Bacteriophage ϕ KP mediated generalized transduction in *Erwinia carotovora* subspecies *carotovora*

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The bacteriophage ϕ KP is capable of generalized transduction in *Erwinia carotovora* subspecies *carotovora* (Ecc) strains SCRI193 and ATCC 39048. ϕ KP is a virulent phage containing double stranded DNA of approximately 46 kb. The frequencies of transduction were established for a number of chromosomal markers and plasmid pHCP2, and conditions for transduction optimized after exposure of the phage lysate to UV irradiation.

Introduction

The soft rot erwinias, *Erwinia carotovora* subspecies *carotovora* (Ecc), *Erwinia carotovora* subspecies *atroseptica* (Eca) and *Erwinia chrysanthemi* (Echr), are plant pathogens responsible for large crop losses throughout the world, estimated to be around \$50–100 million annually (Perombelon & Kelman, 1980). Of the soft rot erwinias, Ecc has probably the widest host range, causing soft rot in a number of crops from both temperate and subtropical regions (Perombelon, 1985).

Ecc strain SCRI193 is presently being used in this laboratory to investigate the role of extracellular enzymes in the economically important soft rot disease of potato. Within the last 5 years this strain has become amenable to genetic manipulation by techniques such as transposon mutagenesis (Hinton & Salmond, 1987; Hinton *et al.*, 1989) conjugation and transformation (Hinton, 1985). Transduction via a generalized transducing phage, however, has not yet been achieved.

To date, generalized transducing phages have been isolated for only a small number of *Erwinia* species, e.g. Erch-12 on Echr strain EC183 (Chatterjee & Brown, 1981), ϕ EC-2 on Echr strain 3937 (Resibois *et al.*, 1984) and phages ϕ 49 and ϕ 59 on both Ecc strain 268 and *Erwinia horticola* strain 450 (Mukvich *et al.*, 1987).

Generalized transduction can be used to carry out a range of genetic manipulations including fine structure mapping, localized mutagenesis, construction of isogenic strains, transposon mutagenesis and plasmid transfer

(Masters, 1985). In this report we describe the isolation of a generalized transducing phage for our genetically amenable strain SCRI193 to complete the range of genetic tools available for this strain.

Methods

Bacterial strains. Bacterial strains are listed in Table 1. All *Erwinia* strains were grown at 30 °C, unless stated otherwise.

Media. Luria broth (LB) and phage buffer (PB) were as described by Maniatis *et al.* (1982). Minimal medium (MM) was as described by Chatterjee *et al.* (1985). Ampicillin, for the selection of pHCP2, and kanamycin, for the selection of Tn5 induced mutants, were used at 50 μ g ml⁻¹ final concentration.

Isolation of ϕ KP. Untreated sewage, activated sludge and effluent (500 ml each) were collected from Finham sewage works, Warwickshire, UK, and pooled. The resulting mixture was centrifuged at 9000 r.p.m. for 15 min at 4 °C. The supernatant was collected and the constituents of LB added. Chloroform (0.5 ml) was added to destroy

Table 1. Strains used in this study

All strains are *Erwinia carotovora* subspecies *carotovora* with the exception of SCRI1043 which is *Erwinia carotovora* subspecies *atroseptica*.

Bacterial strain	Strain characteristics	Strain reference
SCRI193	Wild-type Ecc	Salmond <i>et al.</i> (1986)
HC131	SCRI193(pHCP2)	
HC519	HC131 <i>leu</i> ::Tn5	Hinton <i>et al.</i> (1987)
HC500	HC131 <i>cysB</i> ::Tn5	
HC512	HC131 <i>lac</i> ::Tn5	
HC(8.11)	SCRI193(pFE1) <i>trp</i> ::Tn5	Ellard <i>et al.</i> (1989)
HC(12.19)	SCRI193(pFE1) <i>arg</i> ::Tn5	
HC(17.5)	SCRI193(pFE1) <i>pur</i> ::Tn5	
SCRI1043	Wild-type Eca	Hinton <i>et al.</i> (1989)

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Abbreviation: PB, phage buffer.

bacterial contaminants and the centrifugation step repeated. Supernatant (250 ml) was transferred to a 2 litre flask and 5 ml of an overnight culture of SCRI193 added. After 24–48 h incubation at 25 °C in a shaking waterbath at 250 r.p.m., the culture had reached stationary phase. Chloroform was added and the culture was then shaken for 5 min. The supernatant was recentrifuged and stored over chloroform. After serial dilution the lysate was incubated at 25 °C for 15 min with 300 µl of a culture of SCRI193 and a 0.7% agar overlay made on an LB agar plate which was incubated overnight at 25 °C.

Plaque purification and preparation of high titre lysates. A phage lysate was serially diluted, and incubated with 300 µl of a culture of SCRI193 at 25 °C for 15 min. The mixture was added to 3 ml of 0.7% agar and an overlay made on an LB agar plate. After overnight incubation at 25 °C, individual plaques were removed from the bacterial overlay with a Pasteur pipette and resuspended in 200 µl sterile PB containing 10 µl chloroform. For the preparation of high titre lysates, the soft agar overlay was removed from a single plate showing semi-confluent lysis and resuspended in 0.5 ml sterile PB containing chloroform. Phages were eluted at 4 °C for 4–6 h and residual agar was removed from the lysate by centrifugation at 1000 r.p.m. Lysates of ϕ KP for transduction assays were prepared on wild-type SCRI193 and lysates for plasmid pHCP2 transduction assays were prepared on HC131 (Table 1).

Screening for transduction. A ϕ KP lysate made on SCRI193 was tested for its ability to transduce the *cysB*⁺ marker into the auxotrophic mutant HC500 (*cysB*::Tn5). After overnight incubation at 30 °C, 10 ml of bacterial culture (approximately 1.0×10^8 c.f.u. ml⁻¹) was centrifuged at 5000 r.p.m. for 10 min at room temperature and the pellet resuspended in 1 ml LB. ϕ KP was added at an m.o.i. of 1 (1.0×10^9 p.f.u.) and the suspension incubated at 25 °C for 30 min. The pellet was re-centrifuged, washed, and resuspended in 10 ml LB and incubated at 30 °C for 30 min. The suspension was then re-centrifuged, resuspended in 1 ml MM and 100 µl aliquots were spread onto MM agar plates. Transduction assays were performed in triplicate. For investigations into secondary transposition (re-transposition of Tn5 once inserted into the chromosome) and plasmid transduction, the final suspension was plated onto media containing kanamycin or ampicillin respectively.

UV irradiation. Phage lysates diluted 1 in 10 in PB, to a final volume of 5 ml, were exposed to short-wave UV light ($8 \mu\text{W cm}^{-2} \times 100$). At time intervals of approximately 10 s up to 60 s, 200 µl of the lysate was dispensed into 1.5 ml Eppendorf tubes on ice; 100 µl of each sample was then serially diluted and titrated on SCRI193.

Electron microscopy. Copper grids (3.09 mm) were prepared and negatively stained as described by Bradley (1967). Grids were examined using a Joel electron microscope at 60 kV.

Restriction analysis. The DNA of ϕ KP was extracted by the rapid small scale isolation method (liquid culture) of Maniatis *et al.* (1982) used for the isolation of DNA from bacteriophage λ . Once purified, the DNA was digested with a number of restriction endonucleases and electrophoresed through a 0.7% agarose gel as described by Maniatis *et al.* (1982) (Fig. 1). Restriction fragment sizes were determined by comparison with fragments of phage λ DNA digested with *Hind*III.

Test for lysogenization. Bacteria from the centre of several turbid plaques were streaked to single colonies on LB agar plates; 100 colonies were then re-streaked to single colonies. All 100 of these colonies were tested for the production of phage by growth on LB and spotting onto a lawn of SCRI193. The colonies were also tested for immunity to KP by spotting a ϕ KP lysate onto lawns of each of the colonies.

Host range. A high titre lysate of ϕ KP was spotted onto a number of agar plates with soft agar (0.7%) overlays containing *Erwinia* strains. These strains included 49 *Ecc* strains, 58 *Eca* strains, 5 *Echr* strains, 3

E. uredo strains, 5 *E. rubrifaciens* strains, 4 *E. rhapontici* strains, 3 *E. amylovora* strains, 6 *E. herbicola* strains, 2 *E. quercina* strains and 1 *E. nigrifluens* strain. On signs of reduced cell growth or cell death, caused by the action of phages, the original phage lysate was diluted to obtain individual plaques when re-spotted onto agar plates.

Phage adsorption. A high titre lysate of ϕ KP (1.0×10^9 p.f.u. in 100 µl LB) was added to an overnight suspension of SCRI193 (1.0×10^9 c.f.u. in 10 ml LB). A sample (100 µl) was removed immediately, centrifuged at 3000 r.p.m. for 5 min at 4 °C to remove bacterial cells and adsorbed phage, then the supernatant was diluted and the phage titrated. After incubation at 25 °C, at time intervals of approximately 10 min up to 60 min, further samples were removed, diluted and titrated. Phage adsorption assays were performed in triplicate.

Results and Discussion

Isolation and characterization of ϕ KP

Phage ϕ KP was isolated after enrichment on the host *Ecc* SCRI193. On a 0.7% soft agar overlay after overnight incubation at 25 °C the plaques appeared turbid, had irregular edges, and were approximately 1–2 mm in diameter. The morphology of ϕ KP was determined by electron microscopy. The phage possessed an isometric head of approximately 60 nm and a contractile tail of approximately 120–135 nm. ϕ KP therefore fell into the most commonly isolated phage type, group B of the Bradley classification system (Bradley, 1967). The nucleic acid of ϕ KP was shown to be double-stranded DNA by restriction with restriction endonucleases (Fig. 1). From restriction fragment analysis the genome size was estimated to be approximately 46 kb.

Fig. 1. Restriction endonuclease digestion of ϕ KP DNA and electrophoresis through a 0.7% agarose gel. Lane 2, *Hind*III; lane 3, *Cla*I; lane 4, *Bam*HI; lane 5, *Bgl*II; lane 6, *Eco*RI; lanes 1 and 7, λ DNA restricted with *Hind*III.

Table 2. ϕ KP mediated transduction of various markers from *Ecc* SCRI193, and plasmid pHCP2 from HC131

Frequencies of transduction and standard deviations of transduction frequencies are given per p.f.u., while reversion/spontaneous resistance frequencies are given per c.f.u.

Recipient strain	Transduced marker	Reversion frequency (c.f.u. ⁻¹)	Transduction frequency (p.f.u. ⁻¹)	Standard deviation (p.f.u. ⁻¹)
SCRI193	Ap ^r (pHCP2)	< 10 ⁻⁸ *	2.4 × 10 ⁻⁶	2.6 × 10 ⁻⁷
HC519	<i>leu</i> ⁺	2.0 × 10 ⁻⁸	2.8 × 10 ⁻⁶	1.9 × 10 ⁻⁶
HC500	<i>cysB</i> ⁺	2.9 × 10 ⁻⁷	1.0 × 10 ⁻⁶	3.4 × 10 ⁻⁷
HC512	<i>lac</i> ⁺	< 10 ⁻⁸	2.1 × 10 ⁻⁷	1.2 × 10 ⁻⁷
HC(8.11)	<i>trp</i> ⁺	4.7 × 10 ⁻⁸	1.4 × 10 ⁻⁶	6.2 × 10 ⁻⁷
HC(12.19)	<i>arg</i> ⁺	1.0 × 10 ⁻⁸	1.4 × 10 ⁻⁶	9.7 × 10 ⁻⁷
HC(17.5)	<i>pur</i> ⁺	5.3 × 10 ⁻⁷	2.0 × 10 ⁻⁶	1.1 × 10 ⁻⁶

From over 100 colonies tested for lysogeny after infection with ϕ KP, none showed signs of lysogeny, indicating that ϕ KP is probably a virulent phage.

From 49 *Ecc* strains tested ϕ KP was able to plaque on only two, SCRI193 (on which the phage was originally isolated) and ATCC 39048 (a strain presently being studied in this laboratory for its carbapenem antibiotic production). From the range of other *Erwinia* strains tested no susceptible strains were found.

Screening for transduction

Preliminary crude screening for transduction of the *cysB* mutant HC500 to prototrophy suggested that ϕ KP was capable of transduction. In an attempt to optimize the transduction system we investigated the effects of the multiplicity of infection (m.o.i.), UV irradiation, temperature and adsorption time on the efficiency of transduction.

At an m.o.i. of 1, ϕ KP adsorbed rapidly to SCRI193 over the first 45 min, resulting in adsorption of up to 90% of the phage population. At 30 min, up to 85% of the phage population had adsorbed, and this time period was therefore chosen for further assays, since it allowed a high proportion of the phage population to adsorb and at the same time minimized the time required for the assay. ϕ KP did not adsorb to *Eca* strain SCRI1043, another genetically amenable strain used to study *Erwinia* virulence (Hinton *et al.*, 1989). Although some phages require the presence of divalent cations for adsorption, e.g. λ requires Mg²⁺ for efficient plaque formation (Arber *et al.*, 1983), the addition of Mg²⁺ (10 mM-MgSO₄) did not increase the rate of ϕ KP adsorption to SCRI193. The effects of other possible adsorption factors, e.g. Ca²⁺ were not tested.

The efficiency of transduction at different temperatures was studied. After initial phage adsorption at 25 °C, ϕ KP

failed to produce plaques on SCRI193 at 37 °C. This suggested that post-adsorption incubation could be carried out at 37 °C, thereby reducing host killing due to co-infection or superinfection of transductants with wild-type phage. In practice, however, fewer transductant colonies were produced at 37 °C (2.0×10^{-7} p.f.u.⁻¹) than at 30 °C (6.8×10^{-6} p.f.u.⁻¹). A combination of healthy cell growth and a 65% reduction in the efficiency of plating (e.o.p.) at 30 °C gave the best transduction frequencies.

Transduction frequencies at m.o.i. values of 10, 1 and 0.1, and at temperatures of 25 °C, 30 °C and 37 °C were studied. The results indicated that an m.o.i. of 1 at 30 °C produced the highest transduction frequencies (6.8×10^{-6} p.f.u.⁻¹). A reduced transducing efficiency at an m.o.i. of 10 and 30 °C (1.8×10^{-7} p.f.u.⁻¹) was probably a result of excess phage killing.

Generalized nature of transduction

The ability of ϕ KP to transduce the *cysB*⁺ marker was insufficient evidence for generalized transduction. A number of other markers were selected in SCRI193 derivatives and their transduction, via ϕ KP, was assessed. Recipients were Tn5-induced mutants of SCRI193, i.e. five auxotrophs and one lactose utilization mutant. In the case of plasmid transduction, ϕ KP lysates were prepared on HC131 with SCRI193 as a recipient (Table 1). All experiments were carried out in triplicate and an average transduction frequency taken together with a standard deviation for each marker. Frequencies of transduction, spontaneous ampicillin resistance and reversion to prototrophy and sugar utilization are given in Table 2. All chromosomal markers tested and the plasmid pHCP2 were transduced at similar frequencies to the *cysB*⁺ marker, with the exception of the *lac*⁺ marker which transduced at a frequency 13 times lower. Transducing frequencies of ϕ KP ranged from 2.1×10^{-7} p.f.u.⁻¹ to 2.8×10^{-6} p.f.u.⁻¹ without UV stimulation. (Table 2). These values are generally higher than those of Ech-12 (7.0×10^{-9} to 1.0×10^{-7} p.f.u.⁻¹), ϕ EC2 (1.0×10^{-8} to 1.0×10^{-6} p.f.u.⁻¹) and ϕ 59 (1.1×10^{-8} to 1.8×10^{-6} p.f.u.⁻¹). The above results showed that ϕ KP was a generalized transducing phage. Transduction of a number of Tn5 insertion mutations, e.g. *cysB*::Tn5 into wild-type SCRI193, allowed the percentage of secondary transposition to be calculated for each marker. This was done by comparing the number of kanamycin resistant prototrophic colonies with the number of kanamycin resistant *cysB* auxotrophs. From these studies, the percentage of secondary transposition for each marker was found to be highly variable, ranging from undetectable to 80%, suggesting that secondary transposition of Tn5 into different areas of the *Erwinia* chromosome

Fig. 2. Effect of UV irradiation on the transduction of two markers (*leu*⁺ and *cysB*⁺) via ϕ KP. ◆, Number of *cysB*⁺ transductants of HC500; ■, number of *leu*⁺ transductants of HC519; ★, ϕ KP titre.

varies considerably (as has been found for *E. coli*; e.g. see de Bruijn & Lupski, 1984).

Effect of UV light

The effect of UV irradiation on the transducing efficiency of generalized transducing phages has been well documented (Masters *et al.*, 1984; Newman & Masters, 1980). In an attempt to improve transduction frequencies, the effect of irradiation on the transduction of markers *leu*⁺ and *cysB*⁺ was investigated (Fig. 2). As phage inactivation increased, the number of transductant colonies increased to a maximum, obtained at a level of irradiation causing a 3-log drop in p.f.u. Further inactivation led to a decrease in the number of transductant colonies. Although both the *leu*⁺ and *cysB*⁺ markers showed a maximum transduction frequency after a 3-log fall in phage titre, the frequency of *cysB*⁺ transduction increased by a factor of 3.7 compared to only 1.7 for the *leu*⁺ marker. Without UV irradiation the number of *leu*⁺ transductants was three times that of *cysB*⁺. After UV irradiation however, the number of transductant colonies was approximately equal in both cases (Fig. 2). This result not only suggests a reduction in recipient cell death by irradiated wild-type phage, but also suggests a stimulation in recombination efficiency, possibly reaching a maximum in both cases. Similar transduction results have been seen previously with UV irradiated P1 lysates in *E. coli*, highlighting the fact that recombination activity may be a rate-limiting factor in determining variation in marker transduction frequencies

in *E. coli* (Masters *et al.*, 1984; Newman & Masters, 1980).

Conclusions

A generalized transducing phage, ϕ KP, has been isolated from sewage and characterized. A number of chromosomal markers and the plasmid pHCP2 have been transduced and conditions for transduction optimized. Generalized transduction, via ϕ KP, is now being used extensively in this laboratory for construction of transposon-tagged strains and co-transductional linkage mapping. It also provides a powerful tool for the creation of novel mutations, by localized mutagenesis techniques, as our current studies demonstrate (J. N. Housby & G. P. C. Salmond, unpublished). Recent data show that ϕ KP is also able to transduce in *Ecc* ATCC 39048 (unpublished).

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References

- ARBER, W. (1983). Experimental methods for use with Lambda. In *Lambda II*, pp. 433–466. Edited by R. W. Hendrix, J. W. Roberts, F. W. Stahl & R. A. Weisberg. Cold Spring Harbor, NY: Cold Spring Harbour Laboratory.
- BRADLEY, D. E. (1967). Ultrastructure of bacteriophages and bacteriophages. *Bacteriological Reviews* **31**, 230–314.
- CHATTERJEE, A. K. & BROWN, M. A. (1981). Generalized transduction in the enterobacterial phytopathogen *Erwinia chrysanthemi*. *Journal of Bacteriology* **143**, 1444–1449.
- CHATTERJEE, A. K., ROSS, L. M., McEVROY, J. L. & THURN, K. K. (1985). pULB113, an RP4::mini Mu plasmid, mediates chromosomal mobilization and R-prime formation in *Erwinia amylovora*, *Erwinia chrysanthemi* and subspecies of *Erwinia carotovora*. *Applied and Environmental Microbiology* **50**, 1–9.
- CLEMENT, J. M., PERRIN, D. & HEDGPETH, J. (1982). Analysis of lambda receptor and β -lactamase synthesis and export using cloned genes in a minicell system. *Molecular and General Genetics* **185**, 302–310.
- DE BRUIJN, F. J. & LUPSKI, J. R. (1984). The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids – a review. *Gene* **27**, 131–149.
- ELLARD, F. M., CABELLO, A. & SALMOND, G. P. C. (1989). Bacteriophage lambda-mediated transposon mutagenesis of phytopathogenic and epiphytic *Erwinia* species is strain dependent. *Molecular and General Genetics* **218**, 491–498.
- HINTON, J. C. D. (1985). *Development and application of molecular genetic techniques in Erwinia carotovora subspecies carotovora*. PhD thesis, Warwick University, UK.
- HINTON, J. C. D. & SALMOND, G. P. C. (1987). Use of TnphoA to enrich for extracellular enzyme mutants of *Erwinia carotovora* subsp. *carotovora*. *Molecular Microbiology* **1**, 381–386.
- HINTON, J., PEROMBELON, M. C. M. & SALMOND, G. P. C. (1987). Cloning of the *cysB* gene of *Erwinia carotovora* subspecies *carotovora* and the identification of its products. *Molecular and General Genetics* **207**, 466–470.
- HINTON, J. C. D., SIDEBOTHAN, J. M., HYMAN, L. J., PEROMBELON, M. C. M. & SALMOND, G. P. C. (1989). Isolation and character-

- ization of transposon-induced mutants of *Erwinia carotovora* subsp. *atroseptica* exhibiting reduced virulence. *Molecular and General Genetics* **217**, 141–148.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning, A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MASTERS, M., NEWMAN, B. J. & HENRY, C. M. (1984). Reduction of marker discrimination in transductional recombination. *Molecular and General Genetics* **196**, 85–90.
- MASTERS, M. (1985). Generalized transduction. In *Genetics of Bacteria*, pp. 197–215. Edited by J. Scaife, D. Leach & A. Galizzi. London: Academic Press.
- MUKVICH, N. S., ROMANYUK, L. V. & KISHKO, Y. G. (1987). Gene transfer of the chromosomal markers in *Erwinia horticola* 450 by the temperate phages $\phi 49$ and $\phi 59$. *Mikrobiologichnyi Zhurnal (Kiev)* **49**, 31–35.
- NEWMAN, B. J. & MASTERS, M. (1980). The variation in frequency with which markers are transduced by phage P1 is primarily a result of discrimination during recombination. *Molecular and General Genetics* **180**, 585–589.
- PEROMBELON, M. C. M. (1985). Pathogenesis of pectolytic erwinias. In *Proceedings of the Sixth International Conference on Plant Pathogenic Bacteria* (Maryland, USA), pp. 109–120. The Hague: Martinus Nijhoff.
- PEROMBELON, M. C. M. & KELMAN, A. (1980). Ecology of the soft rot erwinias. *Annual Review of Phytopathology* **18**, 361–387.
- RESIBOIS, A., COLET, M., FAELEN, M., SCHOONEJANS, E. & TOUSSAINT, A. (1984). ϕ EC2, a new generalized transducing phage of *Erwinia chrysanthemi*. *Virology* **137**, 102–112.
- SALMOND, G. P. C., HINTON, J. C. D., GILL, D. R. & PEROMBELON, M. C. M. (1986). Transposon mutagenesis of *Erwinia* using phage lambda vectors. *Molecular and General Genetics* **203**, 524–528.