A Generalized Transducing Phage of *Pseudomonas cepacia*

By HIDEKI MATSUMOTO,* YOSHIFUMI ITOH, SHIN OHTA AND YOSHIRO TERAWAKI

Department of Bacteriology, School of Medicine, Shinshu University, Matsumoto 390, Japan

(Received 19 February 1986; revised 22 April 1986)

A generalized transducing phage, named CP75, was derived from a lysogenic strain of *Pseudomonas cepacia*. The frequency of transduction per phage particle ranged from $1.0 \times 10^{-6}$ to $2.0 \times 10^{-6}$ for a given marker. About half of the 105 *P. cepacia* strains tested were sensitive to the phage. The molecular size of the CP75 genome was approximately 52 kb.

INTRODUCTION

*Pseudomonas cepacia* was initially described as a plant pathogen that causes decay of onion bulbs (Burkholder, 1950; Ballard *et al.*, 1970). In recent years, however, reports of human infection due to this micro-organism have increased, and its aetiological role as an opportunistic pathogen is now well documented (Gilligan & Schidlow, 1984; Mackevitt & Woods, 1984). *P. cepacia* is also a nutritionally versatile organism (Stanier *et al.*, 1966; Ballard *et al.*, 1970). We have attempted to obtain a recombination system in *P. cepacia* by isolating a generalized transducing phage.

METHODS

**Bacterial and phage strains.** Strains PCJ1 and PCT1 were provided by Dr Teruko Nakazawa, School of Allied Health Sciences, Yamaguchi University, Ube, Japan, as clinical isolates of *P. cepacia* labelled JN25 and JN75, respectively. Both strains were aerobic Gram-negative, indophenol oxidase positive, motile rods. Using the 'Minitek differentiation system' (BBL) they gave biochemical reactions which are typical of *P. cepacia* (Gilardi, 1985). Auxotrophic mutants of PCJ used for transduction experiments were PCJ3 (his-20 nal-3), PCJ5 (leu-7 nal-12), PCJ7 (met-15 nal-17) and PCJ10 (trp-36 leu-29 met-26). All were derivatives of PCJ1 (prototroph). Phage CP75 was isolated from strain PCT1 as described below. A number of other *P. cepacia* strains were used in preliminary experiments. Cultures were kept on nutrient agar slants at room temperature and subcultured every 3 to 5 d. Growth was at 30 °C.

**Media.** Complete medium was nutrient broth or nutrient agar. Soft-agar for phage experiments contained 0.7% (w/v) agar and was supplemented with 100 μM-CaCl₂. Minimal medium was that of Ornston & Stanier (1966) but without nitriloacetic acid.

**Isolation of bacterial mutants.** A culture that had been grown for 8 to 10 h at 30 °C on nutrient agar was treated with N-methyl-N′-nitro-N-nitrosoguanidine (50 μg ml⁻¹) for 10 min at 30 °C in nutrient broth, and was incubated subsequently in nutrient broth for 10 h to allow phenotypic expression. The culture was washed three times with saline, suspended in saline to a concentration of $2 \times 10^8$ cells ml⁻¹ and starved for 5 h at 30 °C with shaking. The resting cells were inoculated at a concentration of $5 \times 10^5$ cells ml⁻¹ into minimal medium containing piperacillin (500 μg ml⁻¹) and incubated overnight. The culture was diluted to obtain about 200 colonies per plate and spread on nutrient agar. Auxotrophic mutants were sought by replica-plating. Mutants resistant to nalidixic acid (Nal⁰) were isolated by seeding 0-1 ml of a saline suspension (10⁸ ml⁻¹) of fresh agar culture onto nutrient agar plates containing the drug at a concentration of 80 μg ml⁻¹.

**Isolation of phage.** A saline suspension ($2 \times 10^8$ cells ml⁻¹) of agar-grown culture was exposed to UV light for 20 s, giving 5% survival. The irradiated suspension was mixed with an equal volume of nutrient broth, incubated at 30 °C overnight and then treated with chloroform. The culture was centrifuged at 5000 r.p.m. for 5 min and the clear supernatant was tested for the presence of phage by spotting onto a soft-agar overlay containing indicator cells. A discrete plaque was picked and propagated three times using the soft-agar overlay method to ensure purity of the phage.
Purification of phage. Phage lysate, usually containing 10^9 to 10^{10} p.f.u. ml^{-1}, was centrifuged at 12000 \text{g} for 1 h to remove cell debris. Polyethylene glycol 6000 and NaCl were added to the lysate at concentrations of 10\% (w/v) and 3\% (w/v), respectively. The mixture was left at 2 \degree C for 3 h and then centrifuged at 12000 \text{g} for 1 h at 4 \degree C. The sediment was dissolved in phage buffer solution (Clowes & Hayes, 1968) and centrifuged at 55000 \text{g} for 1 h at 4 \degree C to remove the polyethylene glycol.

Purification of phage DNA. A concentrated phage preparation (10^{12} p.f.u. in 1 ml 10 mm-Tris/HCl buffer, pH 8-0, 10 mm-MgCl_{2}) was treated with RNAase A (Sigma; 100 \mu g) and DNAase I (Sigma; 10 \mu g) at 37 \degree C for 1 h to degrade contaminating host RNA and DNA. Phage particles were collected by centrifugation at 55000 \text{g} for 1 h at 4 \degree C and dispersed in 1 ml of the same buffer. After addition of 20 mm-EDTA and SDS 0-2\% (w/v), the phage suspension was treated three times with the same volume of phenol saturated with TES buffer (10 mm-Tris/HCl, pH 8-0, 1 mm-EDTA, 10 mm-NaCl). The phage DNA was dialysed against TES buffer and subsequently against TE buffer (10 mm-Tris/HCl, pH 8-0, 1 mm-EDTA). The final yield was approximately 50 \mu g DNA.

Digestion with restriction endonucleases and agarose gel electrophoresis. Restriction endonucleases EcoRI, HindIII and BamHI were purchased from Toyobo Co. Ltd, and PvuII from Takara Shuzo Co. Ltd; they were used as recommended by the suppliers. The DNA fragments were electrophoresed on a 0-7\% (w/v) agarose gel prepared in running buffer (89 mm-Tris/HCl, 89 mm-boric acid, 2-5 mm-disodium EDTA) at a constant voltage of 70 V for 16 h. After electrophoresis, the gel was stained with ethidium bromide and photographed.

Neutralization test. Anti-CP75 phage serum was prepared by immunizing a rabbit (New Zealand White) with purified phages. The K value of the serum was estimated as described by Key (1972). The effect of anti-phage serum on the frequency of transduction was tested by mixing 0.5 ml of saline dilution of the serum with an equal volume of phage lysate. After incubation at 37 \degree C for 10 min, 0.3 ml of a saline suspension of recipient cells (10^9 cells ml^{-1}) was added and the mixture was incubated at 30 \degree C for 5 min. Saline (8 ml) was added and centrifuged at 5000 \text{r.p.m.} for 5 min. The sediment was resuspended in 1 ml saline, and 0.1 ml of the suspension was spread in a selective plate. A control was run in which an equal volume of saline was added in place of antiserum.

Electron microscopy. Phage particles in phage buffer solution were negatively stained with 2\% (w/v) potassium phosphotungstate solution at pH 7-0 and observed with a transmission electron microscope (Hitachi, type HU11A).

Transduction. Phage lysate (0.5 ml) and saline suspensions of recipient cells (5 \times 10^8 to 1 \times 10^9 cells ml^{-1}) were mixed, kept at 30 \degree C for 20 min to allow phage adsorption and then centrifuged at 5000 \text{r.p.m.} for 5 min. The pellet was resuspended in an appropriate volume of saline and samples (0.1 ml) were spread on selective plates, which were then incubated at 30 \degree C for 3 to 5 d.

RESULTS AND DISCUSSION

Phages derived from lysogenic strains of \textit{P. cepacia} were examined for their transducing ability. A total of 221 strains of \textit{P. cepacia}, mostly from clinical sources, were tested for their lysogenicity by employing twenty as indicator strains. Twenty-five strains proved to be lysogenic. A phage designated CP75 and isolated from strain PCT1 was found in a preliminary test to mediate transfer of chromosomal genes in \textit{P. cepacia} strain PCJ sublines.

Evidence for the occurrence of transduction by phage CP75. Phage CP75 was propagated on PCJ sublines including a prototroph and three auxotrophic mutants requiring histidine, leucine or methionine. Each of the auxotrophic mutants were then infected crosswise at an m.o.i. of about 2 with phages from different hosts. The markers tested, \textit{his}-20, \textit{leu}-7 and \textit{met}-15, were transduced at a frequency ranging from 4.0 \times 10^{-6} to 2.0 \times 10^{-5} per infected cell, suggesting that CP75 is a generalized transducing phage. Similar results were obtained in two replicate experiments.

Furthermore, co-transduction of \textit{trp}-36 and \textit{leu}-29 from PCJ10 was demonstrated in duplicate experiments. Of 256 \textit{trp}^+ transductants obtained by using the phage propagated in a prototrophic strain, 191 (74\%) inherited the \textit{leu}^+ allele of the donor simultaneously. Of 247 \textit{leu}^+ transductants, 161 (65\%) were \textit{trp}^+. In contrast, \textit{met}-26 from PCJ10 was not co-transducible with either \textit{trp}-36 or \textit{leu}-29.

Relationship between change of m.o.i. and frequency of transduction. As expected, an apparent reduction of frequency of transduction was seen in parallel with decreasing m.o.i. (Table 1). The frequency of transduction per phage particle was estimated to be from 1.0 \times 10^{-6} to 2.0 \times 10^{-6} for a given marker.
Transducing phage of Pseudomonas cepacia

Fig. 1. Electron micrograph of particles of phage CP75. Bar, 100 nm.

Table 1. Effect of m.o.i. and anti-phage serum on frequency of transduction

P. cepacia PCJ1 (prototrophic) was used as the donor.

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Selected marker</th>
<th>M.o.i.</th>
<th>Final dilution of anti-phage serum*</th>
<th>Frequency of transduction†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCJ5, leu-7</td>
<td>leu+</td>
<td>1-1</td>
<td>–</td>
<td>1-1 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-1</td>
<td>–</td>
<td>2-1 x 10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-01</td>
<td>–</td>
<td>&lt;10^-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>No serum added</td>
<td>1-8 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1 : 400</td>
<td>1-4 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1 : 200</td>
<td>9-8 x 10^-8</td>
</tr>
<tr>
<td>PCJ7, met-15</td>
<td>met+</td>
<td>0-8</td>
<td>–</td>
<td>1-9 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-08</td>
<td>–</td>
<td>4-4 x 10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0-008</td>
<td>–</td>
<td>&lt;10^-8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>No serum added</td>
<td>2-5 x 10^-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1 : 400</td>
<td>1-7 x 10^-6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>1 : 200</td>
<td>1-4 x 10^-7</td>
</tr>
</tbody>
</table>

* The K value was 440 min^-1 at 37 °C.
† Number of prototrophic colonies per infected cell. Phage lysate contained 9-6 x 10^-9 p.f.u. ml^-1. Results are means of two separate experiments.

Effect of anti-phage serum on frequency of transduction. A phage lysate of PCJ1 was treated with antiserum against CP75 prior to mixing with recipient cells. The decrease in the number of prototrophic colonies on the selective plates was proportional to the increase in the concentration of anti-phage serum (Table 1), confirming the role of the phage as the vehicle of
the gene transfer. Treatment of the phage lysate with DNAase I (Sigma, type IV; 50 μg ml⁻¹ for 1 h at 37 °C) had no effect on the frequency of transduction (data not shown).

Molecular size of the genome of CP75. The DNA of CP75 was digested with endonucleases BamHI or PvuII, and the fragments were electrophoresed on a gel with standards of phage λ DNA digested with HindIII and/or EcoRI. Digestion with BamHI and PvuII yielded 10 and 16 fragments, respectively, with the following sizes (kb): for BamHI, 10-7, 10-0, 7-0, 5-8, 5-4, 4-05, 3-45, 3-25, 1-48 and 0-57 (51-4 kb in total), and for PvuII, 8-5, 6-9, 5-9, 4-55, 4-25, 3-9, 2-78, 2-4, 2-38, 2-15, 2-02, 1-85, 1-75, 1-55, 0-80 and 0-54 (52-2 kb in total), respectively. Thus, the size of the genome of CP75 was approximately 52 kb. The phage DNA was not cleaved by either EcoRI or HindIII (data not shown).

Virological characteristics of CP75. Plaques of CP75 on a host cell lawn were turbid and about 1 mm in diameter. The phage had a head with a hexagonal outline (57 × 71 nm) and a contractile tail (14 × 98 nm) (Fig. 1). Treatment with chloroform did not inactivate the phage. Among the 105 strains of P. cepacia tested, 46 (44%) were sensitive to this phage. As far as we know, this is the first report of a transducing phage of P. cepacia, and this phage should be useful for the study of the genetics of P. cepacia.

The authors thank Dr Teruko Nakazawa for her interest in this study and for providing many strains. We are also grateful to Dr Naomasa Gotoh, Kyoto College of Pharmacy, and Dr Yoshiyuki Kawakami, Shinshu University Hospital, for providing strains.

REFERENCES


