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Polynucleotide Sequence Relationships Among Ent Plasmids and the Relationship Between Ent and Other Plasmids

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Deoxyribonucleic acid-deoxyribonucleic acid hybridization studies reveal that the plasmids coding for the production of heat stable and heat labile enterotoxins of *Escherichia coli*, regardless of their origin, have a majority of their polynucleotide sequences in common, but are not related in any significant way to those plasmids coding for the synthesis of only ST toxin. The heat stable and heat labile plasmids also share a significant degree of their polynucleotide sequences with plasmids of the FI and FII incompatibility groups, but not with R factors belonging to the I, N, W, P, or X incompatibility groups.

It has become increasingly apparent that many cases of noninvasive gastroenteritis in man and domestic animals are caused by toxigenic strains of *Escherichia coli*. The production of enterotoxin by enteropathogenic *E. coli* strains has been shown in a number of instances to be governed by a plasmid designated Ent (17, 20, 23). Ent plasmids are often accompanied in their enteropathogenic hosts by other plasmids which are uncommon or absent from *E. coli* isolated from asymptomatic individuals. These distinctive plasmids include Hly, a plasmid specifying a hemolysin (18, 22), as well as those coding for the surface antigens K88 (1, 15) and K99 (21) found in *E. coli* isolates from calves and lambs. Although Ent, Hly, K88, and K99 plasmids are independent units of replication and transmission, the total plasmid complement of a strain has important implications for the ability of an organism to produce enteric disease. This has been particularly well documented in the case of porcine *E. coli* strains which contain the Ent and K88 plasmids (13, 19). Thus, K88 and Ent alone are relatively minor additions to the total pathogenic properties of a strain, but together they often permit a host bacterium to be fully enteropathogenic. This appears to be a consequence of the combined selective ability of cells containing K88 to multiply in the anterior small bowel together with the fact that *E. coli* enterotoxins specified by Ent affect only the epithelial cells of this region. Thus, while the mere presence of Ent and the associated biosynthesis of enterotoxin is not sufficient in many cases to permit an organism to produce enteric disease, Ent may be the principal bacterial determinant of virulence under the proper circumstances.

We have recently isolated a series of *E. coli* K-12 sublines identical in all respects except for the presence or absence of a single Ent plasmid (11). Two broad classes of Ent plasmids have been identified. One class specifies the production of a heat stable enterotoxin (Ent ST); the other class specifies the production of both a heat stable and heat labile enterotoxin (Ent ST + LT). The Ent ST plasmids are shown to be heterogeneous in their molecular size and mole fraction guanine + cytosine (G + C). In contrast, the Ent ST + LT plasmids, regardless of origin, appear to be relatively homogeneous in both molecular size and G + C content. In this paper we report the results of deoxyribonucleic acid (DNA) duplex studies designed to more clearly define the relationships among the Ent ST + LT plasmids as well as the relationships between the Ent ST + LT and other plasmid classes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strain 711 (11) was used as the bacterial host for all Ent plasmids, whereas the strain *E. coli* J53 (4) was used as the bacterial host for all other plasmid species. The origin, genetic properties, and the molecular properties of the plasmids used in this study are shown in Tables 1 and 2.

Isolation of labeled plasmid DNA. Plasmid DNA was labeled by growing cells in a minimal medium described by Freifelder and Freifelder (6) with 0.9 μg of [^{3}H]thymine (20 Ci/mmol, New England Nuclear) per 30 ml of culture. The cells were aerated vigorously at 37 C until the density reached 5×10^8 cells per ml, whereupon they were harvested. Plasmid DNA was isolated by the cleared lysate method described by Clewell and Helinski (2). We added 2.4 ml of each of four cleared lysates to 8.5 ml of cesium chloride (1.43 g of cesium chloride per ml of buffer [0.05 M NaCl,

TABLE 1. *Properties of Ent plasmids used in this study*

Plasmid	Origin	Phenotype[a]	Molecular weight $\times^{b}$ 10^6 daltons	Mole fraction[c] G + C
P307	Porcine	ST + LT	60	0.50
P130	Porcine	ST + LT	61	0.50
P155	Porcine	ST + LT	55	0.50
SF119	Human	ST + LT	60	0.50
P95	Porcine	ST	20	0.41
P16	Porcine	ST	25	N.D.

[a] Abbreviations: ST, synthesis of heat stable enterotoxin; LT, synthesis of heat labile enterotoxin; N.D., not done.

[b] The molecular weight of each plasmid species was determined by both sucrose gradient sedimentation (11) and the measurement of contour lengths of open circular DNA molecules in the electron microscope.

[c] The mol fraction of G + C was calculated relative to [^{14}C]thymine labeled λ phage DNA which was taken as 1.709 g/cm^{-3}, 0.49 mole fraction G + C, and assuming that the DNA of these plasmids did not contain any unusual bases.

0.005 M ethylenediaminetetraacetic acid (EDTA), 0.03 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0], and 0.58 ml of ethidium bromide (5 mg/ml in 0.05 M NaCl, 0.005 M EDTA, 0.03 M Tris, pH 8.0 buffer). The refractive index of this solution was adjusted to 1.3925 ± 0.001 and the DNA was centrifuged to equilibrium in a fixed angle type 65 rotor at 129,048 × *g* for 40 h at 15 C. Ten-drop fractions were collected and a portion of every other fraction was spotted on Whatman 3MM filters and counted in a liquid scintillation spectrometer. The fractions corresponding to the plasmid peak were pooled, the ethidium bromide extracted with cesium chloride-saturated isopropanol, and dialyzed against at least 200 volumes of 0.42 M NaCl. The DNA was sheared in a Branson sonic oscillator to 2.5 × 10^6 daltons as described previously (3).

Preparation of unlabeled whole cell DNA. Unlabeled DNA was extracted by a modification of the method by Thomas (25). Cells were grown overnight with aeration in nutrient broth (Difco) and harvested. The pellet was resuspended in a lysing solution consisting of 0.5 M EDTA, 0.05 M Tris-hydrochloride, pH 8.1, 0.1 M NaCl, and 50 μg of Pronase (Calbiochem) per ml. Sodium lauryl sulfate was added to a final concentration of 1%. The suspension was extracted with an equal volume of 1/1 mixture of phenol (Fischer) and chloroform-isoamyl alcohol, the latter mixture at a ratio of 24:1. The DNA was precipitated with cold 95% ethanol and resuspended in 0.01 × SSC (SSC = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0). The solution was adjusted to 0.1 M NaCl, 0.05 M EDTA, 0.05 M Tris-hydrochloride, pH 8.1, and was extracted again with the phenol mixture. The DNA was precipitated with cold ethanol and redissolved in 0.01 × SSC. Acetate-Versene (3.0 M sodium acetate + 0.001 M EDTA, pH 7.0) was added in a ratio of 1 ml of acetate-Versene to 9 ml of DNA solution. Isopropanol was added drop by drop to precipitate the DNA which was redissolved in 0.01 × SSC. The DNA was sheared in a Branson sonic oscillator to 2.5 × 10^6 daltons, dialyzed against 0.42 M NaCl, and passed through a Metricell filter (Gelman) with a pore size of 0.45 μm. The DNA extracted in this manner gave a 260–280 nm optical density ratio of 1.7 and the yield was between 20 to 25 mg per 3 liters of cells.

Preparation of DNA-DNA duplexes and their detection. DNA-DNA duplex formation between labeled plasmid DNA and the whole cellular DNA of *E. coli* containing the appropriate plasmid species was assayed as previously described by Crosa et al. (3).

RESULTS AND DISCUSSION

Polynucleotide sequence relationships among Ent plasmids. The Ent ST + LT plasmid P307 was chosen as a reference for comparison since it has been studied extensively in genetic experiments (9) and its toxin has been the subject of considerable interest (12). The sheared labeled P307 plasmid DNA was denatured and 0.002 μg (3 × 10^5 counts/min per μg of DNA) was mixed with 150 μg of sheared unlabeled, single-stranded DNA from *E. coli* K-12 carrying appropriate Ent plasmids. The DNA of a homogenic *E. coli* K-12 plasmid-

TABLE 2. *Properties of R and other plasmids used in this study*

Plasmid	Compatibility[a]	Resistance markers[b]	Molecular weight[c] × 10^6 daltons	Mole fraction G + C[d]
R144	I α	Tc Km	63	0.50
Rldrd19	FII	Ap Sm Cm Km Su	65	0.51
F	FI		62	0.48
N3	N	Sm Tc Su	32	0.49
Sa	W	Sm Cm Km Su	25	0.62
RP4	P	Ap Tc Km	34	0.59
R6K	X	Ap Sm	25	0.45

[a] The compatibility group of these plasmids has been previously described (S. Falkow, P. Guerry, R. W. Hedges, and N. Datta, J. Gen. Microbiol., in press).

[b] Abbreviations: Ap, ampicillin; Sm, streptomycin; Tc, tetracycline; Cm, chloramphenicol; Km, kanamycin; Su, sulfonamide.

[c] The molecular weight of each plasmid species was determined by both sucrose gradient sedimentation (11) and the measurement of contour lengths of open circular DNA molecules in the electron microscope.

[d] The mole fraction of G + C was calculated relative to [^{14}C]thymine labeled λ phage DNA which was taken as 1.70 g/cm^3, 0.49 mole fraction G + C, and assuming that the DNA of these plasmids did not contain any unusual bases.

less subline (F^-) served as a control. The DNA mixtures were placed at 75 C in 0.42 M NaCl for 16 h at which time the proportion of common polynucleotide sequences between the labeled P307 and the unlabeled Ent DNA was determined with S1 endonuclease (3). Table 3 summarizes the results of these experiments. P307 DNA was found to share roughly 7% of its sequences with the DNA of an *E. coli* K-12 F^- strain. This level of relatedness has been commonly observed between several R factors and host chromosomal DNA (8), but is significantly less than that seen between the F factor and *E. coli* (5). The nature of the sequences held in common between Ent (or other plasmids) and chromosomal DNA is unknown, although the reassociation experiments were carried out at 75 C which sets stringent criteria for the formation of DNA-DNA duplexes and discriminates against imperfectly matched base pair sequences (3). Taking the F^- contribution of P307 DNA into account it can be seen that P307 shared about 87% of its sequences with other Ent ST + LT plasmids of porcine origin and about 55% of its sequences with SF119, an Ent ST + LT plasmid derived from an *E. coli* strain of human origin. No significant level of relatedness was detected between the P307 ST + LT plasmid DNA and any Ent ST plasmid that was examined.

Because of the partial antigenic identity between *E. coli* ST enterotoxin and cholera toxin, there has been speculation that the *E. coli* Ent plasmids might have been directly derived from *Vibrio* (or vice-versa) (10, 24). All toxigenic *V. cholerae* strains that we have examined have contained at least one plasmid, and in some cases up to three distinct plasmids. There is no evidence that toxin biosynthesis in *V. cholerae* is plasmid mediated, however. We were unable to detect any degree of DNA sequence relationship between P307 ST + LT DNA and the DNA extracted from three toxigenic *V. cholerae* strains (<1% relatedness, data not shown). Our data indicate, therefore, that the *E. coli* Ent ST + LT plasmids are quite distinct in their molecular organization from *Vibrio* genetic material.

TABLE 3. *Polynucleotide sequence relationships between Ent plasmid P307 and other Ent plasmids*

Plasmid	Origin	Toxin type	Relatedness with P307 (%)[a]
P307	Porcine	ST + LT	100
P130	Porcine	ST + LT	87
P155	Porcine	ST + LT	88
SF119	Human	ST + LT	55
P95	Porcine	ST	1
P16	Porcine	ST	1

[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]P307 purified plasmid DNA with whole cell *E. coli* P307 DNA was 90%, which was corrected for a 7% reaction between [^{3}H]P307 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.

DNA-DNA duplex studies have also been performed with several ^{3}H-labeled representative Ent ST plasmids of porcine origin. No detailed data will be presented here. It should be noted, however, that they do not share a significant proportion of their nucleotide sequences (2%) with any Ent ST + LT plasmids that have been examined nor with *V. cholerae* DNA (<1% relatedness). The clinical observation that toxigenic isolates from pigs produce either only ST or ST + LT suggested the possibility that the porcine Ent ST plasmid might be an immediate ancestor, or alternatively, a derivative of Ent ST + LT plasmids. Clearly, our observations are not consistent with this view. Of course, the structural genes determining toxin biosynthesis are expected to occupy but a small portion of the plasmid genome. It is possible, therefore, that the ST toxin specified by Ent ST plasmids is closely related to the ST toxin synthesized by strains containing an Ent ST + LT plasmid. Similarly, it is possible that the LT toxin of *E. coli* and cholera toxin have a common ancestral origin. Our data simply indicate that the major genetic sequences of the molecular vehicles carrying the toxin genes are not closely related.

Relationship between Ent ST + LT plasmids and other extrachromosomal elements. Plasmids have been classified in terms of their ability to mutually coexist within the same cell. In general, unrelated plasmids can stably coexist in the same host cytoplasm; they are compatible. On the other hand, related plasmids ordinarily do not coexist within the same host cytoplasm; they are incompatible. Plasmids which are mutually incompatible with each other are said to form an incompatibility group and it has been shown by several laboratories that plasmids of the same incompatibility group share a significant proportion of their polynucleotide sequences in common but are only marginally related to plasmids of other compatibility groups (7, 8). The major exception to this general rule is found among plas-

mids which are genetically compatible with each other but determine similar sex pili. Among the compatible FI and FII groups, for example, DNA-DNA duplex studies have shown that the sequences shared in common are restricted to a continuous stretch of DNA involved in pili biosynthesis and transfer functions. Since the Ent ST + LT plasmids studied were a homogeneous group which shared a significant proportion of their nucleotide sequences in common, it seemed likely that they belonged to the same incompatibility groups. However, genetic tests of compatibility were not possible because of the technical restrictions of dealing with a group of plasmids that have no discernible genetic properties except enterotoxin synthesis. Consequently, we examined the nucleotide relationships between labeled P307 plasmid DNA and the DNA from representatives of several R plasmids that have been previously characterized. Table 4 shows that the DNA of the P307 Ent plasmid did not share a significant level of nucleotide sequences in common with representative I, N, P, W, or X plasmids. There was a significant degree of DNA relatedness between P307 DNA and the DNA of the classic F factor (FI) and the FII R plasmid, R1. Since F and R1 have been shown to share only sequences involved in transfer and pili genes (16), it seems likely that the Ent P307 plasmid contains, at least in part, the genetic information for the biosynthesis of F-like pili. This has been confirmed by showing that the F-specific phage MS2 can be propagated on strains carrying P307 as well as *E. coli* K-12 carrying other Ent ST + LT plasmids. Table 4 shows the results obtained when ^{3}H-labeled R1 plasmid DNA was reassociated with representative Ent plasmids. About 45% of the sequences of R1 were found to be shared by the porcine Ent ST + LT plasmid P307, P155, and P130. The SF119 ST + LT plasmid of human origin was even more closely related and shared 75% of its sequences in common with R1. No significant degree of relatedness was found between R1 and an Ent ST plasmid however. Thus, the Ent ST + LT plasmids we have examined do not appear to represent a unique plasmid group, but rather seem to belong to the F incompatibility complex (7, 14).

The groundwork that has already been established for the detailed structural mapping of F and R factors (16) can be obviously exploited to better define the precise functional similarities between Ent and other F-like plasmids. However, even this preliminary view of the molecular relationships of Ent ST + LT plasmids is sufficient to show that they are but a variation on a common biological theme encountered so often with bacterial plasmids. For example, the differences between the R-factor R1, and the Ent plasmid, SF119, is rather minimal and reflects that basically the same transfer factor has in the former instance acquired drug resistance genes and in the latter instances, genes determining enterotoxin biosynthesis, in response to different selective pressures.

TABLE 4. *Polynucleotide sequence relationships*

Plasmid	Incompatibility class	Homology (%)
Between P307 Ent ST + LT plasmid and plasmids of known incompatibility groups		
P307	?	100[a]
R144	Iα	5
Rldrd	FII	45
F	FI	21
N_3	N	<1
Sa	W	<1
RP4	P	<1
R6K	X	<1
Between Rldrd and Ent ST + LT plasmids		
Rldrd	FII	100[b]
P307	?	46
P155	?	41
SF119	?	75

[a] [^{3}H]thymine P307 DNA was reassociated with whole cell *E. coli* DNA extracted from a strain carrying each of the indicated plasmids of different representative compatibility groups. The degree of duplex formation is expressed relative to the homologous P307 reaction (actual binding 88%) corrected for the *E. coli* F$^-$ contribution (6.5%).

[b] [^{3}H]thymine Rldrd19 plasmid DNA was reassociated with whole cell *E. coli* DNA extracted from a strain harboring each of the indicated plasmids. The degree of duplex formation is expressed relative to the homologous Rldrd19 reaction (actual binding, 92%) corrected for the *E. coli* F$^-$ contribution (13.2%).

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ADDENDUM IN PROOF

Recent genetic experiments have revealed that the Ent plasmid P307 is incompatible with the classical F-*lac*$^+$ factor but can stably coexist in the same host with the R plasmid R1. We assume, therefore, that P307 should be assigned to the FI incompatibility group. In contrast, the Ent plasmid SF119, on genetic grounds, should be assigned to the FII incompatibility group since it can stably coexist with F-*lac*$^+$ but not R1.

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