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ISOLATION OF THE ST ENT PLASMID SPECIFIC BACTERIOPHAGE M124

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SUMMARY For simple detection of enterotoxigenic *E. coli* from patients, attempts were made to find a bacteriophage that specifically proliferate on these bacteria. As a result phage M124 was isolated. Analysis of plasmid DNA together with detection of ST enterotoxin showed that this phage can specifically attack *E. coli* carrying the ST Ent plasmid (80 Mdalton). These results indicate that phage M124 is very useful for detection of ST enterotoxigenic bacteria.

INTRODUCTION

Two virulence factors are generally known to be associated with the enterotoxigenicity of *E. coli*. (Evans et al., 1975). One is enterotoxin and the other is the colonization factor.

Enterotoxigenic *E. coli* produce two peptide enterotoxins that cause diarrhea in man and animals (Gyles, 1971; Sack, 1975). One is a large molecular weight, heat labile enterotoxin (LT) (Clements et al., 1979). The other is a small molecular weight, heat stable enterotoxin (ST) (Chan and Giannella, 1981; Aimoto et al., 1982; Takao et al., 1983). The observed differences between ST and LT with respect to their immunogenicity, mode of action and apparent molecular sizes support the concept that these two enterotoxins are distinct. Some *E. coli* produce only ST enterotoxin, some produce only LT and others produce both ST and LT.

The productions of LT and ST enterotoxin have been shown to be governed by plasmid designated Ent (Skerman et al., 1972). The colonization factor is known to be produced by another plasmid (Evans et al., 1975).

At present ST enterotoxin is detected by the ligated intestinal loop method in animals (Gyles, 1971; Smith et al., 1971) or by its effect on intragastric injection into suckling mice (Dean et al., 1972). LT enterotoxin is

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usually detected by its effect in causing elongation of chinese hamster ovary (CHO) cells (Honda et al., 1976) or increase in permeability of rabbit skin (Craig, 1971). These methods give fairly accurate and reliable results, but the procedures involved are complicated and time consuming and require skill. Therefore we developed a simple, new method. This communication describes the establishment of an easier and more rapid method using a bacteriophage that specifically lyses enterotoxigenic *E. coli*.

MATERIALS AND METHODS

1. *Strains and culture medium*

For screening bacteriophages, standard enterotoxigenic *E. coli* 269–1 (ST^+ LT^+), 240–3 (LT^+) and 53402 (ST^+) were kindly provided by Dr. T. Takeda. A derivative of the K12 strain *E. coli* 20SO (Str^r, Sa^r, Azi^r, Rif^r, Nal^s, Gal^+) (Yokota et al., 1979) was used. Enterotoxigenic *E. coli*, which were collected from patients at Osaka Airport Quarantine Station and provided by Dr. Y. Takeda and Dr. T. Miwatani, were used for tests on sensitivity to bacteriophages. All the above *E. coli* strains were cultivated in L-broth, which consists of 1.0% bactotryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl and 0.1% glucose. The pH of the medium was adjusted to 7.2. T_1 buffer, containing 6 ml of 1 *M* Tris-HCl (pH 7.3), 1 ml of 1% gelatin, 0.25 ml of 1 *M* $CaCl_2$, and 0.5% $MgSO_4$. 7 H_2O per liter, was used for dilution of phage suspensions.

2. *Electron micrograph of partially purified phage*

The bacteriophage was centrifuged on a stepwise density gradient of cesium chloride (Sigma Co.) with gradient of $\rho=1.3$, 1.5 and 1.7 at 24,000 rpm for 2 h in a swinging rotor, Beckman type SW 55–1. Then the phage band was collected by syringe and dialysed against sodium phosphate buffer contained 5.85 g NaCl, 5.2 g NaH_2PO_4. $2H_2O$ and 27.2 g Na_2HPO_4. 12 H_2O in a total volume of 1 liter. After dialysis, the titer of the bacteriophage was checked and adjusted at about 5×10^{11} PFU/ml. A sample was adsorbed on Parlodion covered grids and stained with uranyl acetate. Electron micrographs were taken with a Hitachi Electron Microscope HU-12A at an instrumental magnification of 125,000.

3. *Enterotoxin assays*

i) LT enterotoxin assay

CHO cell assay of LT enterotoxin was carried out as described by Honda et al. (1979). Eagle's minimal essential medium was used in place of F12 medium originally described by Guerrant et al. (1974).

ii) ST enterotoxin assay

The suckling mouse method (Dean et al., 1972) was used for detection of ST enterotoxin.

4. *Preparation and agarose gel electrophoresis of plasmid DNA*

Plasmid DNA was purified by the method of Olsen and Hansen (1978). All procedures were done very gently in order not to break the large sized plasmid. Samples of plasmid DNA were charged on the agarose gel and electrophoresis was performed by the method of Greene et al. (1979).

RESULTS

1. *Isolation of bacteriophage*

Samples of sewage and dusty water were collected from many places in the cities of Jakarta and Bogor in Indonesia. Contaminant bacteria were killed with chloroform, and then the samples were incubated with *E. coli* 269–1 (ST^+ LT^+) to allow selective growth of bacteriophages that proliferate on this strain. Lysates were plated with *E. coli* 269–1 (ST^+ LT^+) to obtain plaques. In this way, we obtained at least 150 independent plaques from dusty water. All the plaques were examined for lytic activity on the standard strains of toxigenic bacteria, *E. coli* 53402 (ST^+), 240–3 (LT^+) and 269–1 (ST^+ LT^+). Fifty phage strains that gave clear plaques on these bacteria were selected for further analysis. First, we examined their effects on 78 strains of *E. coli* obtained from patients that had already been checked for production of ST and LT enterotoxin. Some of the data are shown in table 1. No correlation between toxigenicity and sensitivity to the phages isolated here was found except with M124. The results are summarized in Table 2. All the ST and ST-LT producing *E. coli* tested

TABLE 1. *Sensitivities of E. coli strains*[a] *to phages obtained from dusty water*

Phage strain		Am 11	Am 20	M 124	P 7	S 3
E. coli strain[a]	Toxin					
23-1	ST^-LT^-	+	+	−	−	+
69-1	ST^+LT^-	−	−	+	−	+
70-1	ST^+LT^+	−	+	+	−	−
15-1	ST^+LT^+	−	−	+	+	+
67-1	ST^+LT^-	+	+	+	+	+
12-1	ST^-LT^+	−	−	−	−	−

[a] *E. coli* samples obtained from stools of diarrhea patients at Osaka Airport Quarantine Station in January 1981. +, sensitive (bacterial lysis); −, resistant.

TABLE 2. *Sensitivities of enterotoxigenic E. coli*[a] *to phage M124*

Type of enterotoxin	Number of strains tested	Number of strains lyzed by M124
ST^+	31	31
LT^+	6	0
ST^+LT^+	16	16
ST^-LT^-	25	13

[a] Bacteria isolated from diarrhea patients at Osaka Airport Quarantine Station in January 1981.

were sensitive to M124, whereas non producers of ST enterotoxin seemed to be insensitive to this phage; though some strains not producing any enterotoxin were sensitive. Thus, enterotoxigenic *E. coli* producing ST enterotoxin can easily be detected by screening for sensitivity to M124.

2. *Electron micrograph of M124*

Electron micrography showed that M124 is a typical T-even like bacteriophage, with a icosahedral shaped head and strong tail with a tail plate and fibers (Fig. 1).

3. *Detection of plasmid DNA in enterotoxigenic E. coli*

To obtain more information about the relation between sensitivity to M124 and enterotoxigenicity, we checked for the existence of Ent plasmid DNA (Skerman et al., 1972). Only the *E. coli* producing ST enterotoxin, which were all sensitive to M124, gave a plasmid band. The size of the ST Ent plasmid was about 80 Mdalton (Fig. 2A,B) and did not vary in different strains. This result is not consistent with the results of Gyles et al. (1974) and So et al. (1975). In contrast, the LT Ent plasmid was found to vary in size. Thirteen ST^- LT^- strains as shown in table 2 that were sensitive to M124, gave no Ent plasmid band.

4. *Parallel between M124 sensitivity and existence of the ST Ent plasmid*

To clarify the relation between sensitivity to M124 and existence of the ST Ent plasmid, we tried to obtain ST Ent plasmid-free segregants. After overnight cultivating the bacteria with 50 μg/ml acridine orange, we plated them and allowed them to make single colonies. Then we picked up 3 colonies each from 10 bacterial strains (total 30 colonies) and tested ST enterotoxin production by each in suckling mice. In this way we obtained 6 ST^- segregants (20%). In a control experiment, *E. coli* K12 carrying F'lac plasmid when treated in the same way all lost F'lac (100%). We confirmed that the ST^- segregants gave no Ent plasmid band. It was very interesting that all ST^- segregants became insensitive to M124. This suggests that the ST Ent plasmid contains both the ST enterotoxin gene and a gene(s) for sensitivity to M124.

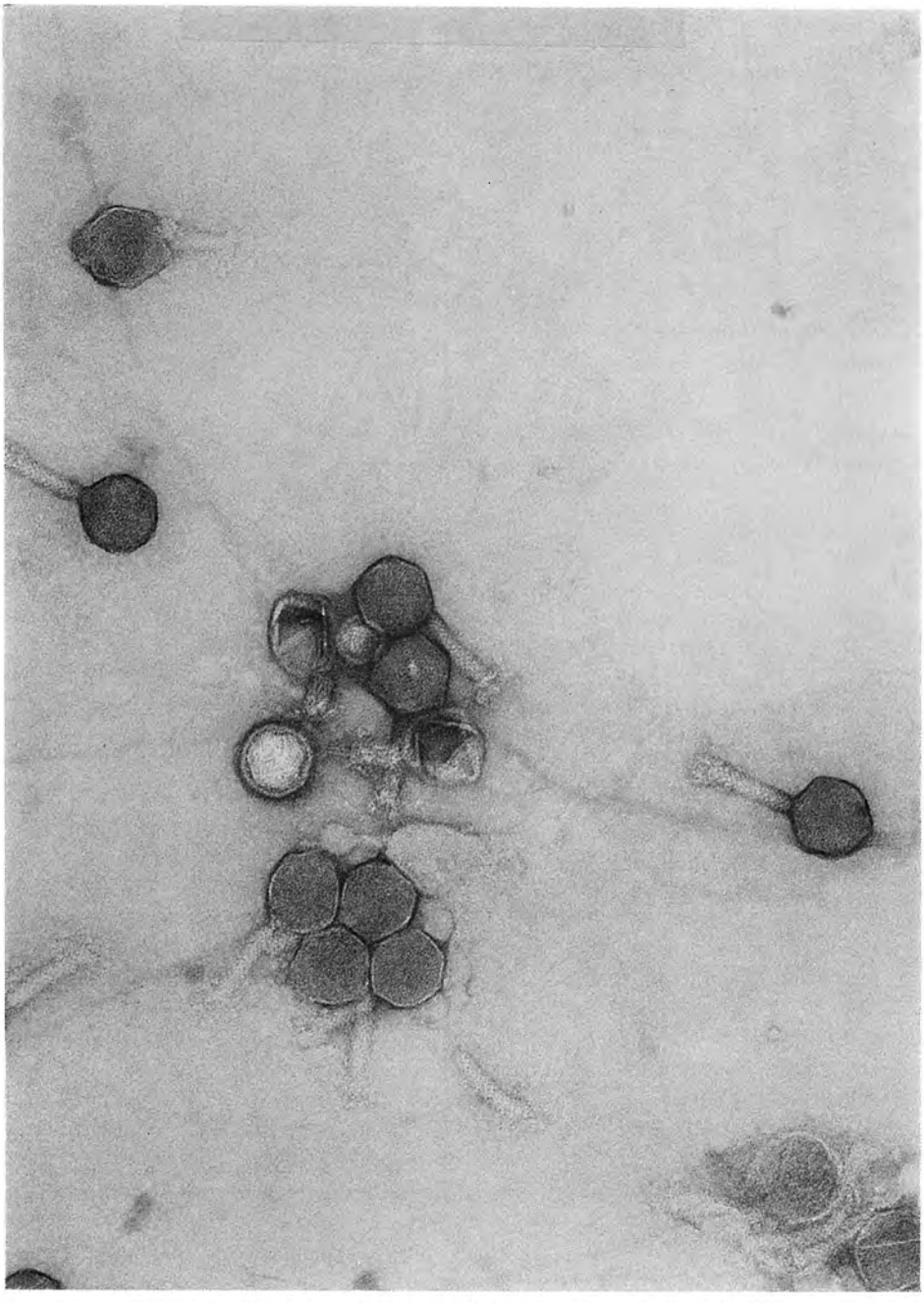

FIGURE 1. Electron micrograph of bacteriophage M124×125,000.

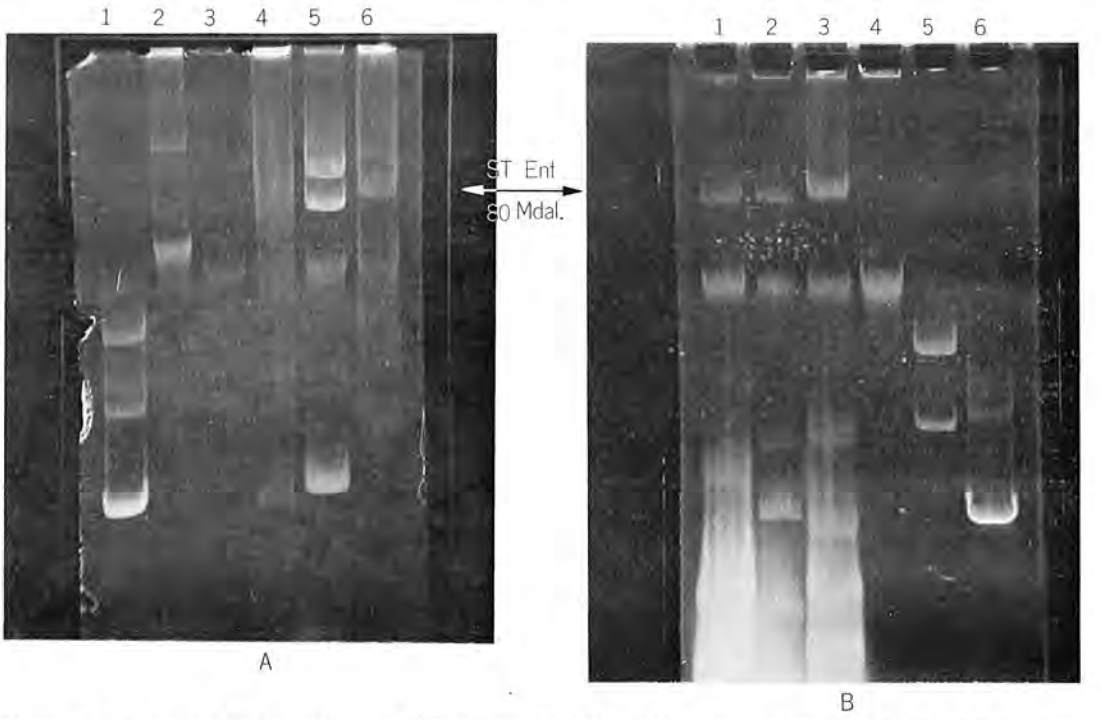

FIGURE 2. Agarose gel electrophoresis of Ent plasmid DNA. A) Line 1: plasmid pBR 322, 2.8 Mdalton, and plasmid RSF 1010, 5.0 Mdalton and its dimer. Line 2: plasmid F'lac_9 200 Mdalton. Line 3: *E. coli* 16–1 ST^- LT^-. Line 4: *E. coli* 20–1 LT^+. Line 5: *E. coli* 114 ST^+ LT^+. Line 6: *E. coli* 103 ST^+. B) Line 1, 2 and 3 are *E. coli* 105 ST^+, 106 ST^+ and 116 ST^+, respectively. All have molecular weights of about 80 Mdalton. Line 4: *E. coli* 20SO. Line 5: plasmid RSF 1010, 5.0 Mdalton and its dimer. Line 6: plasmid pBR 322, 2.8 Mdalton and its dimer.

DISCUSSION

A more advanced, easier and faster method was required for detecting enterotoxigenic *E. coli* in the laboratory.

So et al. (1975) pointed out that a kind of Ent plasmid, P 307, is related to the F (FI) or RI (FII) compatibility group of plasmid, and that the RNA phage MS2 can be adsorbed to P 307 pili as well as F pili. This finding prompted us to use this phage to screen enterotoxigenic bacteria. However, no *E. coli* producing enterotoxin obtained from patients was sensitive to RNA phage, R17 (closely related to MS2) or single-stranded DNA phage, ZD (data not shown). Accordingly, we attempted to find a new phage that specifically lysed enterotoxigenic *E. coli*.

By examination of more than 150 phages with enterotoxigenic *E. coli*, we finally obtained an ST Ent plasmid specific phage, M124. The specificity of M124 was confirmed by showing that M124 can proliferate on *E. coli* containing the ST Ent plasmid but not on ST^- segregants. Thus, we conclude that the Ent plasmid contains both the ST enterotoxin gene and a gene(s) for sensitivity to M124.

These lines of evidence indicate that this M124 will be very useful in first step screening of *E. coli* harbouring the ST Ent plasmid. Further studies are necessary on some false positive cases (Table 2), which were sensitive to M124 but contained no ST Ent plasmid.

Since the ST enterotoxin gene and a gene(s) for sensitivity to M124 might be separable, we are planning to clone these genes separately from Ent plasmid DNA.

E. coli K12 containing a transferred ST Ent plasmid was not sensitive to M124 (data not shown). Thus the mechanism for lysis of *E. coli* containing the ST Ent plasmid may not be simple.

In this article, we described the isolation and characterization of a specific phage for the ST Ent plasmid. We must also isolate a specific phage for the LT Ent plasmid. When this is accomplished, it will be possible to check all types of enterotoxigenic *E. coli* by means of phage sensitivity.

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