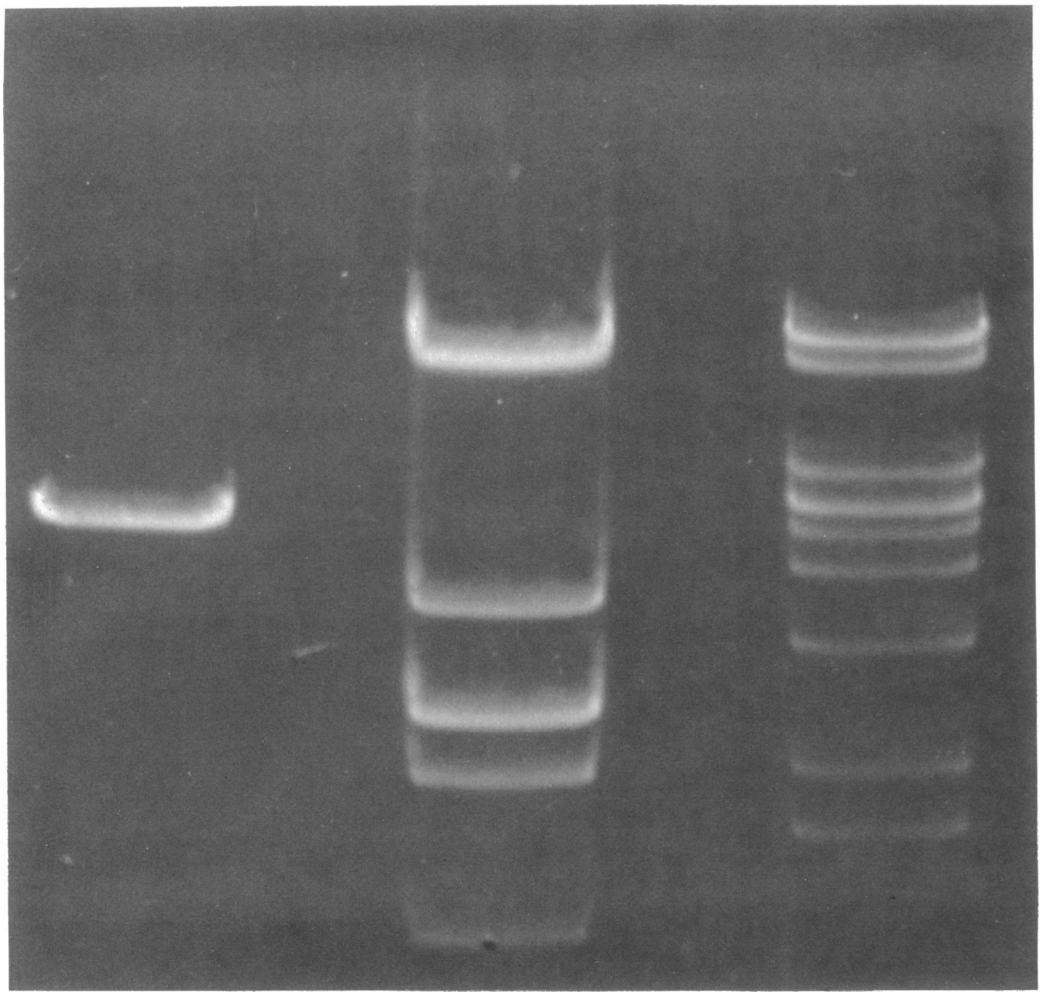

FIG. 1. *Migration in 0.7% agarose gel of EcoRI-cleaved pSC101 DNA (5.8 Mdal), lambda DNA (from top to bottom: 13.70, 4.71, 3.7 and 3.5 [appear as single band under these electrophoresis conditions], 3.03, and 2.09 Mdal), and ESF0041 DNA (extrapolated molecular weights, from top to bottom: 15.5, 13.2, 7.3, 6.2, 6.2, 5.6, 5.1, 4.3, 3.4, 2.4, and 0.4 Mdal). Electrophoresis was carried out at 100 V for 2.45 h.*

Heteroduplex molecules (Fig. 4) prepared between pSC101 and Ent 3000 show, as expected, that Ent 3000 contains a complete pSC101 genome and an additional segment of DNA, presumably contributed by Ent 41. This assumption was confirmed by preparing [^{3}H]thymine-labeled Ent 3000 DNA and showing by DNA-DNA hybridization that it shared approximately 50% of its sequences in common with pSC101 and approximately 50% with Ent 41 (data not shown).

The Ent 3000 plasmid was transformed into a variety of *E. coli* sublines to confirm that a structural gene for ST resided on this plasmid. In every instance, the acquisition of ESF3000 was associated with the biosynthesis of ST that was produced at a level comparable to that of the wild-type *E. coli* B41 toxigenic strain (Table 2). It seems fair to conclude from these data that the structural gene(s) associated with ST biosynthesis has been cloned as part of a 5.7-Mdal fragment into pSC101.

Amplification of ST production. To simplify the analysis of ST, it seemed worthwhile to clone the DNA fragment carrying ST with a plasmid such as ColE1, which could replicate as a multicopy gene pool within host cells, and its numbers could be further amplified in the presence of chloramphenicol. For these experiments, the plasmid RSF2124 was employed. This 7.3-Mdal plasmid is a derivative of ColE1 that has received the translocation sequence

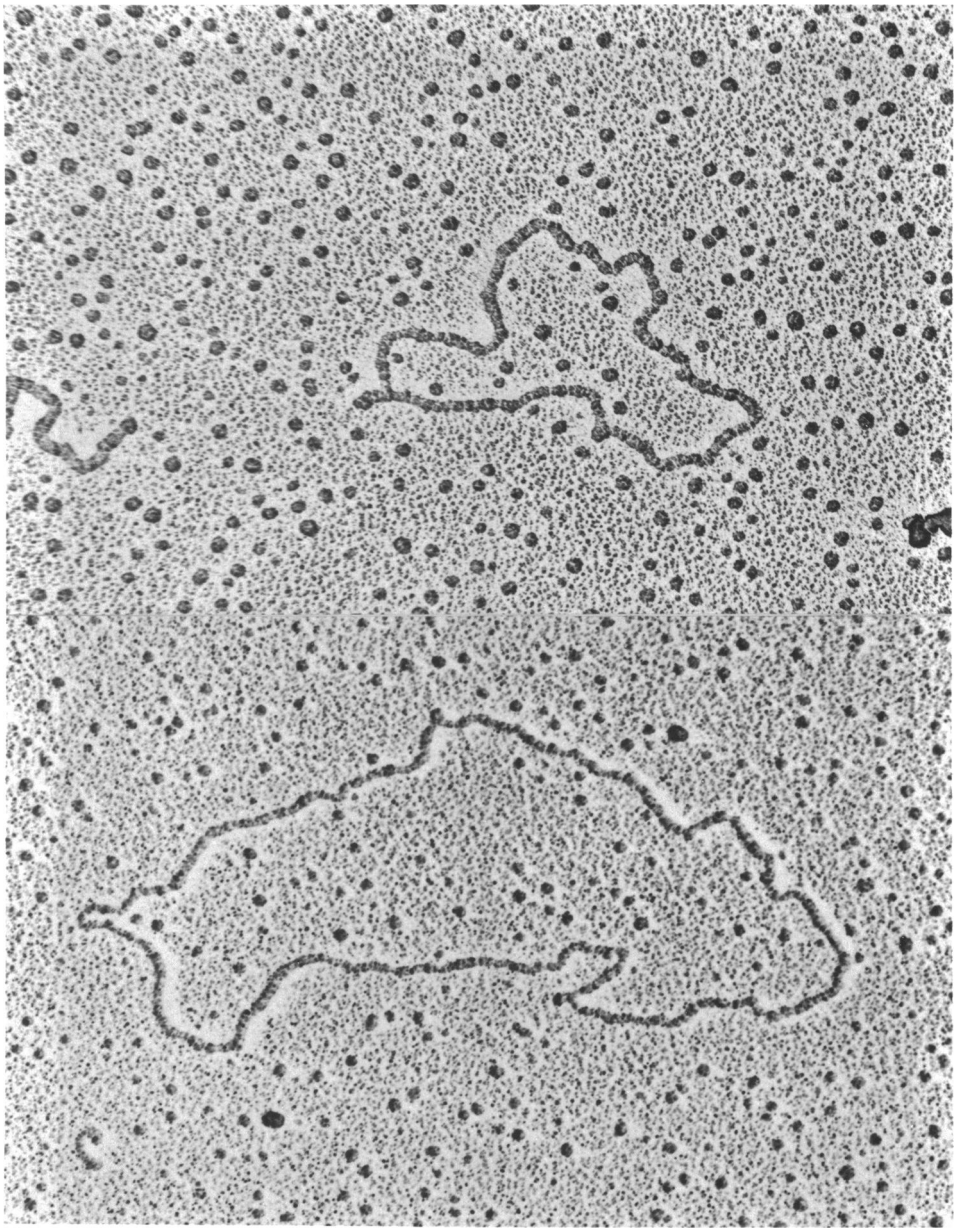

FIG. 2. *(Top) Electron micrograph of relaxed circular pSC101 plasmid DNA; (Bottom) electron micrograph of relaxed circular plasmid DNA, designated ESF3000, isolated from the Tc^r ST$^+$ clone.*

TnA, carrying the structural genes for ampicillin resistance (27). The differentiation of wild-type RSF2124 (Ap^r *col*$^+$) from RSF2124 carrying an inserted fragment of DNA (Ap^r *col*$^-$) is a relatively straightforward matter (27).

A mixture of [^{3}H]thymine-labeled ESF3000 DNA and ^{14}C-labeled RSF2124 DNA was prepared, digested with *Eco*RI, and ligated with *E. coli* polynucleotide ligase. The ligation productions were sedimented in a linear 5 to 20% neutral sucrose gradient. Aliquots of each fraction were counted for radioactivity. Those fractions containing coincident ^{3}H and ^{14}C peaks were dialyzed and used to transform *E. coli* K-12 strain C600 with selection for Ap^r. The Ap^r transformants were tested for colicin E1 pro-

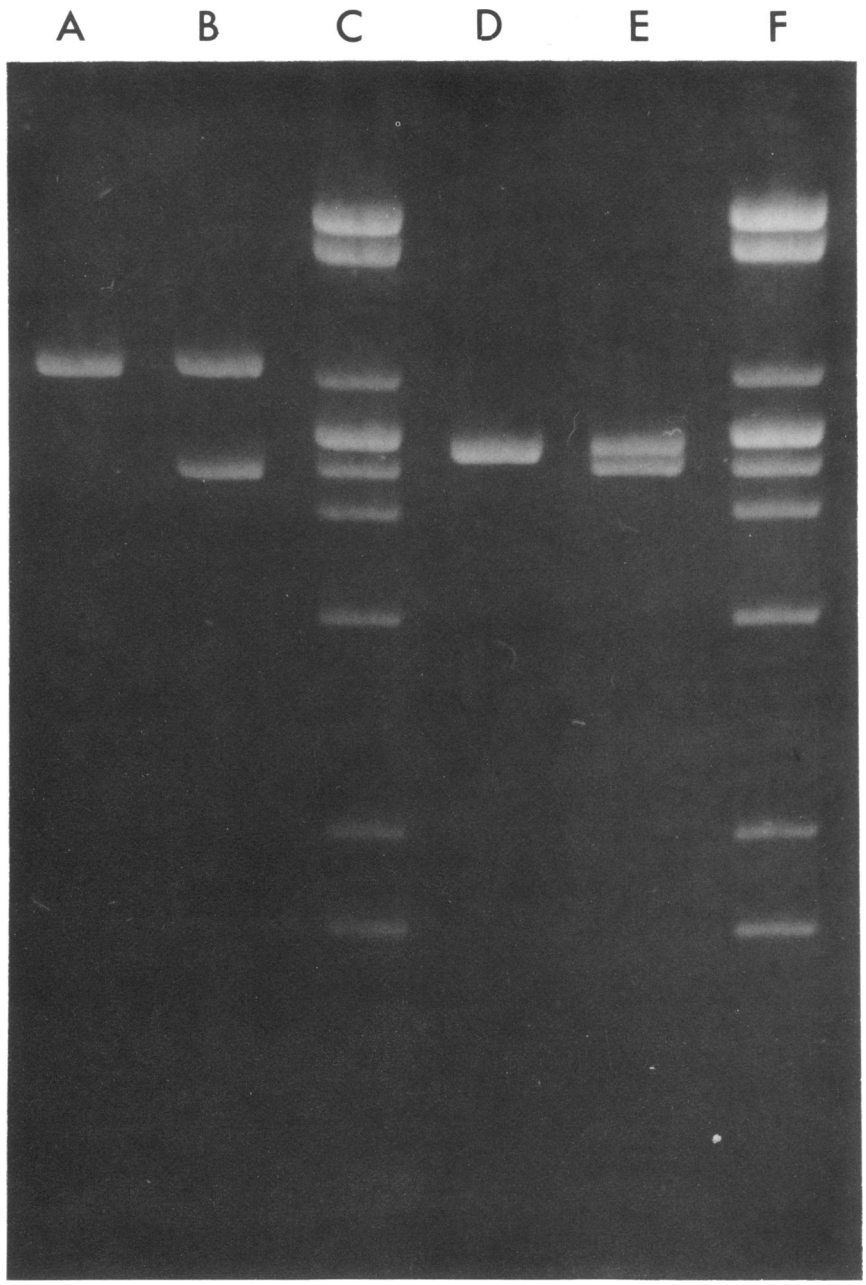

FIG. 3. *Migration patterns in 0.7% agarose of EcoRI-cleaved (A) RSF2124, (B) ESF3001, (C) and (F) ESF0041, (D) pSC101, and (E) ESF3000. The DNA fragments were electrophoresed at 60 V and 15 mA for 20 h.*

duction, and only those clones with the phenotype Ap^r *col*$^-$ were selected for further study. Of the first seven Ap^r *col*$^-$ clones examined, four were Ap^r Tc^r *col*$^-$ ST$^-$, indicating that a hybrid pSC101-RSF2124 plasmid was present within these cells. This was expected, since ESF3000 itself contains the pSC101 genome. Two of the remaining Ap^r *col*$^-$ clones were Tc^s but ST$^+$, indicating that they contained a hybrid plasmid composed of RSF2124 and the 5.7-Mdal Ent 41 fragment. The remaining Ap^r Col$^-$ clone examined was Ap^r *col*$^-$ Tc^s ST$^-$ and was not analyzed further.

The two Ap^r Tc^s *col*$^-$ ST$^+$ clones were se-

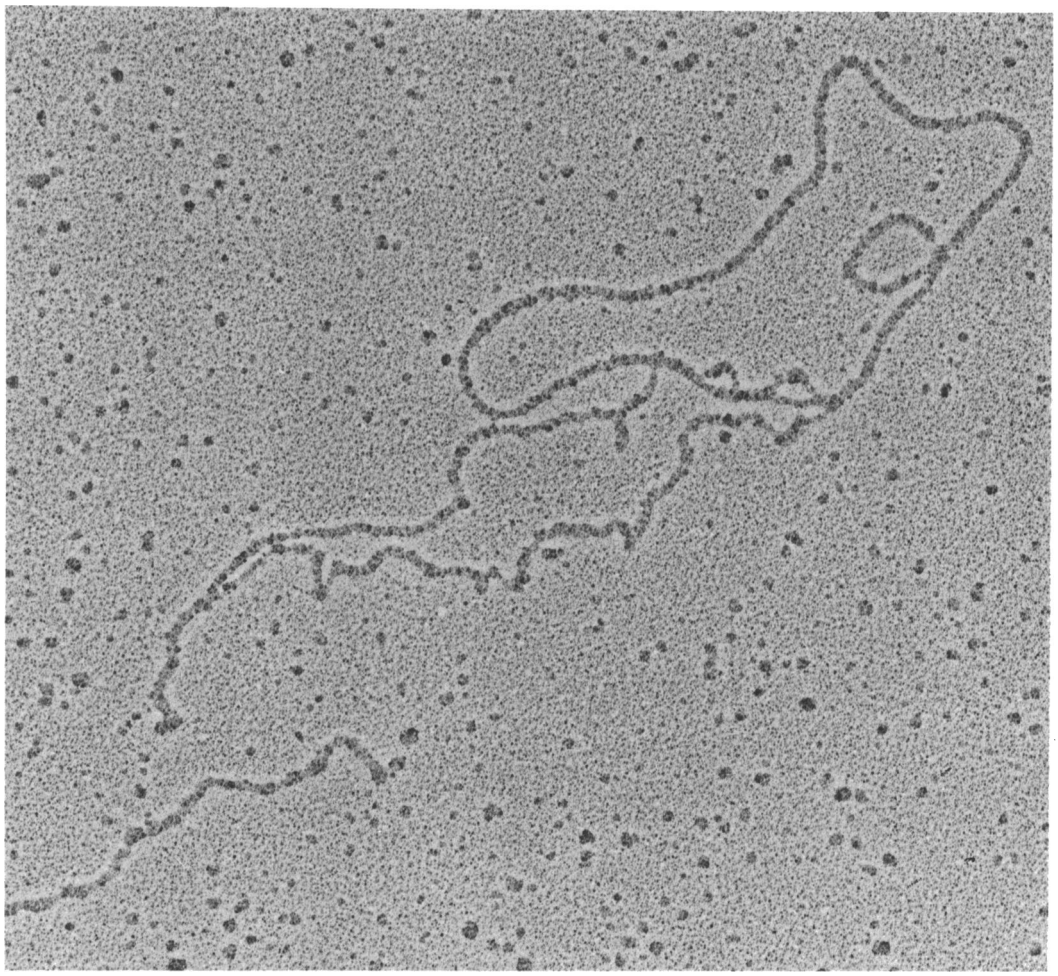

FIG. 4. *Heteroduplex molecule of pSC101 and ESF3000.*

TABLE 2. *Toxicity in mice of E. coli strains harboring the Ent ST determinant*

Strain	Derivation	No. of assays	Ratio of gut wt to body wt
E. coli B41	Clinical isolate	31	0.126 ± 0.006
E. coli K-12	Plasmid$^-$, tox$^-$	19	0.064 ± 0.006
E. coli K-12 (pSC101)	By transformation with pSC101	10	0.065 ± 0.005
E. coli K-12 (ESF0041)	By conjugation from *E. coli* B41	10	0.122 ± 0.013
E. coli K-12 (ESF3000)	By transformation of ligated mixture of pSC101 and Ent 41	10	0.130 ± 0.023
E. coli B	Plasmid$^-$, tox$^-$	10	0.065 ± 0.007
E. coli B (ESF3000)	By transformation	10	0.124 ± 0.004

lected for further study. Each of the clones contained a single plasmid species 13.7 Mdal in size. Upon *Eco*RI cleavage, these plasmids, designated ESF3001 and ESF3002, yielded two linear fragments: one 7.5 Mdal and one 5.7 Mdal. Figure 3B shows the migration pattern of *Eco*RI cleaved ESF3001. Electron microscope heteroduplexes revealed that the 7.5-Mdal fragment corresponds to RSF2124, whereas the 5.7-Mdal fragment corresponds to the Ent 41 fragment carrying the determinant for ST biosynthesis (data not shown). ESF3001 and ESF3002, like the cloning plasmid RSF2124, were present in approximately 20 to 30 copies per cell. Consequently, we expected that the levels of ST toxin produced by cells harboring these plasmids

might be significantly increased. Unfortunately, the measurement of ST toxin is, at best, semiquantitative. Nevertheless, when comparable populations of *E. coli* strain B41, *E. coli* K-12 (Ent 3000) and *E. coli* K-12 (Ent 3001) were prepared, the supernatant fluid from *E. coli* K-12 (Ent 3001) appeared to contain at least three times the amount of toxin as compared with cells containing the wild-type B41 plasmid or the ESF3000 plasmid (Fig. 5).

DISCUSSION

Plasmids encoding for a heat-stable enterotoxin, ST, have been found in *E. coli* isolated from piglets and calves and recently in travelers visiting Mexico (Merson et al., in press) as well as nursery epidemics (K. Wachsmuth, personal communication). In our experience, all toxigenic *E. coli* from calves produce only ST, whereas, for example, *E. coli* ST$^+$ strains comprise only about 20% of the toxigenic isolates from human disease. In the present study, we have been successful in isolating a 5.7-Mdal segment containing the genetic determinant for ST biosynthesis from a large Ent plasmid of bovine origin and joining it to nonconjugative plasmids pSC101 and RSF2124. The isolation of these hybrid plasmids containing the ST gene(s) provides us with a useful tool with which to study the nature and possible origin of the *E. coli* ST toxin. These hybrids segregate readily into *E. coli* minicells, providing an excellent model for studying the transcription and translation of the ST toxin. The amplification of the ST DNA fragment and the coincident increase in toxin levels should simplify toxin isolation. Indirectly, this should also materially aid in the determination of the mode of action of ST on the small bowel as well as the establishment of the nature of the ST cellular receptor. The cloned ST fragment also can be usefully employed in nucleic acid hybridization experiments to examine the origin of the ST structural gene(s) found in various toxigenic isolates. In this latter context, we presume that the ST specific sequences (structural gene(s) and any controlling elements) comprise at least 1 Mdal of the plasmid DNA. Thus, the ST contribution to the 5.7-Mdal fragment of DNA would account for some 20% of the total DNA, as compared with a contribution by the same genes to the 69-Mdal wild-type plasmid genome of only about 2%.

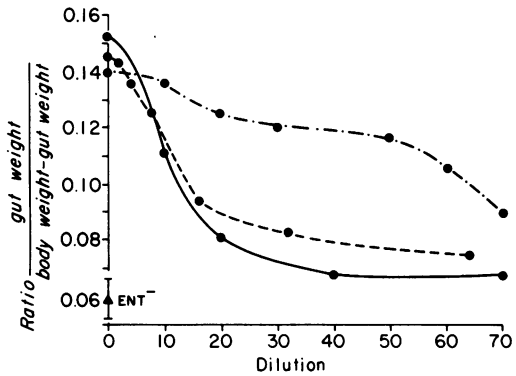

FIG. 5. *Effect of the dilution of ST$^+$ supernates on toxicity in infant mice (each value represents data from three to four mice). Cells were grown at 37°C for 20 h in 5-ml cultures of PF medium. The supernatants were filtered through 0.45-μm filters and diluted in sterile PF medium. Dilutions were assayed in suckling mice by the method of Dean et al. (6). Symbols: - - -, supernatant from E. coli K-12 (ESF0041); ——, supernatant from E. coli K-12 (ESF3000); -·-·, supernatant from E. coli K-12 (ESF3001).*

The research presented here is, to our knowledge, the first attempt to clone a genetic determinant concerned with bacterial pathogenicity. Because of the concerns raised by Berg et al. (2) and the Asilomar Conference on the potential biohazards associated with the construction of DNA recombinant molecules, it seems useful to review some of the criteria we employed in assessing the potential biohazards associated with this research. At the outset, a group of concerned scientists suggested in a letter (1) the postponement of certain experiments and specifically indicated that ". . . (construction of new) autonomously replicating bacterial plasmids that result in the introduction of genetic determinants for antibiotic resistance or bacterial toxin formation into bacterial strains that do not presently carry such determinants . . ." should be deferred. Subsequently, the summary statement of the Asilomar Conference on recombinant DNA molecules (2) stated that experiments involving the generation of recombinant DNA molecules within, and whose parental genomes are native to, prokaryotic agents known to naturally exchange genetic information can be performed in minimal risk containment facilities—essentially the operating procedures recommended for clinical microbiological laboratories. It was our feeling that cloning a genetic determinant found naturally as part of a conjugative *E. coli* plasmid totally within *E. coli* did not present any more potential hazard than examining natural clinical isolates of enteropathogenic *Escherichia* strains. In addition, we chose to use the 65-Mdal Ent 41 plasmid because of the following reasons. (i) It has been transmitted to *E. coli* K-12 as a single unit. (ii) It was isolated from nature in conjunction with a plasmid encoding for Tc^r. Conse-

quently, in attempting to join the ST genes of Ent 41 with the pSC101 Tc^r plasmid, we reasoned that the combination did not materially differ from that found naturally. Subsequently, when the ST determinant was associated with the ampicillin resistance determinant after ligation with RSF2124, we were cognizant that our survey of enterotoxigenic strains of domestic animals and humans showed that roughly 10% of the Ent^+ clinical isolates contained R-Ap^r plasmids. More recently, we have shown that Ent plasmids co-resident within the same cell as R-Ap^r plasmids receive the TnA translocation at a relatively high rate (M. So, F. Heffron, and S. Falkow, unpublished data). (iii) In vivo studies in the large-animal isolation facility at Fort Collins, Colorado, has established that *E. coli* K-12 (Ent 41) could not cause clinical disease in calves of any age, even if accompanied by the specific colonization antigen K99 (11). Moreover, it is clear that normal fecal *E. coli* isolates infected with an Ent plasmid alone are ordinarily incapable of causing overt disease (30). (iv) The ST toxin of B41 was active in vivo against calves and lambs, but not against swine or rabbits (32). (v) By working solely within *E. coli,* the host range of the plasmid would not be materially changed; that is, we would be working with an *E. coli* plasmid within *E. coli*. In terms of potential biohazard, therefore, it appeared to us that working with an *E. coli* K-12 subclone containing a nonconjugative plasmid-ST recombinant plasmid probably presented less of a hazard than working with human toxigenic isolates. Nevertheless, there are two parameters that should be considered from the safety standpoint. First, one must recognize that although ST production in and of itself is not sufficient to elicit a clinical response, *E. coli* K-12 carrying Ent 41 survive to a better extent in calves than does the parental *E. coli* K-12 strain (11). Although this finding is restricted solely to survival experiments in bovines, it serves to illustrate that such *E. coli* strains may not be considered totally innocuous, but rather require careful monitoring and adherence to clinical microbiological practice. Second, the amplification of toxin production by linkage of the ST determinant with ColE1 might be considered more of a potential hazard than wild-type Ent plasmids. The *E. coli* heat-stable enterotoxin is, however, of relatively low toxicity as compared, for example, with the *Shigella dysenteriae* type I enterotoxin (23) or even cholera toxin (12). Indeed, in excess of 10^{12} viable cells of *E. coli* K-12 (Ent 41; K99) cells may be fed to calves without overt clinical symptoms or even subclinical symptoms (11; Falkow, unpublished data). Nevertheless, prudence dictates that such strains and their recombinant plasmids be treated with the same procedures applied to known invasive enteric pathogens as well as *Vibrio cholerae* and other known toxigenic clinical isolates. The isolation of determinants of pathogenicity of *E. coli* such as the ST toxin by molecular cloning within *E. coli* appears to be no more hazardous (and possibly less so) than that which we normally associate with clinical isolates from diseased animals or human patients. It would be a mistake, however, to assume that such *E. coli* K-12 derivatives were a priori of no hazard so that they would be subsequently treated haphazardly in the laboratory.

In the main, the pathogenicity of enteric species or particular enteropathogenic strains cannot be attributed to a single determinant, but more often than not is a reflection of a constellation of unlinked bacterial genes (both chromosomal and extrachromosomal) and a multitude of specific and relatively nonspecific animal host factors. The use of molecular cloning to isolate specific determinants of bacterial pathogenicity holds great promise for our better basic understanding of bacterial pathogenesis and may be extremely useful at the practical level for the construction of vaccine strains.

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