INFECTION AND IMMUNITY, Sept. 1983, p. 1056–1061
0019-9567/83/091056-06$02.00/0

Vol. 41, No. 3

Synthesis of Plasmid-Coded Heat-Labile Enterotoxin in Wild-Type and Hypertoxinogenic Strains of *Escherichia coli* and in Other Genera of *Enterobacteriaceae*

ROGER J. NEILL,† E. M. TWIDDY, AND R. K. HOLMES*

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Received 14 April 1983/Accepted 14 June 1983

The effect of host determinants on expression of plasmid-coded heat-labile enterotoxin (LT) was examined. A collection of LT plasmids was introduced into isogenic strains of *Escherichia coli* K-12 strains containing the wild type or hypertoxinogenic (*htx-2*) allele. For each plasmid tested, production of LT increased by approximately 1.5- to 3-fold in the host containing *htx-2*, indicating that the *htx-2* allele affects a regulatory function for LT production that is common to many different enterotoxin plasmids. LT plasmids from *E. coli* were also introduced into strains of *Shigella flexneri*, *Shigella sonnei*, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, and *Salmonella typhimurium*. The plasmids were stably maintained and determined production of LT in those genera, although the amounts of LT produced varied by more than 50-fold. These observations demonstrate that host factors have an important role in determining the level of expression of plasmid-coded LT genes and support the hypothesis that interspecific, conjugal transfer of enterotoxin plasmids may confer enterotoxigenicity to a wide variety of potentially pathogenic enteric bacteria.

Recent studies have characterized the subunit structure (10), mechanism of action (11), and genetic organization (5, 34) of the plasmid-coded heat-labile enterotoxin (LT) of *Escherichia coli*. Although DNA-DNA hybridization studies did not reveal significant differences between LT genes from independently isolated, LT-producing strains of *E. coli* (26, 27), the LTs produced by enterotoxigenic *E. coli* strains of porcine and human origin are antigenically cross-reacting, but not identical (9, 17; R. K. Holmes, E. M. Twiddy, and R. J. Neill, *in* Y. Takeda, ed., *Bacterial Diarrheal Disease: an International Symposium*, in press). These antigenic differences indicate some degree of divergence among LT structural genes in *E. coli*. It is not known to what extent there may also be divergence in regulatory genes that control LT production. The experiments reported here were performed to investigate effects of host genetic determinants on expression of plasmid-coded LT genes. We analyzed the expression of LT genes of several independently isolated enterotoxin plasmids in isogenic *E. coli* strains containing different alleles that affect LT production. We also studied expression of plasmid-coded LT genes in a variety of bacterial genera within the family Enterobacteriaceae.

(A preliminary report of these findings was presented at the Seventeenth Joint U.S.-Japan Conference on Cholera, Baltimore, Md., 26 through 28 October 1981.)

MATERIALS AND METHODS

Media and conditions of cultivation. All cultures were incubated at 37°C, and liquid cultures, except for conjugal matings, were agitated on a rotary shaker. LB broth (6) was used for liquid cultures, and meat extract agar (28) was used for growth on solid medium unless other media are specified. Glucose-syncase medium was modified from syncase medium (7) and contained glucose (2.5 mg/ml) instead of sucrose, plus tryptophan (40 μg/ml), adenine (40 μg/ml), thiamine (10 μg/ml), and nicotinic acid (10 μg/ml). Media were supplemented as required with tetracycline-hydrochloride (5 μg/ml), kanamycin sulfate (30 μg/ml), ampicillin (100 μg/ml), rifampin (100 μg.ml), nalidixic acid (25 μg/ml), or streptomycin sulfate (100μg/ml).

Bacterial strains and plasmids. The bacterial strains and enterotoxin plasmids used in our studies are listed in Tables 1 and 2. EWD299 is a nonconjugative multicopy plasmid containing the cloned LT genes from plasmid p307. Plasmid EWD299 DNA was introduced into *E. coli* strains KL320 and HE22 by transformation (6). All of the other enterotoxin plasmids are conjugative plasmids. Conjugal transfer of these plasmids was performed by using mixtures containing equal numbers of donor and recipient bacteria either in

† Present address: Division of Biochemistry, Walter Reed Army Institute of Research, Washington, DC 20307.

TABLE 1. Bacterial strains

Strain	Species	Characteristics[a]	Source (reference)
KL320	*Escherichia coli*	*his lac met pro rpsL trp nal*^{r}	W. Maas (31)
LS289	*E. coli*	*his ilv pro thi trp*, donor host for plasmid pCG86	W. Maas (31)
NH1	*E. coli*	*rif*^{r} derivative of LS289, donor host for plasmid pTD2Tc	This study
711	*E. coli*	*his lac phe pro trp nal*^{r}	S. Falkow (15)
Throop D	*E. coli*	LT_h, ST, *tet*^{r} *str*^{r}	R. Finkelstein (8)
HE12	*E. coli*	Hypertoxinogenic mutant (*htx-2*) of KL320(pCG86)	Our laboratory (3)
HE22	*E. coli*	Derivative of HE12 cured of pCG86	Our laboratory (3)
C600	*E. coli*	*thr lac leu thi supE*, donor host for plasmid EWD299	W. Maas (33)
21R868	*E. coli*	*rpsL*, donor host for plasmid TP252Km	M. McConnell
14R525	*E. coli*	*nal*^{r}, donor host for plasmids TP213Tc, TP235Km, TP236Tc, TP237Tc	M. McConnell
NH2	*E. coli*	Donor host for plasmid p307Ap, derived from 711(p307)	This study
NH3	*Shigella flexneri*	*rif*^{r} derivative of *S. flexneri* 2457	This study (21)
NH4	*Shigella sonnei*	*rif*^{r} derivative of *S. sonnei* 53G	This study (22)
NH5	*Citrobacter freundii*	*rif*^{r} derivative of a clinical isolate	This study
NH6	*Enterobacter cloacae*	*tet*^{s} *rif*^{r} derivative of *E. cloacae* 264	This study (25)
NH7	*Klebsiella pneumoniae*	*tet*^{s} *rif*^{r} derivative of *K. pneumoniae* 355	This study (25)
LT2	*Salmonella typhimurium*	Prototroph	B. Ames (1)

[a] Abbreviations: ST, production of heat-stable enterotoxin; LT, production of LT; LT_p and LT_h, LT encoded by a plasmid from an *E. coli* strain isolated from porcine and human sources, respectively (LT_p and LT_h are antigenically cross-reacting but not identical [9, 17; Holmes et al., in press]. Resistances to antibiotics or heavy metals: ampicillin (*amp*^{r}), kanamycin (*kan*^{r}), mercury (*mer*^{r}), nalidixic acid (*nal*^{r}), rifampicin (*rif*^{r}), streptomycin (*str*^{r} *and rpsL*), sulfonamides (*sul*^{r}), and tetracycline (*tet*^{r}). Susceptibility to tetracycline (*tet*^{s}). Inability to ferment lactose (*lac*). Requirements for nutrients or vitamins for growth; histidine (*his*), isoleucine and valine (*ilv*), leucine (*leu*), methionine (*met*), phenylalanine (*phe*), proline (*pro*), thiamine (*thi*), and tryptophan (*trp*). Amber suppressor (*supE*).

LB broth cultures without aeration or on meat extract agar (11a). Transconjugants were isolated by plating samples from the mating mixtures onto meat extract agar containing appropriate antibiotics to select for resistance determinants of the bacterial recipient and the plasmid from the donor strain.

Plasmid p307Ap, a derivative of p307 containing the ampicillin transposon Tn*801*, was constructed by introducing the temperature-sensitive plasmid pMR5 from *E. coli* strain UB1636 into 711(p307) by conjugation (29, 30). The transconjugant 711(p307, pMR5) was then treated as described previously (29) to select for derivatives in which pMR5 was eliminated and Tn*801* was transposed onto p307. Plasmid pTD2 is an LT enterotoxin plasmid isolated from *E. coli* Throop D (R. J. Neill and R. K. Holmes, unpublished data). The tetracycline transposon Tn*10* was transposed into pTD2 by infecting *E. coli* strain KL320(pTD2) with a defective λ phage containing Tn*10* (18). The defective λ phage used in this experiment, λ 561 (*c*I171::Tn*10* Δ*b*221 *Oam29 Pam80*), was constructed by N. Kleckner. A tetracycline-resistant colony was isolated that transferred both pTD2 and Tn*10* simultaneously by conjugation into strain NH1.

Assays for LTs. The antigenic variants of LT encoded by plasmids from *E. coli* strains of porcine and human origin were designated LT_p and LT_h, respectively. LT_p was purified from *E. coli* HE12, and LT_h was purified from *E. coli* HE22(pTD2) by modifications of published methods (4, 16). Briefly, bacteria grown to the stationary phase in glucose-syncase medium were collected by centrifugation and disrupted by sonication. After particulate debris was removed by centrifugation, the LTs released from the bacterial

TABLE 2. Bacterial plasmids

Plasmid	Characteristics[a]	Source (reference)
pCG86	LT_p, ST, *tet*^{r}, *sul*^{r}, *str*^{r}, *mer*^{r}	W. Maas (14)
p307	LT_p, ST	S. Falkow (15)
p307Ap	p307 containing *amp*^{r} transposon Tn*801*	This study
EWD299	LT_p genes from p307 cloned into pBR313	W. Maas (5)
TP213Tc	LT_h, ST, *tet*^{r} transposon Tn*10*	M. McConnell (24)
TP235Km	LT_h, *kan*^{r} transposon Tn*5*	M. McConnell (24)
TP236Tc	LT_h, *tet*^{r} transposon Tn*10*	M. McConnell (24)
TP237Tc	LT_h, *tet*^{r} transposon Tn*10*	M. McConnell (24)
TP252Km	LT_h, *kan*^{r} transposon Tn*5*	M. McConnell (24)
pTD2	LT_h plasmid from strain Throop D	This study
pTD2Tc	pTD2 containing *tet*^{r} transposon Tn*10*	This study

[a] See footnote *a* of Table 1.

cells were adsorbed onto agarose, eluted with D-galactose, dialyzed, and subjected to ion-exchange chromatography on phosphocellulose. Analysis of the purified LT_p and LT_h enterotoxins by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed only their A and B subunits without contaminating polypeptides (Holmes et al., in press). Hyperimmune antiserum A45 was prepared in a rabbit immunized with repeated doses of purified LT_p in complete Freund adjuvant. Enterotoxin antigen was measured quantitatively by a modification (11a) of a solid-phase radioimmunoassay reported previously (3). Concentrations were calculated by using an LT_p standard curve for enterotoxin encoded by plasmids from porcine strains of *E. coli* and an LT_h standard curve for LTs encoded by plasmids from human isolates of *E. coli*. Enterotoxin activity was measured in Y1 adrenal cell assays (23), and concentrations were calculated by comparison with the activity of purified LT_p or LT_h.

Preparation of samples for enterotoxin assays. Bacteria were grown overnight to the stationary phase at 37°C with shaking in glucose-syncase medium containing an appropriate antibiotic: tetracycline-hydrochloride for strains containing pCG86, TP213Tc, TP236Tc, or pTD2Tc; kanamycin sulfate for strains containing TP235Km or TP252Km; and ampicillin for strains containing EWD299 or p307Ap. The extracellular culture fluid was separated from bacterial cells by centrifugation and then sterilized by filtration, through Millex 0.45-μm filters (Millipore Corp., Bedford, Mass.). Cells were washed twice with phosphate-buffered saline, suspended in 1/10 of the original culture volume in phosphate-buffered saline, and disrupted by sonication. Debris was removed from sonic extracts by centrifugation at 27,000 × *g* for 30 min. The bacterial extracts were then sterilized by filtration as described above. Assays for extracellular LT were performed with the culture supernatants, and assays for cell-associated LT were performed with the bacterial extracts. Assays for protein were performed by the method of Bradford (2).

RESULTS AND DISCUSSION

Our laboratory has previously described hypertoxinogenic (*htx*) mutants of the *E. coli* K-12 strain KL320 containing plasmid pCG86 (3). The chromosomal allele *htx-2* resulted in a several-fold increase in production of the LT encoded by plasmid pCG86. We wished to determine whether the hypertoxinogenic phenotype was specific for the interaction of *htx-2* with pCG86 or whether it could also be expressed when other enterotoxin plasmids were introduced into an *E. coli* host containing the *htx-2* allele. We prepared a set of the isogenic *E. coli* strains KL320 and HE22 that differed only at the *htx-2* locus and contained several different enterotoxin plasmids derived from *E. coli* strains of porcine or human origin from several geographic locations. Each of these strains was assayed quantitatively for LT antigen; representative data are shown in Table 3. More than 97% of the LT in each strain was cell associated. The amount of LT that was produced varied over a range of approximately 30-fold when different enterotoxin plasmids were present in a single bacterial host strain. For each of the plasmids studied, however, the amount of LT produced was approximately 1.5- to 3-fold greater in the *htx-2* host HE22 than in the wild-type host KL320. Thus, the effect of the *htx-2* allele was not specific for pCG86. We conclude that the *htx-2* allele affects a regulatory function for LT production that is common to many different wild-type enterotoxin plasmids and to the hybrid plasmid EWD299.

TABLE 3. Production of LTs encoded by various enterotoxin plasmids in isogenic strains of *E. coli* K-12 differing at the *htx-2* locus

Plasmid[a]	Cell-associated LT antigen[b]			
	Strain KL320 (*htx*$^+$)		Strain HE22 (*htx-2*)	
	μg/ml of culture	μg/mg of protein	μg/ml of culture	μg/mg of protein
pCG86	1.7	3.0	4.9	11.1
p307Ap	0.40	0.85	0.70	1.5
EWD299	7.0	19.4	25.0	47.2
TP213Tc	0.70	1.2	1.3	2.9
TP235Km	11.0	20.4	22.0	52.4
TP236Tc	3.2	5.2	5.6	11.7
TP237Tc	2.7	5.9	8.5	18.9
TP252Km	2.8	4.7	5.6	11.9
pTD2Tc	1.1	1.8	3.9	8.1

[a] Conjugal transfer of plasmids was accomplished by bacterial matings in liquid media. Plasmids TP213Tc, TP235Km, TP236Tc, and TP237Tc were first transferred from *E. coli* 14R525 into strain NH1 and then from strain NH1 into KL320 or HE22. All other conjugative plasmids were transferred directly from the donor host indicated in Table 1 into KL320 or HE22.

[b] Less than 3% of the LT antigen from each strain was extracellular. The results of assays with culture supernatants are not shown.

LTs antigenically related to *E. coli* LT have been reported in other genera of gram-negative enteric bacteria collected from patients with diarrhea (13, 19, 20). The production of LT was an unstable trait that was lost during laboratory passage of some strains (12), consistent with the possibility that LT production was determined by unstable plasmids. However, the role of plasmids in determining LT production has not been studied in detail in *Enterobacteriaceae* other than *E. coli*.

We wished to determine whether conjugative LT plasmids could be transferred from *E. coli* into other enteric bacteria and be maintained and expressed in them. The recipient strains included avirulent derivatives of *Shigella flexneri* and *Shigella sonnei*, clinical isolates of *Citrobacter freundii*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*, and a laboratory strain of *Salmonella typhimurium*. We used the conjugative enterotoxin plasmids pCG86 and TP237Tc with selectable antibiotic resistance markers and tested for production of LT by radioimmunoassays and Y1 adrenal cell assays. These methods enabled us to isolate rare transconjugants and to measure low levels of toxin production. These sensitive techniques were not available in an earlier study which reported the transfer of an enterotoxin plasmid from *E. coli* into *Salmonella* spp., but not into other genera of *Enterobacteriaceae* (32).

Each plasmid was successfully transferred into each recipient strain by conjugation on agar plates. Plasmids pCG86 and TP237Tc were maintained in each of the bacterial hosts during continuous exponential growth in glucose-syncase medium for 40 generations at 37°C and during weekly passage in nutrient agar slants for 1 month at 22°C. No tetracycline-susceptible segregants were detected among 100 colonies of each strain that were tested after passage under these conditions in the absence of tetracycline.

The results of assays for extracellular and cell-associated LT produced by the various bacteria containing pCG86 or TP237Tc are presented in Table 4. Although none of these enteric bacteria produced LT in the absence of enterotoxin plasmids (data not shown), all of the plasmid-bearing strains produced LT. Most of the LT was cell associated in each of the strains tested. The yields of LT measured by Y1 adrenal cell bioassays were comparable to the amounts of LT antigen determined by radioimmunoassays, indicating that the LT was biologically active. For each specific bacterial host strain tested, the amounts of LT antigen produced by derivatives containing pCG86 or TP237Tc differed by less than eightfold. In contrast, the amounts of LT antigen produced by different bacterial host strains containing the same enterotoxin plasmid varied by more than 50-fold.

The primarily cell-associated distribution of LT in *E. coli* and other genera of *Enterobacteriaceae* was in striking contrast with our recent observation that wild-type *Vibrio cholerae* strains containing pCG86 produced LT and secreted more than 95% of it into the culture supernatant (27a). These observations are consistent with the hypothesis that *V. cholerae* has a mechanism for secretion of LT into the extracellular milieu that is not present in any of the *Enterobacteriaceae* tested.

In summary, we have evaluated the role of several genetic factors on the expression of LT genes. In isogenic *E. coli* strains containing a variety of Ent plasmids, the yields of LT were greater when the chromosomal mutation *htx-2* was present than in wild type *htx*$^+$ strains,

TABLE 4. Synthesis of LT by several species of *Enterobacteriaceae* containing plasmid pCG86 or TP237Tc

Bacterial host[a]		pCG86 LT (μg/ml of culture)			TP237Tc LT (μg/ml of culture)		
		Cell associated		Extracellular activity[b]	Cell associated		Extracellular activity[b]
Strain	Species	Antigen	Biological activity		Antigen	Biological activity	
KL320	*E. coli*	0.71	0.40	0.005	1.7	4.0	0.020
NH3	*S. flexneri*	0.14	0.10	–	0.20	0.81	0.020
NH4	*S. sonnei*	0.054	0.02	0.001	0.032	0.040	0.020
NH5	*C. freundii*	0.014	0.010	0.001	0.11	0.32	0.001
NH6	*E. cloacae*	0.31	0.10	0.001	0.26	0.40	0.010
NH7	*K. pneumoniae*	0.10	0.023	–	0.048	0.10	0.001
LT2	*S. typhimurium*	NT[c]	NT	NT	6.9	10.1	0.040

[a] Conjugal transfer of plasmids into these host strains was performed by matings on solid media. Plasmid TP237Tc was first transferred from strain 14R525 into an *rif*^{r} purine auxotroph of *S. typhimurium* LT2 and was subsequently transferred from that intermediate host into strain LT2.

[b] The amounts of extracellular enterotoxin were below the limits of detection of radioimmunoassay. –, No detectable activity in the Y1 adrenal cell assay (≤0.0005 μg of LT per ml of culture supernatant).

[c] NT, Not tested.

suggesting that a regulatory feature common to all the plasmids was controlled by the *htx* locus. The plasmids pCG86 and TP237Tc were maintained in a variety of gram-negative bacteria within the family *Enterobacteriaceae*. In each of these strains, the plasmid-coded LT genes were expressed, and most of the LT remained cell associated. Our observations support the concept that interspecific, conjugal transfer of enterotoxin plasmids may be an important mechanism by which a wide variety of potentially pathogenic enteric bacteria can acquire the ability to produce LT.

ACKNOWLEDGMENTS

We are grateful to B. Ames, S. Falkow, R. A. Finkelstein, D. Kopecko, W. Maas, M. McConnell, and M. Robinson for providing bacterial strains used in the study. We are indebted to D. Court for phage λ 561 and to J. Newland and B. Green for providing purified plasmid EWD299 DNA. We thank I. Dinger for typing of this manuscript.

This work was supported by Public Health Research grant 5 R22 AI14107 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Alper, M. D., and B. N. Ames.** 1978. Transport of antibiotics and metabolite analogs by systems under cyclic AMP control: positive selection of *Salmonella typhimurium cya* and *crp* mutants. J. Bacteriol. **133**:149–157.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Bramucci, M. G., E. M. Twiddy, W. B. Baine, and R. K. Holmes.** 1981. Isolation and characterization of hypertoxinogenic (*htx*) mutants of *Escherichia coli* KL320(pCG86). Infect. Immun. **32**:1034–1044.
4. **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.
5. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139**:850–858.
6. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics, p. 140–141 and 201. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Finkelstein, R. A., P. Atthosampumna, M. Chulasamaya, and P. Charunmethee.** 1966. Pathogenesis of experimental cholera: biological activities of purified procholeragen A. J. Immunol. **96**:440–449.
8. **Finkelstein, R. A., M. J. Vasil, J. R. Jones, R. A. Anderson, and T. S. Barnard.** 1976. Clinical cholera caused by enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **3**:382–384.
9. **Geary, S. J., B. A. Marchlewicz, and R. A. Finkelstein.** 1982. Comparison of heat-labile enterotoxin from porcine and human strains of *Escherichia coli*. Infect. Immun. **36**:215–220.
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. **Gill, D. M., and S. H. Richardson.** 1980. Adenosine diphosphate-ribosylation of adenylate cyclase catalyzed by heat-labile enterotoxin of *Escherichia coli*: Comparison with cholera toxin. J. Infect. Dis. **141**:64–70.

11a. **Green, B. A., R. J. Neill, W. T. Ruyechan, and R. K. Holmes.** 1983. Evidence that a new enterotoxin of *Escherichia coli* which activates adenylate cyclase in eucaryotic target cells is not plasmid mediated. Infect. Immun. **41**:383–390.

12. **Guerrant, R. L., M. D. Dickens, R. P. Wenzel, and A. Z. Kapikian.** 1976. Toxigenic bacterial diarrhea: nursery outbreak involving multiple bacterial strains. J. Pediatr. **89**:885–891.
13. **Guerrant, R. L., R. A. Moore, P. M. Kirschenfeld, and M. A. Sande.** 1975. Role of toxigenic and invasive bacteria in acute diarrhea of childhood. N. Engl. J. Med. **293**:567–573.
14. **Gyles, C. L., S. Palchaudhuri, and W. K. Maas.** 1977. Naturally occurring plasmid carrying genes for enterotoxin production and drug resistance. Science **198**:198–199.
15. **Gyles, C. L., M. So, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**:40–48.
16. **Holmes, R. K., M. G. Bramucci, and E. M. Twiddy.** 1980. Genetic and biochemical studies of heat-labile enterotoxin of *Escherichia coli*, p. 189–201. *In* Proceedings of the 15th Joint Conference on Cholera. National Institutes of Health publication 80-2003. National Institutes of Health, Bethesda, Md.
17. **Honda, T., T. Tsuji, Y. Takeda, and T. Miwatani.** 1981. Immunological nonidentity of heat-labile enterotoxin from human and porcine enterotoxigenic *Escherichia coli*. Infect. Immun. **34**:337–340.
18. **Kleckner, N., D. F. Barker, D. G. Ross, and D. Botstein.** 1978. Properties of the translocatable tetracycline-resistance element Tn10 in *Escherichia coli* and bacteriophage lambda. Genetics **90**:427–461.
19. **Klipstein, F. A., and R. F. Engert.** 1977. Immunological interrelationships between cholera toxin and the heat-labile and heat-stable enterotoxins of coliform bacteria. Infect. Immun. **18**:110–117.
20. **Klipstein, F. A., and R. F. Engert.** 1978. Immunological relationships of different preparations of coliform enterotoxins. Infect. Immun. **21**:771–778.
21. **Kopecko, D. J., J. Holcombe, and S. B. Formal.** 1979. Molecular characterization of plasmids from virulent and spontaneously occurring avirulent colonial variants of *Shigella flexneri*. Infect. Immun. **24**:580–582.
22. **Kopecko, D. J., O. Washington, and S. B. Formal.** 1980. Genetic and physical evidence for plasmid control of *Shigella sonnei* form I cell surface antigen. Infect. Immun. **29**:207–214.
23. **Maneval, D. R., Jr., R. R. Colwell, S. W. Joseph, R. Gray, and S. T. Donta.** 1980. A tissue culture method for the detection of bacterial enterotoxins. J. Tissue Cult. Methods. **6**:85–90.
24. **McConnell, M. M., H. R. Smith, G. A. Willshaw, S. M. Scotland, and B. Howe.** 1980. Plasmids coding for heat-labile enterotoxin production isolated from *Escherichia coli* O78: comparison of properties. J. Bacteriol. **143**:158–167.
25. **Minshew, B. H., R. K. Holmes, J. P. Sanford, and C. R. Baxter.** 1974. Transferrable resistance to tobramycin in *Klebsiella pneumoniae* and *Enterobacter cloacae* associated with enzymatic acetylation of tobramycin. Antimicrob. Agents Chemother. **6**:492–497.
26. **Moseley, S. L., P. Echeverria, J. Seriwantana, C. Tirapat, W. Chaicumpa, T. Sakuldaipeara, and S. Falkow.** 1982. Identification of enterotoxigenic *Escherichia coli* by colony hybridization using three enterotoxin gene probes. J. Infect. Dis. **145**:863–869.
27. **Moseley, S. L., K. Huq, A. R. M. A. Alim, M. So, M. Samadpour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142**:892–898.

27a. **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.

28. **Parker, C., and W. R. Romig.** 1972. Self-transfer and genetic recombination mediated by P, the sex factor of *Vibrio cholerae*. J. Bacteriol. **112**:707–719.
29. **Quackenbush, R. L., and S. Falkow.** 1979. Relationship

between colicin V activity and virulence in *Escherichia coli*. Infect. Immun. **24**:562–564.
30. **Robinson, M. K., P. M. Bennett, S. Falkow, and H. M. Dodd.** 1980. Isolation of a temperature sensitive derivative of RP1. Plasmid **3**:343–347.
31. **Silva, M. L. M., W. K. Maas, and C. L. Gyles.** 1978. Isolation and characterization of enterotoxin-deficient mutants of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:1384–1388.
32. **Smith, H. W., and S. Halls.** 1968. The transmissible nature of the genetic factor in *Escherichia coli* that controls enterotoxin production. J. Gen. Microbiol. **52**:319–334.
33. **So, M., W. S. Dallas, and S. Falkow.** 1978. Characterization of an *Escherichia coli* plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. Infect. Immun. **21**:405–411.
34. **Yamamoto, T., T. Yokota, and A. Kaji.** 1981. Molecular organization of heat-labile enterotoxin genes originating in *Escherichia coli* of human origin and construction of heat-labile toxoid-producing strains. J. Bacteriol. **148**:983–987.