Interspecific Plasmid Transfer and Modification of Heat-Stable Enterotoxin Expression by *Klebsiella pneumoniae* from Infants with Diarrhea

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ABSTRACT. We have previously described two heat-stable enterotoxins (ST) produced by *Citrobacter freundii* and by *Klebsiella pneumoniae*. To see whether transfer of toxigenic ability may occur between different bacterial species, conjugal mating experiments were performed using strains isolated from children. Donors and recipients were incubated together. ST production by the recipients was tested by the suckling mouse assay, which is the standard biologic method for detecting ST, and also by an ELISA test. The latter is based on MAb directed to *Escherichia coli* 18- or 19-amino acid toxin. *Citrobacter* and *E. coli* strains producing an ELISA-positive ST were used as plasmid donors. Recipients were *E. coli*, *Klebsiella* P89, and *Klebsiella* AL55. The latter had been previously shown to be capable of producing an ELISA-negative ST, but had spontaneously lost this ability. After conjugation, all strains gave positive results in the suckling mouse assay. However, *E. coli* and *Klebsiella* P89 ST were positive in the ELISA test, whereas *Klebsiella* AL55 ST was negative. The same three strains were transformed by inserting the plasmid pSLM004 encoding an ELISA-positive ST. *Klebsiella* P89 and *E. coli* became capable of producing an ELISA-positive ST, whereas *Klebsiella* AL55 cells were positive in the suckling mouse assay but negative in the ELISA. To assess whether the toxigenicity acquired was itself transmissible, the new ST-producing strains were incubated with a nontoxigenic *Citrobacter* strain. The latter became capable of producing an ELISA-positive ST in all cases. In conclusion: 1) different enterobacteria may transmit ST plasmids by conjugation; 2) the ability to produce ST is acquired together with the ability to transfer it to other bacteria; and 3) selected strains of *Klebsiella* are capable of modifying toxin expression through a posttranslational, yet unknown, mechanism. (*Pediatr Res* 33: 205–208, 1993)

Abbreviations

ST, heat-stable enterotoxin
SMA, suckling mouse assay

Escherichia coli that produce heat-stable toxins are among the most common causes of infantile diarrhea both in developing (1) and in industrialized (2) countries.

The *E. coli* ST1 or STa comprises a family of low-molecular-weight peptides capable of inducing diarrhea. The genes for ST1

are usually located in plasmids (3). Two prototypes of ST1 exist, with minor differences in gene and amino acid sequences (4). The first is an 18-amino acid peptide and is named ST1a (or STp). The second, ST1b (or STh), is a 19-amino acid peptide. However, ST of larger molecular size have also been described, and they probably represent pretoxin forms (5). Both ST1a and ST1b induce diarrhea in man through a multistep process involving the binding of the toxin to receptors located on enterocytes (6) and the coupled stimulation of guanylate cyclase activity (7). ST-like enterotoxins may be produced by bacteria other than *E. coli*, including nonagglutinating *Vibrio* (8) and *Yersinia enterocolitica* (9). These toxins share with *E. coli* ST1 a segment of 13 amino acids, which contains six cysteine residues and is responsible for the biologic activity (10). We have previously described two ST-like enterotoxins produced by *Citrobacter freundii* (11) and by *Klebsiella pneumoniae* (12). The first is identical to the 18-amino acid *E. coli* ST1a (13). *Klebsiella* toxin is less characterized: its physicochemical features are similar to those of *E. coli* ST1a; like *E. coli* ST1a, *Klebsiella* ST stimulates guanylate cyclase activity and induces net intestinal secretion of water and chloride (12). However, its time-course effect is shorter than that of *E. coli* ST. Furthermore, *Klebsiella* ST does not react with MAb raised against pure *E. coli* 18-amino acid ST1a in an ELISA assay (12). Both toxigenic *Citrobacter* and *Klebsiella* strains were isolated from infants with acute watery diarrhea (11, 12). A toxigenic *Citrobacter* strain was isolated from a patient also harboring an ST-producing *E. coli* strain (11). This raises the possibility of *in vivo* transfer of toxigenic ability among different bacterial species.

To find evidence of an interspecific transfer of virulence properties, bacterial conjugal mating as well as transformation experiments were carried out using wild strains isolated from children.

MATERIALS AND METHODS

The following strains were used as ST plasmid donors in bacterial mating experiments: 1) *C. freundii* AG2ST capable of producing an 18-amino acid ST, isolated from a child with diarrhea (11); 2) *E. coli* R18 (producing an 18-amino acid ST), isolated from a child with diarrhea; and 3) *E. coli* R19 (producing a 19-amino acid ST) (14).

Recipients strains were: 1) *E. coli* K12 Rif^r Lac[–] (resistant to rifampicin and nonlactose fermenting); 2) *K. pneumoniae* P89 isolated from the stools of a healthy child; 3) *K. pneumoniae* AL55, isolated from a child with secretory diarrhea (12), and 4) *C. freundii* strain FA29, isolated from a healthy child. *K. pneumoniae* AL55 had been previously shown to produce an ELISA-negative ST, but subsequently had spontaneously lost this ability. *Citrobacter* strain was also used as recipient in the remating experiments.

Experimental procedure. Donor and recipient cultures, grown in L broth (10 g Tryptone, 5 g yeast extract, and 5 g NaCl/L, pH 7.2) at 37°C, in shaking incubation, were mixed in a volume

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ratio of 1:2 and incubated for 2 h at 37°C. Mixed culture was plated onto xylose lysine desoxycholate agar. This was supplemented with 100 µg/mL of rifampicin when *E. coli* K12 Rif^r Lac⁻ was used as the recipient. The other recipients were selected on the basis of morphology and lactose fermentation and/or H₂S production. Identification was confirmed by a multitest system (API 20 E). At least 20 pools, each containing five different colonies, were obtained from each mating and tested for enterotoxigenicity. ST production was tested by the SMA (15); the SMA was performed by killing the animals 150 min after jejunal inoculation of culture supernatant (12). ST-positive and ST-negative control strains were used in each experiment. Results were considered positive when the gut weight/body weight ratio exceeded 0.083 (15). ST production was also tested by an ELISA test, which uses MAb raised against pure 18-amino acid *E. coli* ST (16). This ELISA test is capable of detecting both 18- and 19-amino acid ST.

Transfer frequencies were expressed as the ratio of the number of transformed (ST-positive) cells to the number of recipient cells. This was done by testing serial 5-fold dilutions of recipient cells for the production of ST by the SMA.

Transformation experiments were performed as described (17), by incubating competent *Klebsiella* cells with the plasmid pSLMOO4 from *E. coli* HB101, carrying a gene encoding for 19-amino acid ST1 and a gene for ampicillin resistance (14). Transformed cells were selected by their growth on LB agar supplemented with 200 µg/mL of ampicillin, to which *Klebsiella* AL55 was originally sensitive, and successively tested for ST production. Physicochemical characteristics of ST toxins, including heat stability, pH inactivation, methanol solubility, and mercaptoethanol sensitivity, were determined as previously described (12). Molecular weight was estimated by testing filtrate and retentate fractions of ultrafiltration membranes (PM-10 and UM-2) in the SMA (12).

Stability of enterotoxin production was tested each month on culture supernatant prepared from strains kept in nutrient agar at room temperature in the dark.

Culture media were purchased from Difco s.a.s., Milan, Italy. API system 20 E was obtained from Ayerst Italiana, Milan, Italy. Ultrafiltration membranes were purchased from Amicon Corp., Lexington, MA. Chemicals were obtained from Sigma Chemical Co., St. Louis, MO. Anti-ST MAb were a gift from Dr. R. A. Giannella, Division of Digestive Diseases, University of Cincinnati, OH. *E. coli* R18 was obtained from Dr. A. Caprioli, I.S.S. Rome, Italy. *E. coli* HB 101 containing the plasmid pSLMOO4 was obtained from Dr. A. Fasano, University of Reggio Calabria, Italy. This strain was renamed R19. Serotyping of *Klebsiella* strains was kindly performed by Fritz and Ida Orskov, International Escherichia and *Klebsiella* Centre, Statens Serum Institute, Copenhagen, Denmark.

RESULTS

Conjugal transfer of ST production. Results of bacterial conjugal matings are reported in Table 1. All matings resulted in the transfer of toxigenic ability to recipients. However, *E. coli*, *C. freundii* FA 29, and *K. pneumoniae* P89 became capable of elaborating an ELISA-positive ST. On the contrary, *K. pneumoniae* AL55 showed positive results in the SMA, but not in the ELISA test. Transfer frequency ranged from approximately 1×10^{-2} to 1×10^{-1} .

To see whether the acquired ability to produce ST was itself transmissible to other bacterial species, the new ST-positive strains *E. coli*, *K. pneumoniae* P89, and *K. pneumoniae* AL55 were used as donors in the remating experiments. Results are reported in Table 2. All matings gave origin to enterotoxigenic strains. Interestingly, the recipient incubated with *K. pneumoniae* AL55, which produced an ELISA-negative ST, became capable of producing an ELISA-positive ST.

Transformation experiments. Competent cells of *E. coli* K12,

Table 1. Results of mating experiments

Donor (ST+)	Recipient (ST-)	Recipient after mating		Transfer frequency
		SMA	ELISA	
<i>C. freundii</i> AG2ST	<i>E. coli</i> K12 Lac - rif ^r	+	+	13×10^{-2}
<i>C. freundii</i> AG2ST	<i>K. pneumoniae</i> P89	+	+	1×10^{-2}
<i>C. freundii</i> AG2ST	<i>K. pneumoniae</i> AL55	+	-	7×10^{-2}
<i>E. coli</i> R18	<i>C. freundii</i> FA29	+	+	8×10^{-2}
<i>E. coli</i> R18	<i>K. pneumoniae</i> P89	+	+	5×10^{-2}
<i>E. coli</i> R18	<i>K. pneumoniae</i> AL55	+	-	9×10^{-2}
<i>E. coli</i> R19	<i>C. freundii</i> FA29	+	+	1×10^{-2}
<i>E. coli</i> R19	<i>K. pneumoniae</i> P89	+	+	1.5×10^{-2}
<i>E. coli</i> R19	<i>K. pneumoniae</i> AL55	+	-	2×10^{-2}

Table 2. Results of remating experiments

Donor (ST+)	Recipient (ST-)	Recipient after mating		Transfer frequency
		SMA	ELISA	
<i>E. coli</i> K12	<i>C. freundii</i>	+	+	9×10^{-2}
<i>K. pneumoniae</i> P89	<i>C. freundii</i>	+	+	5×10^{-2}
<i>K. pneumoniae</i> AL55	<i>C. freundii</i>	+	+	6×10^{-2}

Table 3. Results of transformation experiments

Transformed strain	SMA	ELISA
<i>K. pneumoniae</i> AL55	+	-
<i>K. pneumoniae</i> P89	+	+
<i>E. coli</i> K12	+	+

K. pneumoniae P89, and *K. pneumoniae* AL55 were transformed by inserting the ST-encoding plasmid pSLMOO4. Transformed *E. coli* and *K. pneumoniae* P89 cells were positive in both the SMA and the ELISA test, whereas *K. pneumoniae* AL55 showed positive results only in the SMA (Table 3).

Physicochemical characteristics. The physicochemical characteristics of enterotoxigenic activities of *K. pneumoniae* P89 and AL55, *E. coli* R18, *E. coli* R19, and *C. freundii* AG2ST are reported in Table 4. All toxins showed identical behavior under the conditions tested.

Time course. The time-course effect of ST produced by different enterobacteria is reported in Figure 1. Toxins produced by all strains, with the exception of *K. pneumoniae* AL55, gave positive results in the SMA over a period of 6 h. *K. pneumoniae* AL55 ST showed a short-acting enterotoxigenic activity, with a sharp peak at 2.5 h postinoculation and a rapid normalization of the gut weight/body weight ratio thereafter.

Stability of acquired enterotoxigenic activity. ST production acquired by all recipients, with the exception of *K. pneumoniae* AL55, was stable over a range of 3 to 6 mo. Enterotoxigenic activity produced by *K. pneumoniae* AL55 was lost in 30 d by the majority of the strains tested, but some strains were found to be positive up to 1 y after conjugation.

Serotyping of *Klebsiella* strains. *K. pneumoniae* P89 showed positivity for K54 antigen; *K. pneumoniae* AL55 was related to K60, K61, and K15 antigens.

DISCUSSION

The interspecific transfer of virulence properties has been previously demonstrated using laboratory-constructed plasmids (18). We used wild strains, isolated from children with acute diarrhea of secretory type (11, 12), as donors in bacterial conjugal mating. Also, the recipients were clinical isolates.

All the strains used in the conjugation experiments were able to transfer their toxigenic ability. This suggests that a plasmid exchange network exists among different enterobacteria. The same results obtained with conjugal mating experiments were

Table 4. Characteristics of enterobacterial ST

	<i>E. coli</i> ST 18 amino acid	<i>E. coli</i> ST 19 amino acid	<i>C. freundii</i> AG2ST	<i>K. pneumoniae</i> AL55	<i>K. pneumoniae</i> P189
Molecular weight	1 972	2 000	2 000	2 000–10 000	2 000–10 000
Heat stability	+	+	+	+	+
pH inactivation	>8	>8	>8	>8	>8
Methanol solubility	+	+	+	+	+
2-Mercaptoethanol sensitivity	+	+	+	+	+

Fig. 1. Time course of enterotoxigenic activities of *E. coli* R18, *Klebsiella* P89, *Klebsiella* AL55, and *Citrobacter* AG2ST. Culture supernatant (0.1 mL) was injected intragastrically into mice. The animals were killed at various times and the gut weight/body weight ratio (GW/BW) determined. A GW/BW ratio greater than 0.083 was considered positive. Each point represents the mean of three separate experiments.

also obtained when ST-negative bacterial cells were transformed by inserting a plasmid carrying the gene for ST. This provides reliable evidence for ST gene transfer among different enterobacteria rather than the transfer of some activity that regulates expression of ST gene.

The fact that all the strains used were able to transfer indicates the lack of specific restriction systems among wild strains with regard to toxigenic transfer. The transfer frequencies were in the same range for all the strains used, i.e. approximately 1×10^{-2} . Such frequency is in the upper range of that reported using strains transformed with laboratory-constructed plasmids (18). Recipients acquiring the ability to produce ST were also capable of transferring the toxigenic ability to other strains. This suggests that toxin conjugal transfer should be regarded as an infectious characteristic of enterobacteria.

It has been reported that toxins produced by other enterobacteria may be less stable than that produced by *E. coli* (19). This appears not to be an absolute finding, based on our results. It should be noted, however, that stability of ST production may depend on storage conditions of individual bacterial species.

Like the other strains, *K. pneumoniae* AL55 acquired the ability to elaborate an enterotoxigenic activity by conjugation, but the phenotype of such ST was different from that produced by the donor, as it was not recognized by MAb. When *K. pneumoniae* AL55 was used as donor in the remating experiments, the recipient strain became capable of elaborating an ELISA-positive ST. This suggests that the modification might occur during the processing and/or exporting of the toxin, because it does not involve the transfer of genetically encoded activities capable of modifying the toxin expression from the donors.

The modification did not affect the main physicochemical characteristics of ST, which were identical for all the strains and compatible with that of ST1 or STa. However, the time-course effect of *K. pneumoniae* AL55 ST was markedly different from

that of ST produced by all the other bacteria, showing a sharper peak of shorter duration. This observation has also been reported in the original work describing the toxin produced by *K. pneumoniae* AL55 (12). It is therefore clear that the modification of ST structure, probably occurring at the site recognized by the MAb, results in variations of the kinetics of *Klebsiella* enterotoxigenic activity.

Only one of the two strains of *Klebsiella* used in the experiments showed the ability to modify the toxin, suggesting that this is a property of selected strains. As a consequence, different strains of *K. pneumoniae* can produce different types of ST.

In conclusion, transfer of toxigenic ability may occur *in vitro* between different enterobacteria. Selected *Klebsiella* strains may modify toxin expression.

These findings may have relevant implications in the field of basic knowledge related to ST production, but they are also of clinical interest. First, the transfer of toxigenic capability may explain the presence of multiple toxigenic strains in a child with diarrhea. Furthermore, the production of a modified ST toxin, as in the case of *K. pneumoniae* AL55, suggests that diagnostic kits based on the use of MAb may give false-negative results. Finally, the possibility exists that a specific immune response, evoked by vaccine against classic ST, may not be protective versus strains elaborating modified toxic activities.

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Announcement

The National Council of Jewish Women Center for the Child, in collaboration with the Society for Research in Child Development, has been awarded the contract by The Administration on Children, Youth and Families to hold the second national Head Start research conference, entitled "Translating Research into Practice: Implications for Serving Families with Young Children," on November 4-7, 1993 in Washington, DC. Following the success of the first Head Start research conference, "New Directions in Child and Family Research: Shaping Head Start in the Nineties," the major goals of the second conference are to identify and disseminate current research in early childhood and families, discuss child and family issues, and identify new research and program needs. A special focus will be on translating research into practice in an effort to help practitioners serving low-income families gain a better understanding of effective early childhood interventions and programming. *For additional information, contact* Faith Lamb Parker, Ph.D., Project Director, Head Start Research Projects, NCJW Center for the Child, 53 West 23rd Street, New York, NY 10010, (212) 645-4048.