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Characterization of an R-Plasmid Associated with Ampicillin Resistance in *Shigella dysenteriae* Type 1 Isolated from Epidemics

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Ampicillin-resistant strains of *Shigella dysenteriae* type 1 isolated in epidemics in Mexico, Central America, and Bangla Desh were examined for the presence of plasmid deoxyribonucleic acid (DNA) by gel electrophoresis. All strains contained a heterogeneous population of plasmids. Transfer experiments to *Escherichia coli* K-12 indicated that the ampicillin resistance determinant (Ap^r) was located on a 5.5-megadalton (Mdal) plasmid identical in all Shiga strains examined, as judged by DNA hybridization and by its molecular properties. This 5.5-Mdal plasmid contained the ampicillin transposon (TnA) sequences. There was not a high degree of homology between the Shiga Ap^r plasmid DNA and DNA obtained from Ap^r *Salmonella typhi* strains isolated from typhoid epidemics in Mexico, previous to the dysentery outbreaks. Although low, the degree of reassociation observed indicated that probably part of the TnA sequence was present in *S. typhi* DNA. The DNA hybridization experiments showed, in addition, that there was a high degree of homology among Ap^r plasmids isolated from different enterobacteria, and this identity was confirmed by restriction endonuclease activity. These results together with their similarities in molecular and replicative properties indicate that the Ap^r plasmids, as was suggested for the Sm^r Su^r plasmids, possibly evolved once and then epidemiologically spread in the *Enterobacteriaceae*.

Shigella dysenteriae type 1 was the causative organism in the 1970 epidemics of dysentery in Central America (12) and Mexico (16). The isolated Shiga bacillus strains were resistant to chloramphenicol, tetracycline, streptomycin, and sulfonamides, but uniformly susceptible to ampicillin. Subsequently, an outbreak of dysentery occurred in Mexico in a hospital ward lodging children under treatment for tuberculosis. This time the causative organism was Shiga bacillus, which was resistant to ampicillin in addition to the other drugs (14). In the period between 1972 and 1974 several ampicillin-resistant strains of *S. dysenteriae* type 1 were also isolated in outbreaks that occurred in Central America (17) and Bangla Desh (18). In at least two cases (14, 17) the ampicillin resistance could be transferred independently from the other drug resistances from *S. dysenteriae* to *Escherichia coli* K-12, suggesting that an independent R-plasmid might be involved. Our present communication is to report the isolation, molecular characterization, and epidemiological implications of the R-plasmid associated with ampicillin resistance in *S. dysenteriae* type 1.

MATERIALS AND METHODS

Bacterial strains. The ampicillin-resistant strains of *S. dysenteriae* type 1 were: strain 51B isolated in a Mexican epidemic, strain 762 isolated in a Costa Rican outbreak, and strain CDC 6986 (obtained from the Center for Disease Control, Atlanta, Ga.) isolated in a Bangla Desh epidemic. *Salmonella typhi* strains JM and P12 and *E. coli* K-12 W 1485-1 thy^- Nx^r (SF 148) were described previously (6, 14).

Bacterial conjugation. Crosses between multiply resistant *S. dysenteriae* type 1 and *E. coli* K-12 Nx^r were carried out by mixing 5 ml of log-phase cultures of both donor and recipient into 25 ml of Pennassay broth and incubating at 37°C without shaking for 10 h. Samples were plated on MacConkey agar containing the appropriate antibiotics at a concentration of 20 μg/ml. Colonies were restreaked several times on antibiotic plates before further processing. When needed, the antibiotic minimal inhibitory concentration was determined by the method of Bauer et al. (3).

Determination of plasmid DNA content. The

presence of plasmid deoxyribonucleic acid (DNA) was determined by an agarose electrophoretic method (13). Strains were grown overnight in 30 ml of brain heart infusion broth. Lysates were prepared (13) and after digestion with ribonuclease and extraction with phenol (once) and chloroform (twice), the clear aqueous phase was adjusted to 0.3 M sodium acetate and precipitated with twice the volume of precooled ethanol (−20°C) overnight at −20°C. After centrifugation at −10°C at 10,000 rpm for 20 min, the precipitate was resuspended in 0.2 ml of TES buffer [50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-5 mM ethylenediaminetetraacetate (EDTA)-50 mM NaCl (pH 8)]. A sample was electrophoresed in a 0.7% agarose gel in Tris-borate buffer (89 mM Tris base-2.5 mM disodium EDTA-89 mM boric acid). DNA was visualized by staining with ethidium bromide and viewing the fluorescence produced by the intercalated dye when illuminated with an ultraviolet light source. The preparation of ^{3}H-labeled plasmid DNA and unlabeled whole-cell DNA as well as the analysis of plasmid DNA in cesium chloride-ethidium bromide and sucrose density gradients was described previously (7, 8).

DNA hybridization. DNA-DNA hybridizations were carried out essentially as described previously (6). Approximately 0.01 μg of ^{3}H-labeled, sheared, denatured plasmid DNA was incubated with 150 μg of unlabeled, sheared, denatured whole-cell DNA from each of the indicated organisms. DNA reassociation was performed in 0.42 M NaCl at 70°C for 22 min.

The degree of homology was assessed by the S1 nuclease method of Crosa et al. (6).

RESULTS

Molecular characterization of plasmids in *S. dysenteriae* type 1. Resistance to ampicillin (Ap^r) was reported to be independently transferred from resistance to chloramphenicol (Cm^r), tetracycline (Tc^r), streptomycin (Sm^r), and sulfonamides (Su^r) from *S. dysenteriae* type 1 to *E. coli* K-12 (14, 17). These results suggested that the Ap^r determinant was carried on an independent plasmid from the one carrying the Cm Tc Sm Su determinants. When both plasmids in *E. coli* K-12 were tested for their transfer properties to other *E. coli* K-12 strains, it was found that the plasmid carrying the Cm Tc Sm Su determinants was conjugative, whereas the Ap^r plasmid was not conjugative but mobilizable by the conjugative R plasmid (unpublished data).

To analyze the molecular nature of the *Shigella* plasmids, we began by examining agarose gel electrophoresis patterns of DNA isolated from the Shiga and exconjugant *E. coli* strains isolated from the conjugal crosses with *Shigella*. The rationale for this method is that covalently closed circular (CCC) plasmid DNA molecules band in agarose gels according to their size, whereas the chromosome DNA (which is sheared due to manipulations during its preparation) forms a broader band at a specific region (13).

All three multiple resistant *Shigella* strains show quite a number of bands in addition to that of the chromosome (Fig. 1), which indicates the presence of several different plasmids in each strain. By comparison with a molecular weight standard, it is possible to calculate the molecular weight of the Shiga plasmids that are listed in the legend to Fig. 1. Although there are several different plasmid sizes in each Shiga strain, there is only one plasmid band that all three Shiga strains have in common, that corresponding to a molecular species of 5.5 megadaltons (Mdal). When the DNA obtained from the *E. coli* exconjugants is analyzed, it becomes easier to assign drug resistance determinants to plasmids. The 80-Mdal band is identified as the multiple drug resistance conjugative plasmid, and the 5.5-Mdal band is the nonconjugative Ap^r plasmid. The Bangla Desh strain does not show the 80-Mdal band, but it has two bands of 62 and 52 Mdal instead. The rest of the *Shigella* plasmids remain cryptic in the phenotypic sense.

To further characterize the Shiga Ap^r plasmid, ^{3}H-labeled plasmid DNA obtained from exconjugants 1 and 2 was purified on a CsCl-ethidium bromide density gradient and fractions from the CCC peak were pooled and rerun in a second CsCl-ethidium bromide gradient. This time the fractions corresponding to the CCC peak were pooled, isopropanol treated to remove the ethidium bromide and then dialyzed against 6 mM Tris-0.1 mM EDTA (pH 7.5). The purified plasmid DNA was then analyzed on 5 to 20% sucrose density gradients and by electron microscopy to determine the molecular weight. The results are in close agreement with those obtained by gel electrophoresis of partially purified plasmid DNA (Table 2), indicating that the Ap^r plasmid is 5.5 Mdal in size in all of these strains.

Polynucleotide sequence relationships among Ap^r plasmids. We used the S1 nuclease method (6) to assess the DNA homology among the Ap^r plasmid isolated from different sources.

We hybridized ^{3}H-labeled Ap^r plasmid DNA with unlabeled DNA extracted from the *Shigella* strains and from the *E. coli* exconjugants containing either the Ap^r plasmid, the conjugative R plasmid, or both together. DNA extracted from the *Shigella* strains, independent of the geographical source of the epidemic, shared a high level of homology with the Ap^r plasmid isolated from the Mexican strain. This is also true when DNAs from *E. coli* exconju-

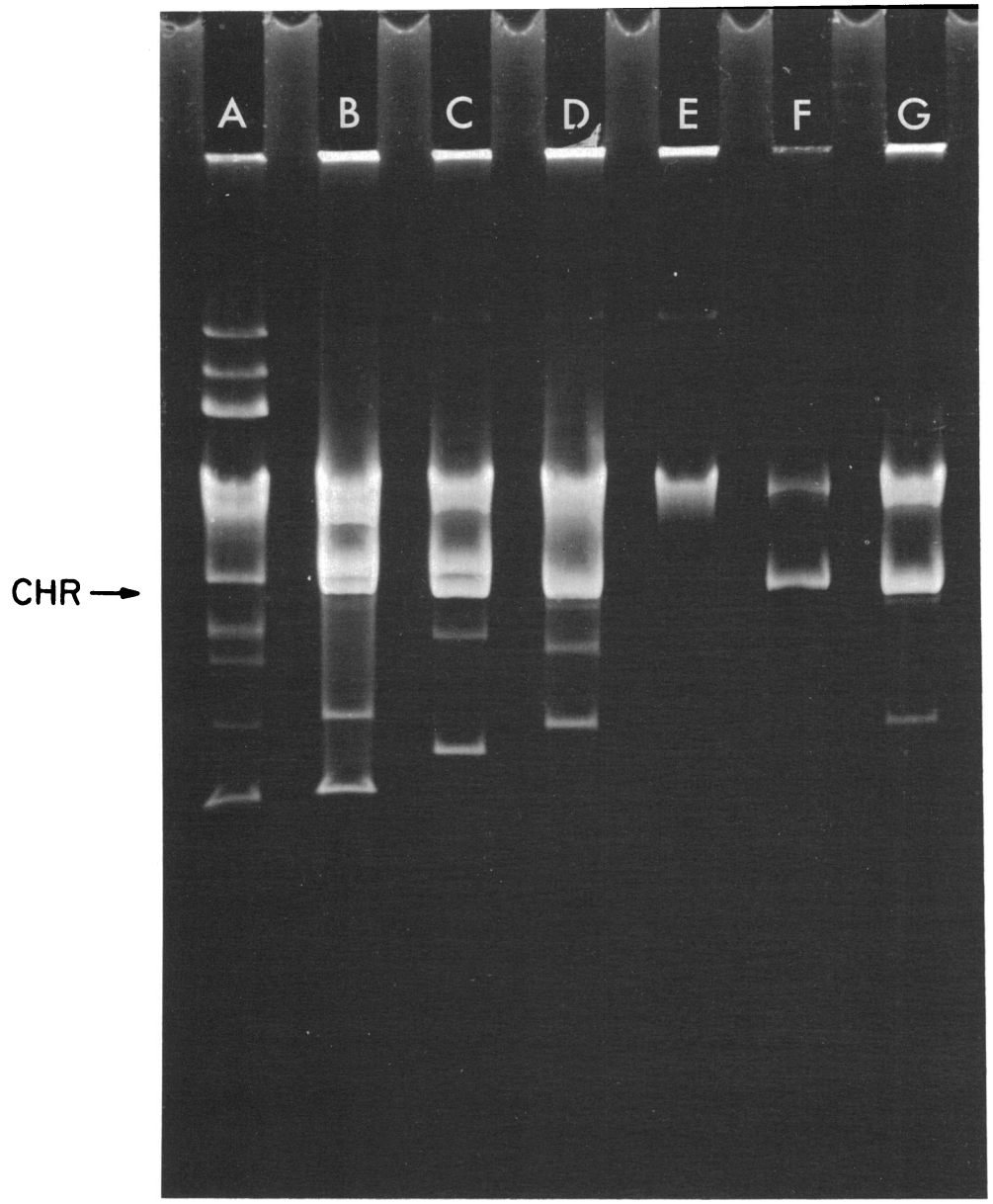

FIG. 1. *Agarose gel electrophoresis of ethanol-precipitated DNA from bacterial lysates. (A) Standard plasmid DNAs ranging in molecular weights from 62 Mdal (uppermost band) to 1.8 Mdal (lowest band). CHR, Band position of the chromosomal DNA. (B) Lysate from S. dysenteriae type 1 strain M6982 Cm^r Tet^r Sm^r Su^r Ap^r isolated in the Bangla Desh epidemics. Molecular weights of plasmids present (in Mdal): 62, 52, 6.1, 5.5, 4.85, 2.65, and 1.85. (The 62-, 52- and 4.85-Mdal bands are barely visible.) (C) Lysate from S. dysenteriae type 1 strain 762 Cm^r Tet^r Sm^r Su^r Ap^r isolated in Costa Rica. Molecular weights of plasmids present (in Mdal): 80, 6.1, 5.5, 4.4, 4.1, and 2.2. (D) Lysate from S. dysenteriae type 1 strain 51B Cm^r Tc^r Sm^r Su^r Ap^r isolated in Mexican epidemic. Molecular weights of plasmids present (in Mdal): 80, 5.5, 4.6, 3.7, and 2.5. (E) Lysate from an E. coli K-12 (exconjugant no. 1; obtained from cross between S. dysenteriae type 1 (Mexico) and E. coli K-12 W1485-1 Nx^r) Cm^r Tet^r Sm^r Su^r and Ap^s. Molecular weights of plasmids present (in Mdal): 80. (F) Lysate from an E. coli K-12 (exconjugant no. 5; obtained from cross between S. dysenteriae type 1 (Mexico) and E. coli K-12 W1485-1 Nx^r) Ap^r (Cm^s Tc^s Sm^s Su^s). (G) Lysate from an E. coli K-12 (exconjugant no. 25; obtained from cross between S. dysenteriae type 1 (Mexico) and E. coli K-12 W1485-1 Nx^r) Cm^r Tet^r Sm^r Su^r Ap^r. Molecular weights of plasmids present (in Mdal): 80, 5.5, and 4.6. The band present at the position of 20 Mdal corresponds to form II (open circular) DNA of the 5.5-Mdal plasmid.*

TABLE 1. *Hybridization between ^{3}H-labeled RJHC5 Ap^r plasmid DNA and whole-cell DNA*

Source of unlabeled DNA	Relative DNA sequence homology with ^{3}H-labeled RJHC5 Ap^r DNA[a] (%)
E. coli K-12 exconjugant 1 (Ap^r Mexico)	100
E. coli K-12 exconjugant 2	0
E. coli K-12 exconjugant 3	89
E. coli K-12 exconjugant 4 (Ap^r Costa Rica)	98
E. coli K-12 exconjugant 5 (Ap^r Bangla Desh)	94
S. dysenteriae type 1 (Mexico)	91
S. dysenteriae type 1 (Costa Rica)	87
S. dysenteriae type 1 (Bangla Desh)	91
E. coli K-12 (RSF1030) (originally from *S. panama*)	98
E. coli K-12 (RJHC2) (originally from *C. freundii* Ind 6A)	93
E. coli K-12 (Sa-1)	51
E. coli K-12 (Sa)	0
S. typhi JM	23
S. typhi P12	30
E. coli K-12 W1485-1	0

[a] The degree of DNA-DNA duplex formation was assayed by the S1 endonuclease method (6). The actual extent of reassociation for the homologous reaction was in average 87%. All other reactions were normalized to this value set at 100%. Each value shown is an average of three separate determinations.

gants containing only the Ap^r plasmid from either the Mexican, Costa Rican, or Bangla Desh epidemics are analyzed. DNA extracted from *E. coli* exconjugants containing the *Shigella* conjugative R-plasmid shared only 1.5% of its sequences with the Ap^r plasmid, whereas exconjugants containing both conjugative and nonconjugative Ap^r R-plasmids were as expected, highly related to the Ap^r plasmid.

The DNA-DNA hybridization experiments show not only the identity among the different *Shigella* Ap^r plasmids but also their high relation to RSF1030 and RJHC2, two Ap^r nonconjugative, 5.5-Mdal plasmids carrying determinants for Ap^r and isolated, respectively, from *Salmonella panama* (4) and *Citrobacter freundii* (unpublished data). It is now well established that, among R-plasmids present in the *Enterobacteriaceae*, the gene specifying TEM β-lactamase resides upon a 3×10^6-dalton sequence of DNA that is capable of excising itself from one plasmid and inserting itself into another (5, 10, 11). To determine whether any or all of the TnA segment was present in the ampicillin-resistant strains of *S. dysenteriae*, DNA-DNA hybridization experiments with Sa-1, a W plasmid containing the TnA sequences, and its counterpart, Sa, which does not carry the TnA sequences (10, 11), were carried out. Results indicate that about 50% of the Ap^r plasmid sequences are shared in common with Sa-1, but no homology was detected with Sa; i.e., about 3 Mdal of the 5.5-Mdal Shiga Ap^r plasmid

TABLE 2. *Comparison of replication properties of the Ap^r plasmids*

Origin of Ap^r plasmid	Designation	Mol wt (Mdal)[a]	No. of copies in *E. coli* K-12[b]	Replication in chloramphenicol[c]	Polymerase I requirement[d]
S. dysenteriae type 1 (Mexico)	RJHC5	5.5	39	90	+
S. dysenteriae type 1 (Costa Rica)	RJHC8	5.5	31	101	+
S. dysenteriae type 1 (Bangla Desh)	RJHC7	5.5	30	97	+
S. panama	RSF1030	5.5	35	100	+
C. freundii	RJHC2	5.5	35	110	+

[a] Molecular weights were determined by centrifugation on 5 to 20% neutral sucrose gradients as described previously (7).

[b] Number of copies per chromosome equivalent was determined as described previously (8).

[c] *E. coli* K-12 W1485-1 containing an Ap^r plasmid was prelabeled with [^{14}C]thymine (0.6 μCi/ml; 3.6 μg/ml) in minimal medium (7) until a concentration of 5×10^8 cells/ml was achieved. At this time cells were centrifuged at 25°C and resuspended in minimal medium without any label and containing 170 μg of chloramphenicol per ml. After 2.5 h in the presence of chloramphenicol, [^{3}H]thymine (55 μCi/ml; 2 μg/ml) was added and incubation was continued for 10 h. Cleared lysates were prepared and spun in a CsCl-ethidium bromide gradient (8). The ratio of ^{3}H to ^{14}C in the plasmid peak for RSF1030 was taken arbitrarily as 100%, and the rest of the values were normalized to this figure.

[d] The requirement of polymerase I for replication was determined by transformation of plasmid DNA into *E. coli* K-12 MM383, a thermosensitive *polA*$^-$ mutant, and by determining the amount of plasmid replicated at the nonpermissive temperature.

DNA must be the TnA sequences. There is not a high degree of homology between the Shiga Ap^r plasmid and DNA obtained from Ap^r *S. typhi* strains isolated from typhoid epidemics previous to the dysentery outbreaks in Mexico. Although low, the degree of reassociation observed would indicate that probably part of the TnA sequences are present in ampicillin-resistant *S. typhi*. Figure 2 shows the identical *Hin*cII restriction endonuclease banding pattern for the 5.5-Mdal Ap^r plasmids from *S. panama*, *C. freundii*, and *S. dysenteriae*, confirming the homology of all three plasmids.

Replication properties of the Ap^r plasmids. Table 2 shows a comparision of the replication properties of the *Shigella* Ap^r plasmid with those of RSF1030 and RJHC2. All Ap^r plasmids replicate as a multicopy pool in *E. coli* cells. They also replicate in the absence of protein synthesis, accumulating RNA-containing CCC DNA. All of them require for their replication a functional chromosomal *polA* product.

DISCUSSION

Ampicillin has been considered the drug of choice for treatment of patients seriously ill with dysentery. The finding of high-level ampicillin-resistant *S. dysenteriae* type 1 strains in geographically widespread dysentery epidemics is of great concern when one considers the serious nature of this disease.

Genetic evidence for the presence of two plasmids, one carrying determinants for resistance to chloramphenicol, tetracycline, sulfonamides, and streptomycin and transfer properties and a separate one with determinants for ampicillin resistance, was presented recently (14, 17). We have now shown that all *S. dysenteriae* type 1 strains have a different and heterogeneous plasmid population, although a unique nonconjugative 5.5-Mdal plasmid has been associated with the ampicillin-resistant strains, irrespective of the geographical source of the epidemics. The rest of the drug resistance determinants are carried (at least in the Costa Rican and Mexican strains) on an 80-Mdal conjugative R-plasmid. Thus, the ampicillin resistance in *S. dysenteriae* type 1 is part of a class 2 transfer system (1).

Interestingly, our DNA-DNA hybridization studies show not only the identity among the different *Shigella* Ap^r plasmids but also their high relation to RSF1030 and RJHC2, two nonconjugative 5.5-Mdal plasmids isolated, respectively, from *S. panama* and *C. freundii* (unpublished data). This identity is confirmed by the restriction endonuclease analysis and by the similarities of the replicative properties of all Ap^r plasmids.

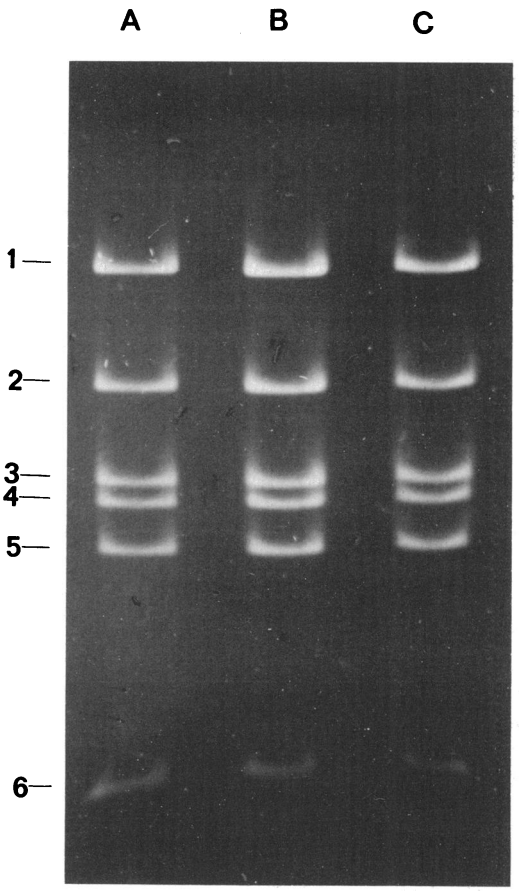

FIG. 2. *HincII restriction endonuclease patterns obtained with Ap^r plasmids from different enterobacteria. Reactions were carried out in 10 mM Tris-hydrochloride pH 7.9, 7 mM $MgCl_2$, 6 mM 2-mercaptoethanol, 60 mM NaCl and purified plasmid DNA in a final volume of 30 μl. Reactions were initiated by the addition of 1 μl of HincII restriction endonuclease (New England Biolab, 2,000 U/ml), incubated at 37°C for 90 min, and halted by adding 5 μl of a mixture containing 7% sodium dodecyl sulfate and 33% glycerol plus 0.04% bromophenol blue. The gel system consisted of 1.5% agarose in a Tris-borate buffer, pH 8.3 (see Materials and Methods). The gels were run at 100 V, 50 mA, at room temperature until the bromophenol blue reached the bottom of the gel. The gel was removed, stained, and photographed as described in Materials and Methods. HincII restrictions patterns for: (A) Citrobacter freundii Ap^r plasmid (RJHC 2); (B) Shigella dysenteriae Ap^r plasmid (RJHC 5); and (C) Salmonella panama Ap^r plasmid (RSF 1030).*

Our results with the Ap^r plasmids are similar to those obtained with the Sm^r Su^r plasmids, which were interrelated (2) but not phylogenetically related to the Ap^r plasmids (Crosa, unpublished data). It is highly likely, then, that the Ap^r plasmids, as it was suggested for the

Sm^r Su^r plasmids (2), have also evolved once and then epidemiologically spread.

It has been established that certain drug resistance genes reside upon sequences of DNA that have the capacity of translocating themselves from one plasmid to another (5, 10, 11). The ability of drug resistance genes to migrate between DNA molecules helps explain the rapid evolution of R-plasmids that possess a varied antibiotic resistance phenotype. Among the enterics, the structural gene for TEM-type β-lactamase is situated on a 3×10^6-dalton translocatable segment of DNA, so-called TnA (5, 10, 11). Our DNA-DNA hybridization experiments show that the TnA segment is present in the Ap^r plasmid. The translocation of Ap^r to an ancestor cryptic plasmid and then mobilization due to their coexistence with drug resistance transfer factors would furnish an additional explanation for the ubiquity of the Ap^r plasmid. There is not a high degree of homology between the Shiga Ap^r plasmid and DNA obtained from the Ap^r *S. typhi* strains isolated from typhoid epidemics previous to the dysentery outbreaks in Mexico. Although low, the degree of reassociation observed indicates that some sequences are held in common between the Shiga Ap^r plasmid and the *Salmonella* DNA (probably part of the TnA sequence).

It is noteworthy that plasmids carrying part of the enteric TnA translocon were also found to be responsible for the ampicillin resistance of recently isolated strains of nonenteric bacteria, such as *Haemophilus influenzae* (9) and *Neisseria gonorrhoeae* (L. Elwell, M. Roberts, L. Mayer, and S. Falkow, manuscript submitted for publication). We are presently investigating whether the same segment of TnA is present in all three bacteria.

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