*Acta virol.* **29**: 73—78, 1985

# ISOLATION AND PARTIAL CHARACTERIZATION OF TEMPERATE BACTERIOPHAGES FROM ENTEROPATHOGENIC STRAINS OF *ESCHERICHIA COLI* 0111 : K58

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*Received March 5, 1984*

*Summary.* — We report studies on ten strains of *Escherichia coli* 0111:K58 isolated from children with acute diarrhea. Our results show that these *E. coli* strains do not produce the pathogenic factors of enterotoxic *E. coli* (ETEC) and are lysogenic for phages belonging to two groups that differ for host range, kinetics of thermal inactivation, antigenicity and morphology. These data support the hypothesis that these phages may in vivo contribute to reduction of the number of common *E. coli* strains by lytic infection favouring the development of the enteropathogenic strain of *E. coli*.

*Key words: E. coli 0111:K58; enteropathogenic E. coli; temperate phages; lysogeny*

## *Introduction*

*E. coli* strains causing acute diarrhea in man may be classified in three different groups: enteroinvasive strains of *E. coli* (EIEC) which rapidly invade colonic mucosal cells producing a Shigella-like disease, the enterotoxic *E. coli* (ETEC) and the enteropathogenic *E. coli* (EPEC) (Pickering, 1979). Strains of ETEC produce two enterotoxins: a heat-labile toxin (LT) and a heat-stable toxin(ST) along with two kinds of colonization factor antigens (CFA/I and CFA/II) (Sack, 1975). Enteropathogenic *E. coli* belong to the specific 0 serogroups associated with childhood diarrheas (Orskov *et al.*, 1977) but rarely they produce enterotoxins detectable with the usual methods (Klipstein *et al.*, 1980).

In our previous observations we pointed out that, in the faeces of children with acute diarrhea, the common strains of *E. coli* were almost completely replaced by the enteropathogenic strain and we showed the frequent lysogeny of *E. coli* strains belonging to EPEC serogroups 026 : K60, 0125 : K70 and 0111 : K58 (Bortolon C. *et al.*, 1977; Sezzano *et al.*, 1983). In this paper we report data concerning the host range, the kinetics of thermal inactivation, the antigen correlation and the morphology of phages released by 10 strains of *E. coli* isolated from children with acute diarrhea.

## *Materials and Methods*

*Bacteria.* Ten enteropathogenic strains (numbered from 1 to 10) belonging to serogroup 0111 : K58 have been isolated in our laboratory from rectal swabs of children with acute diarrhea. The serogroup was determined by slide agglutination test with polyvalent *E. coli* OK antisera and the confirmation of the 0 antigen content was done by using the specific *E. coli* 0 antiserum at 1 : 320 in a tube agglutination test (Difco, USA).

As indicators for phages were used *E. coli* B, *E. coli* C, *E. coli* C600 and *E. coli* K12 strains from culture collection of the Institute of Medical Microbiology. Bacterial cultures were stored in nutrient agar at 4 °C until they were subcultured for testing.

*Media and solutions.* The composition of media and solutions used in our experiments were reported in our previous paper (Neri *et al.*, 1966).

*Phage isolation, purification and assay.* Phages were isolated from supernatant of broth cultures of the *E. coli* 0111 : K58 strains. Bacterial cultures, at early stationary phase in nutrient broth, were centrifuged 10 min at 3,000 g, the supernatants were filtered through 0.22 μm pore size membrane (Millipore, U.S.A.) and spotted on plates seeded with indicator strains. The lytic areas obtained were picked, diluted and replated. Phages stocks (numbered from ø 1 to ø 10) were prepared by confluent lysis on soft agar (Adams, 1959). The concentration and the purification of phages suspensions were obtained by three cycles of low (6,000 g, 15 min) and high (35,000 g, 1 hr) speed centrifugation in a preparative centrifuge (Spinco L5—50) at 4 °C (Ceppellini *et al.*, 1963). The phage assay was performed by using the soft agar overlayer technique suggested by Adams (1959).

*Host range.* The host range was determined by standard procedures using preparations at routine test dilution (RTD) (Morris *et al.*, 1973). The RTD corresponded to a concentration of $10^4 - 5 \times 10^4$ PFU/ml determined by titration on the sensitive *E. coli* strain. Host range was determined by spotting RTD on plates seeded with different *E. coli* strains and scoring plaques after overnight incubation at 37 °C.

*Thermal inactivation.* Phage suspensions in nutrient broth were treated at different temperatures (62 °C, 65 °C, 66,5 °C, 68 °C, 70 °C and 72 °C). Percent of survival was determined after 10, 20, 30, 40, 50, 60 min of treatment evaluating the number of PFU in the phage suspension samples (Turri *et al.*, 1964).

*Neutralization test.* Antisera were obtained from rabbits injected i.m. every 15 days (for three times) with 1 ml of purified phage suspensions containing about $10^{10}$ PFU/ml. Serum was collected one week after the last injection and stored at —20 °C until use. The neutralization tests were performed by adding diluted phage suspensions ($10^7$ PFU) to the different sera diluted 1 : 100; 1 : 200; 1 : 1,000; 1 : 2,000; 1 : 4,000. At intervals ranging from 5 to 40 min incubation at 37 °C, the phage-serum mixtures were collected, rapidly diluted and plated for the PFU test.

*Morphology.* Bacteriophage morphology was studied by negative staining method of Bradley (1962) using 1% potassium phosphotungstate, pH 7. Photographs were taken with electron microscope Siemens Elmiskop 1.

## *Results*

### *Host range*

Based on their host-range phages isolated from the 10 strains of *E. coli* 0111 : K58 may be arranged into two groups (Table 1). The host range of phages ø 1, ø 2 and ø 3 was identical. They produced lysis on *E. coli* B and *E. coli* C cultures yielding small, clear and well-defined plaques. Phages from the ø 4 to the ø 10 were able to produce lysis only on *E. coli* C cultures and gave large and turbid plaques.

### *Thermal inactivation*

The kinetics of thermal inactivation performed on phages ø 1, ø 3, ø 7 and ø 10. (Fig. 1) showed a similar pattern of inactivation characterized by

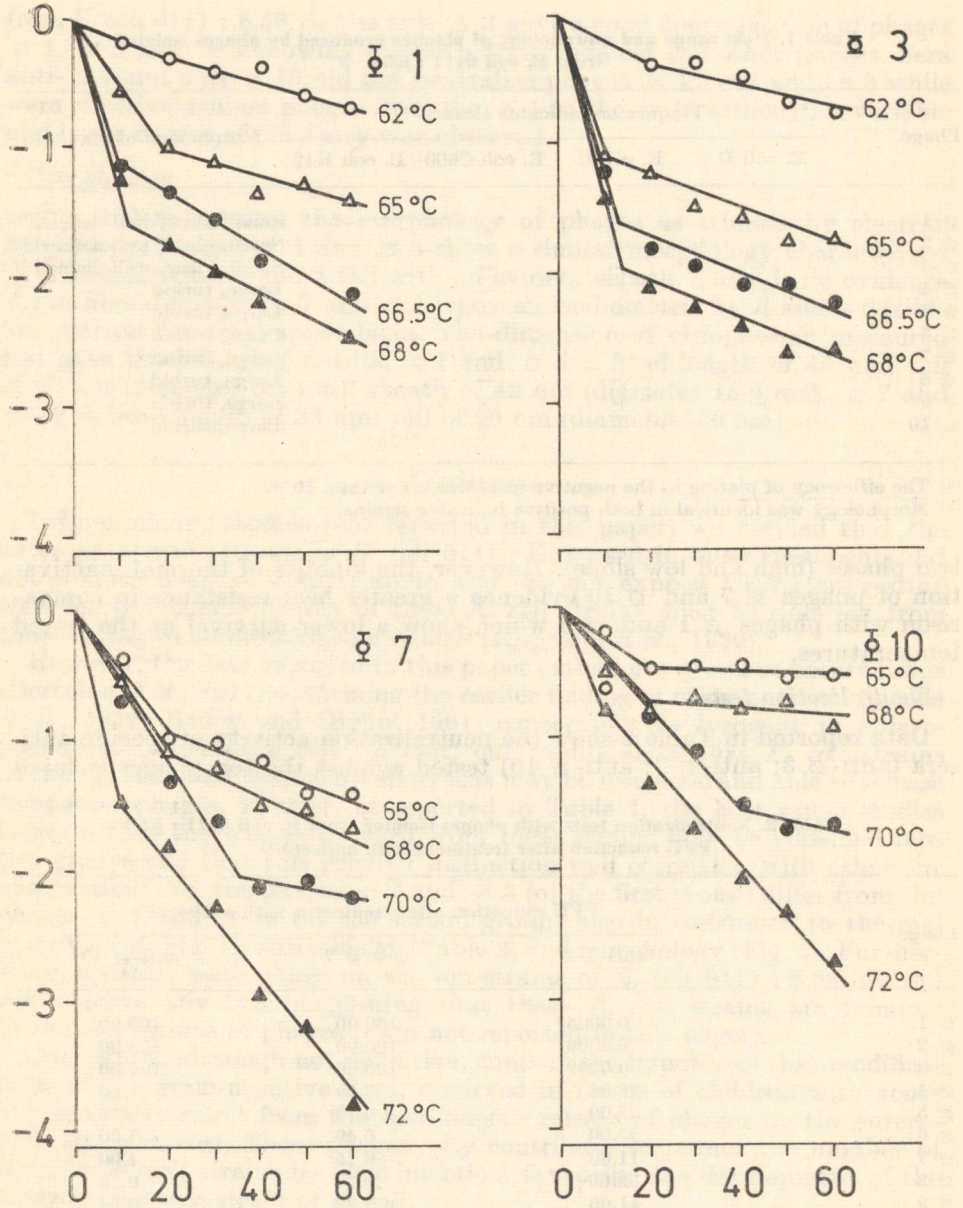


**Fig. 1.**

Kinetics of thermal inactivation of phages ⌀ 1, ⌀ 3, ⌀ 7 and ⌀ 10.
Survival is expressed as log $N/N_0$ (N = number of PFU in the heated sample of phage suspension; $N_0$ = number of PFU in the unheated sample of phage suspension).
Abscissae: time (min); ordinate: $\log_{10} N/N_0$.

**Table 1. Host range and morphology of plaques produced by phages isolated from E. coli 0111 : K58**

| Phage | Plaques on indicator strains[a] | | | | Morphology of plaques |
|---|---|---|---|---|---|
| | E. coli B | E. coli C | E. coli C600 | E. coli K12 | |
| ø 1 | + | + | — | — | Small, clear, well-defined[b] |
| ø 2 | + | + | — | — | Small, clear, well-defined[b] |
| ø 3 | + | + | — | — | Small, clear, well-defined[b] |
| ø 4 | — | + | — | — | Large, turbid |
| ø 5 | — | + | — | — | Large, turbid |
| ø 6 | — | + | — | — | Large, turbid |
| ø 7 | — | + | — | — | Large, turbid |
| ø 8 | — | + | — | — | Large, turbid |
| ø 9 | — | + | — | — | Large, turbid |
| ø 10 | — | + | — | — | Large, turbid |

[a] The efficiency of plating in the negative tests was lower than $10^{-4}$.
[b] Morphology was identical in both positive indicator strains.

two phases (high and low slope). However, the kinetics of thermal inactivation of phages ø 7 and ø 10 evidence a greater heat-resistance in comparison with phages ø 1 and ø 3 which show a lower survival at the tested temperatures.

*Neutralization tests*

Data reported in Table 2 show the neutralization activity of specific antisera (anti-ø 3; anti-ø 7; anti-ø 10) tested against the ten phages isolated

**Table 2. Neutralization tests with phages isolated from E. coli 0111 : K58 PFU reduction after treatment with antisera[a]**

| Phage | PFU reduction after treatment with antisera[a] | | |
|---|---|---|---|
| | anti-ø 3 | anti-ø 7 | anti-ø 10 |
| ø 1 | 0.0005* | 100.00 | 100.00 |
| ø 2 | 0.0009 | 100.00 | 100.00 |
| ø 3 | 0.0002 | 100.00 | 100.00 |
| ø 4 | 22.00 | 0.29 | 1.20 |
| ø 5 | 32.00 | 0.12 | 0.12 |
| ø 6 | 25.00 | 0.49 | 1.00 |
| ø 7 | 11.00 | 0.25 | 1.00 |
| ø 8 | 32.00 | 0.36 | 0.15 |
| ø 9 | 41.00 | 0.32 | 0.54 |
| ø 10 | 22.00 | 0.14 | 0.25 |

[a] The treatment was performed at 37 °C for 10 min. with serum diluted 1 : 100 (for details see Materials and Methods).
* Per cent plaque reduction.

from *E. coli* 0111 : K58. Serum anti- ø 3 gave a good neutralization of phages ø 1, ø 2 and ø 3 showing a poor activity against the other phages. Sera anti- ø 7 and anti- ø 10 did not neutralize phages ø 1, ø 2 and ø 3 while were effective against phages from the ø 4 to the ø 10 although a variable and lower degree of efficiency was observed.

*Morphology*

Fig. 2 demonstrates the morphology of phages as studied by electron microscopy. Phages ø 1 and ø 3 show a similar morphology characterized by a *icosaedric* head and a tail with an evident sheath. There is no evidence of tail fibers. Phages ø 7 and ø 10 show an isodiametric head and a flexible tail without terminal appendages. The dimension of virions were measured and gave the following results: *ø 1* and *ø 3* = head length of 45 nm; tail of 97 nm (diameter 5.1 nm); sheath of 42 nm (diameter 15.9 nm), *ø 7* and *ø 10* = head length of 39 nm; tail of 90 nm (diameter 5.0 nm).

## *Discussion*

In preliminary studies (not reported in this paper) we verified that the enteropathogenic strains of *E. coli* 0111 : K58 used in our experiments did not produce heat-labile enterotoxin and did not express CFA/I suggesting that the pathogenic factors of these *E. coli* strains must be different from those acting in enterotoxigenic strains (Klipstein *et al.*, 1980).

However, the data reported in this paper extend our previous observations (Bortolon *et al.*, 1977) confirming the earlier finding of other authors (Nicolle *et al.*, 1952; Bailey and Glynn, 1961) concerning the lysogeny in enteropathogenic *Escherichia coli*. In fact, our results show that *E. coli* strains 0111 : K58 isolated from acute diarrheas may be lysogenic and able to release temperate phages. Further, as reported in Table 1, the host range studies indicate that the phages released from these strains may be classified into two groups and that this peculiar distinction well correlates with other our observations. So, the phages ø 1 and ø 3 (of the first group) differ from the phages ø 7 and ø 10 (of the second group) also in resistance to thermal inactivation (Fig. 1), antigenicity (Table 2) and morphology (Fig. 2). Furthermore, spotting each phage on the ten strains of *E. coli* 0111 : K58, we did not observe any lysis suggesting that these *E. coli* strains are immune to the two groups of phages (data not reported in this paper).

Our results, although not definitive, support the hypothesis that modifications of the gram-negative flora, observed in faeces of children with acute diarrhea, may result from the spontaneous release of phages by the enteropathogenic strains. These phages may contribute to reduce the number of common *E. coli* strains by lytic infection, favouring the development of the enteropathogenic strain of *E. coli*.

Experiments are in progress to elucidate this hypothesis, because of the interest to isolate phages active against *E. coli* strains that cause infections and to use phages for typing pathogenic strains of *E. coli* (Smith and Huggins, 1983).

*Explanation of Electron Micrographs* (Plate XVI):

*Fig. 2.* Electron micrographs of phages ø 1, ø 3, ø 7 and ø 10.
Negative staining with potassium phosphotungstate. ø 1 = magn. 121,000 ×; ø 3 = magn. 110,000 ×; ø 7 = magn. 84,000 ×; ø 10 = 110,000 ×.

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