INFECTION AND IMMUNITY, July 1980, p. 140-143
0019-9567/80/07-0140/04$02.00/0

Vol. 29, No. 1

Transfer of a CFA/I-ST Plasmid Promoted by a Conjugative Plasmid in a Strain of *Escherichia coli* of Serotype O128ac:H12

M. HENRIQUETA L. REIS,[1] M. HELOIZA T. AFFONSO,[1] LUIZ R. TRABULSI[1] ANTHONY J. MAZAITIS,[2] RENATA MAAS,[2] AND WERNER K. MAAS[2]

Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, Sao Paulo,[1] and Department of Microbiology, New York University School of Medicine, New York, New York 10016[2]

Escherichia coli strains belonging to serotype O128ac:H12 and producing heat-stable enterotoxin (ST) and colonization factor CFA/I were found in Sao Paulo in children with diarrhea, but not in normal children. Segregants occurred in such strains with a frequency of about 10%, which have lost the ability to produce ST and CFA/I at the same time. From one strain, both properties were transferred jointly in matings to an *E. coli* K-12 strain. All such ST^+ CFA/I^+ progeny had received two plasmids of length 97 and 64 kilobases in the matings. Insertion of a transposon, Tn*5*, carrying a gene for kanamycin resistance, into the two plasmids enabled us to select for kanamycin-resistant progeny in further matings. Analysis of such progeny strains in terms of plasmid content and production of ST and CFA/I revealed that the larger plasmid carries the genes for ST and CFA/I and is not self-transmissible, whereas the smaller plasmid does not carry any recognizable phenotypic traits, but is conjugative and promotes cotransfer of the larger plasmid with a frequency of about 30%.

Escherichia coli serogroup O128ac was first implicated in intestinal infections in 1955 (15) and has since then been considered as one of the classical enteropathogenic serogroups associated with infantile diarrhea. Recently we have shown that most Brazilian strains belonging to group O128ac produce a heat-stable enterotoxin (ST) (11). These strains were found to belong to several O:H types. Strains of serotype O128ac:H12 regularly produce colonization factor CFA/I in addition to ST (12). During a study of the association between CFA/I and ST in O128ac:H12 strains, we observed that segregants that lost CFA/I always lost ST. Furthermore, in one strain, CFA/I and ST production were transferred jointly in matings with an *E. coli* K-12 strain. These findings suggested that the genes for CFA/I and ST are located on a single plasmid. In the present paper we have analyzed the plasmids transferred to *E. coli* K-12. As will be shown, two plasmids are transferred, one nonconjugative with the genes for ST and CFA/I, the other conjugative and able to mobilize transfer of the CFA/I ST plasmid.

MATERIALS AND METHODS

Bacterial strains. Strain TR 438/1 was isolated in Sao Paulo from a child with acute diarrhea. It produces ST and gives a positive test for CFA/I (see below). It gives negative tests for heat-labile enterotoxin (LT), colicin, and hemolysin. It is sensitive to sulfonamides, mercury, and commonly used antibiotics. Identification of its serotype as O128ac:H12 was carried out according to Edwards and Ewing (7). Strain MS 101 is a nalidixic acid-resistant (Nal^r) *E. coli* K-12 strain. Strain MA3640 is a lambda-resistant, streptomycin-resistant (Str^r) auxotroph (*pro trp his ilv*) of *E. coli* K-12. Strain MA3456 is a *met* auxotroph of *E. coli* C.

Media, growth conditions, and mating conditions. The minimal medium used was medium A (3) with 0.5% glucose. Enriched media were tryptic soy broth (TS) and tryptone yeast extract broth (TYE), both of which are Difco products. Solid media were prepared by the addition of agar to a final concentration of 2%. The conditions for growing the cells, testing for phenotypes, and carrying out matings have been described previously (5). Donor and recipient cells were mixed in equal proportions (each about 2×10^8/ml) and incubated for 2 h at 37°C with slow shaking, before plating on selective media. Frequencies of recombinant colonies are expressed as the percentage of the total number of recipient bacteria in the mating mixture.

Transposition of Tn*5* into plasmids. The source of Tn*5*, which contains a gene for kanamycin resistance (Km^r), was a defective lambda phage carrying Tn*5*. This phage, λ *b221 rex*::Tn*5* *c*I857, was a gift of Douglas Berg, and we followed his procedure for infecting bacteria and isolating Km^r transductants (2). In such transductants Tn*5* is inserted either into the chromosome or into a resident plasmid. Plates containing 300 or more Km^r transductants were harvested, suspended in 10 ml of TYE, and incubated at 37°C for 18 h. These cultures were then mated with strain

MA3640, and Km^r Str^r transconjugants were selected. It is known that in such matings mainly plasmid but not chromosomal transfer occurs, and thus only those Tn*5* insertions that are in plasmids are obtained.

Scoring of CFA/I. Presence or absence of CFA/I was scored by hemagglutination as previously described (8). We modified the procedure by using a 5% suspension instead of a 25% suspension of washed group A human erythrocytes with or without mannose. Results were confirmed by bacterial agglutination with anti-CFA/I serum prepared by inoculating rabbits with strain H10407. For this test we used slide agglutination at room temperature with bacterial suspensions of about 10^{10} cells per ml in Dulbecco phosphate-buffered saline (GIBCO Laboratories, Grand Island, N. Y.).

Tests for ST. ST production was determined by the suckling mouse assay (4) and in intestinal loops of piglets (9).

Demonstration of plasmids and preparation of plasmid deoxyribonucleic acid (DNA). The strains were tested for the presence of plasmids by the gel-electrophoretic method of Eckhardt (6) using 0.8% agarose. This procedure, which is rapid and simple, gives a good indication of the sizes of different plasmids present and the amount of each plasmid. The procedure for the extraction and preparation of plasmid DNA for electron microscopy has been described previously (10). Length measurements of plasmid molecules were made with an electronic calculator. For an internal standard for length measurements, we used the double-stranded replicative form of phage ϕX 174, length = 5.38 kilobases (kb).

RESULTS

Insertion of Tn*5* into plasmids of ST CFA/I strains. Strain TR438/1 was mated with *E. coli* K-12 strain MS101, and the mating mixture was plated on TS agar containing nalidixic acid (100 μg/ml), which is selective for the recipient strain MS101. Colonies were tested for CFA/I and ST, as described in Materials and Methods. The frequency of CFA/I$^+$ ST$^+$ progeny was about 3% among the colonies, and the two properties were always inherited together. Five colonies were selected for further studies.

Strain TR438/1 contains at least four plasmid bands (Fig. 1, track 1). Two of these plasmid bands (A and B) were seen in the MS101 progeny strains (Fig. 1, track 2). Both TR438/1 and the MS101 derivatives gave rise to CFA/I$^-$ ST$^-$ segregants with a frequency of about 10%. In such strains, band A was usually absent (Fig. 1, track 3).

To study the relationship between the plasmid bands and CFA/I and ST further, we obtained insertions of Tn*5* into these plasmids, as described in Materials and Methods. Tn*5* carries a gene for Km^r. The five MS101 derivatives were infected with λ Tn*5*, and Km^r transductants were collected. These were then mated with strain MA3640, and Km^r Str^r conjugants were selected. They appeared with a frequency of 0.1% in terms of added recipient cells. From each mating, 50 colonies were scored for CFA/I. The frequency of CFA/I$^+$ colonies was 5 to 10%, among the Km^r Str^r conjugants. Both CFA/I$^+$ and CFA/I$^-$ offspring were tested for the presence of plasmids. All CFA/I$^+$offspring had two plasmid bands, whereas all CFA/I$^-$ offspring had one plasmid band (Fig. 1, tracks 4 and 5). The mobilities of these plasmid bands were about the same as those in the parental strains.

These findings suggested that band A is associated with CFA/I, and presumably ST, whereas band B is associated with its own conjugal transfer, as well as mobilization of the CFA/I ST plasmid. On this assumption, insertion of Tn*5* into the transfer plasmid followed by conjugation would result either in transfer of this plasmid alone or cotransfer of the transfer plasmid with the CFA/I plasmid. Insertion of Tn*5* into the CFA/I ST plasmid followed by mating would result in the obligatory transfer of both plasmids, assuming that the CFA/I ST plasmid is not transferable by itself.

Plasmid transfer from Tn*5* insertion strains. To test the above mentioned hypothesis, 14 CFA/I$^+$ progeny strains from the five matings with strain MA3640 were mated with

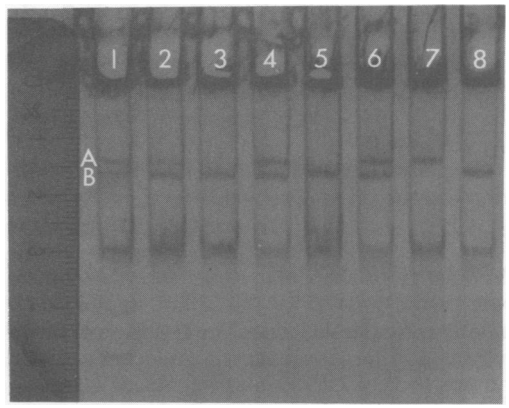

FIG. 1. *Agarose gel electrophoresis of plasmid DNA from CFA/I$^+$ ST$^+$ and CFA/I$^-$ ST$^-$ strains. For each slot, 0.5 ml of an overnight culture in TYE medium was used. (Track 1) strain 438/1; (track 2) strain MS101 31; (track 3) CFA/I$^-$ ST$^-$ segregant of MS101 31; (track 4) CFA/I$^+$ ST$^+$ transconjugant from mating MS101 31 × MA3640; (track 5) CFA$^-$ ST$^-$ transconjugant from mating MS101 31 × MA3640; (track 6) CFA$^+$ ST$^+$ transconjugant from mating of CFA/I$^+$ ST$^+$ MA3640 derivative 1 × MA3456; (track 7) CFA/I$^+$ ST$^+$ transconjugant from mating of CFA/I$^+$ ST$^+$ MA3640 derivative 2 × MA3456; (track 8) CFA/I$^-$ ST$^-$ transconjugant from mating of CFA/I$^+$ ST$^+$ MA3640 derivative 17 × MA3456.*

strain MA3456 and Km^r progeny were selected. These were tested for CFA/I. In agreement with our hypothesis, we found that for some matings the frequency of CFA/I$^+$ was 100% (in six matings) among the Km^r progeny, whereas in the other matings it was about 30%. Again all CFA/I$^-$ progeny had a single plasmid band (band B), whereas all CFA/I$^+$ progeny, with one exception, had two bands, A and B (Fig. 1, tracks 8 and 6).

The one CFA/I$^+$ strain with only one plasmid band obtained from a mating with 100% CFA/I$^+$ cotransfer proved to be important for our hypothesis, since it carried only band A (Fig. 1, track 7). It was mated with strain MA3640, but no Km^r progeny could be obtained. This is consistent with the notion that the CFA/I-ST plasmid is not self-transmissible. In contrast, Km^r conjugative progeny from the other CFA/I$^+$ strains and the CFA/I$^-$ strains were obtained with the usual frequency of 0.1% of the added recipient cells.

Physical characterization of the two plasmid bands. Plasmid DNA was isolated from the CFA/I$^+$ transconjugant with only band A as well as from a CFA/I$^-$ transconjugant with only band B, obtained in a mating with 30% CFA/I$^+$ cotransfer, and characterized by electron microscopy, as described in Materials and Methods. In the DNA from the strain with only band A, a single plasmid species of length 102 ± 1 kb was found. The CFA/I$^-$ strain with only band B contained a single species of length 69 ± 1 kb. Since both plasmids carry Tn*5* insertions, and Tn*5* is 5.3 kb long, the lengths of plasmid A and B were about 97 kb and 64 kb, respectively.

Coinheritance of CFA/I and ST. To substantiate the previous conclusion that the genes for CFA/I and ST are present on the same plasmid, CFA/I$^+$ and CFA/I$^-$ progeny strains obtained from various matings described above were tested for ST by the infant mouse assay and in pig intestinal loops. The results are shown in Table 1. There is a perfect correlation between the presence of CFA/I and ST, as measured in both assays.

TABLE 1. *Characteristics of Km^r-selected transconjugants*

Transconjugant derived from mating of:		Plasmid bands	CFA/I	ST	
CFA/I$^+$ ST$^+$ Kan^r donor	Recipient			Mouse	Pig
MS101 31	MA3640	A, B	+	+	+
MS101 31	MA3640	B	−	−	−
MS101 32	MA3640	A, B	+	+	+
MS101 32	MA3640	B	−	−	−
MS101 33	MA3640	A, B	+	+	+
MS101 33	MA3640	B	−	−	−
MA3640 derivative 1[a]	MA3456	A, B	+	+	+
MA3640 derivative 2[a]	MA3456	A	+	+	+
MA3640 derivative 17[b]	MA3456	A + B	+	+	+
MA3640 derivative 17	MA3456	B	−	−	−

[a] Tn*5* insertion is in plasmid A, as judged by 100% cotransfer of plasmids A and B in matings.

[b] Tn*5* insertion is in plasmid B, as judged by 30% cotransfer in matings.

DISCUSSION

In this paper we have analyzed an enterotoxigenic strain of *E. coli* and found that it carries two plasmids that are readily transmissible by conjugation to other *E. coli* strains. One is a nonconjugative plasmid and contains genes for the production of ST and CFA/I. The other is a conjugative plasmid, without any recognizable phenotypic markers, which is able to mobilize the transfer of the first plasmid. The conjugative plasmid resembles the Δ transfer factor described by Anderson (1) which can mobilize the transfer of R plasmids. This type of mobilization is an efficient method for the dissemination of enterotoxin (Ent) plasmids, since the frequency of cotransfer is high (about 30%) and the conjugative plasmid can presumably move from strain to strain and mobilize other nonconjugative Ent plasmids.

The linkage of ST with CFA/I on the same plasmid is typical of strains of serotype O128:H12 found in Brazil that produce diarrhea in children (M. H. L. Reis and L. R. Trabulsi, unpublished data). The same linkage between CFA/I and ST on plasmids of similar sizes as ours has been reported in strains of serogroup O78 isolated in other parts of the world (13). It seems reasonable that such a linkage would be advantageous for a strain's ability to produce diarrhea, since both the toxin and the colonization factor are necessary for pathogenicity. It is interesting that in studies with animal strains no such genetic linkage between a colonization factor and ST has been demonstrated. In addition, no single plasmids with LT and a colonization factor have been reported in either human or animal strains.

We now have strains that carry each of the two plasmids alone. This will make it easier to carry out physical characterization of these plasmids. Moreover, having Tn*5* insertions in these plasmids and being able to isolate more Tn*5* insertions in different plasmid regions will be very helpful for such studies. We have recently used this approach to construct a physical map of a naturally occurring recombinant Ent plas-

mid of porcine origin (A. J. Mazaitis, R. Maas, and W. K. Maas, unpublished data). We are especially interested in looking for "stem-loop" DNA structures, indicative of transposons. In our studies of the porcine plasmid we found the ST gene to be located on such a structure. Moreover, in a bovine ST-only Ent plasmid, the ST gene, located on a stem-loop structure, was shown to be transposable to another plasmid (14).

Having a Tn*5* insertion in the conjugative plasmid enables us now to study CFA/I-ST plasmids in the other Brazilian isolates that did not transfer these plasmids to *E. coli* K-12. Since Tn*5* carries a gene for kanamycin resistance, we can attempt to introduce the mobilization plasmid into these strains by selecting for Kan^r transconjugants. Subsequently, we can try to effect transfer of the CFA/I-ST plasmids to *E. coli* K-12 for further analysis.

ACKNOWLEDGMENTS

We thank Helen McKeon and Thomas Eckhardt for valuable technical assistance and Carlton L. Gyles for testing our strains for ST by the pig intestinal loop assay.

This work was supported by grant PCM77-25134 from the National Science Foundation (NSF), by a joint grant from NSF and the Brazilian National Research Council under the Cooperative Science Program for Latin America, by a grant from Financiadora de Estudos e Projetos, and by a grant from Fundacao de Amparo a Pesquisa de SP.

LITERATURE CITED

1. **Anderson, E. S.** 1968. The ecology of transferable drug resistance in the Enterobacteria. Annu. Rev. Microbiol. **22**:131-180.
2. **Berg, D. E.** 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn*5*, p. 205-212. *In* A. I. Bukhari, J. A. Shapiro, and S. L. Adhya (ed.), DNA insertion elements, plasmids, and Episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Davis, B. D., and E. S. Mingioli.** 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. J. Bacteriol. **60**:17-28.
4. **Dean, A. G., Y. C. Ching, R. G. Williams, and L. B. Harden.** 1972. Test for *Escherichia coli* enterotoxin using infant mice: application to a study of diarrhea in children in Honolulu. J. Infect. Dis. **125**:407-411.
5. **Dubnau, E., and W. K. Maas.** 1968. Inhibition of replication of an F'lac episome in Hfr cells of *Escherichia coli*. J. Bacteriol. **95**:531-539.
6. **Eckhardt, T.** 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid **1**: 584-588.
7. **Edwards, P. R., and W. H. Ewing.** 1972. Identification of Enterobacteriaceae, 3rd ed., p. 67-107. Burgess Publishing Co., Minneapolis.
8. **Evans, D. G., and D. J. Evans, Jr.** 1978. New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups O6 and O8. Infect. Immun. **21**:638-647.
9. **Gyles, C. L.** 1979. Limitations of the infant mouse test for *Escherichia coli* heat stable enterotoxin. Can. J. Comp. Med. **43**:371-379.
10. **Palchaudhuri, S., E. Bell, and M. R. J. Salton.** 1975. Electron microscopy of plasmid deoxyribonucleic acid from *Neisseria gonorrhoeae*. Infect. Immun. **11**:1141-1146.
11. **Reis, M. H. L., A. F. P. Castro, M. R. F. Toledo, and L. R. Trabulsi.** 1979. Production of heat-stable enterotoxin by the O128 serogroup of *Escherichia coli*. Infect. Immun. **24**:289-290.
12. **Reis, M. H. L., D. P. Matas, A. F. Restana de Castro, M. R. F. Toledo, and Luiz R. Trabulsi.** 1980. Relationship among enterotoxigenic phenotypes, serotypes, and sources of strains in enterotoxigenic *Escherichia coli*. Infect. Immun. **28**:24-27.
13. **Smith, H. R., A. Cravioto, G. A. Willshaw, M. M. McConnell, S. M. Scotland, R. J. Gross, and B. Rowe.** 1979. A plasmid coding for the production of colonization factor antigen I and heat-stable enterotoxin in strains of *Escherichia coli* of serogroup O78. FEMS Microbiol. Lett. **6**:255-260.
14. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding for heat stable toxin is a bacterial transposon. Nature (London) **277**:453-456.
15. **Taylor, J., and R. E. Charter.** 1955. *Escherichia coli* O128 causing gastroenteritis in infants. J. Clin. Pathol. **8**:276-281.