Generalized transduction in the potato blackleg pathogen *Erwinia carotovora* subsp. *atroseptica* by bacteriophage ϕ M1

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Using enrichment methods, a new bacteriophage (ϕ M1) was isolated, which is capable of generalized transduction in *Erwinia carotovora* subsp. *atroseptica* (*Eca*) strain SCRI1043. ϕ M1 is probably a virulent phage and contains double-stranded DNA of approximately 43 kb. Transduction frequencies for a number of chromosomal markers and plasmid pHCP2 were established, and conditions for transduction optimized. UV irradiation of the lysates prior to transduction enhanced the transduction frequency. ϕ M1 infected over 25% of *Eca* strains tested and so may be useful both for the genetic analysis of a number of *Eca* isolates and for the transductional transfer of selectable markers between strains.

Keywords: *Erwinia carotovora* subsp. *atroseptica*, generalized transduction, bacteriophage, ϕ M1, potato blackleg

INTRODUCTION

The soft-rot erwinias, *Erwinia carotovora* subsp. *carotovora* (*Ecc*), *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and *Erwinia chrysanthemi* (*Echr*), are bacterial plant pathogens responsible for large crop losses throughout the world (Perombelon & Kelman, 1980). *Echr* and *Ecc* tend to show a broad plant host range. In contrast, *Eca* appears to be restricted almost exclusively to potato in temperate regions (Perombelon, 1992; Perombelon & Kelman, 1980; Perombelon & Salmond, 1995). Although both *Eca* and *Ecc* can be responsible for soft rotting of tubers in storage, *Eca* is of particular interest because, unlike *Ecc*, it is also capable of causing a systemic infection of the growing plant in the field – potato blackleg (stem rot) (Perombelon, 1992; Perombelon & Kelman, 1980, 1987; Perombelon & Salmond, 1995). In both soft rot and blackleg diseases, extracellular plant cell-wall-degrading enzymes (includ-

ing multiple pectate lyases, polygalacturonases and cellulase) are considered to be the major, but not sole, virulence determinants elaborated by the pathogens (Barras *et al.*, 1994; Collmer & Keen, 1986; Kotoujansky, 1987; Perombelon & Salmond, 1995).

We are currently trying to elucidate the precise roles of the multiple virulence determinants of *Eca* strain SCRI1043 in blackleg disease of potato. This molecular biological analysis is enabled by the use of a series of genetic tools, including coliphage λ and plasmid-mediated transposon mutagenesis, conjugation and transformation, which we previously developed for this strain (Hinton, 1985; Salmond *et al.*, 1986). However, the lack of a generalized transduction system has acted as a significant impediment to some forms of routine genetic analysis in SCRI1043.

Generalized transduction can be used to carry out a range of genetic manipulations, including transposon mutagenesis, plasmid transfer, construction of isogenic strains for comparative virulence assays, localized mutagenesis, fine-structure mapping and, perhaps of greatest importance, movement of transposon insertions and gene fusions into 'clean' genetic backgrounds for regulation studies (Masters, 1985). Consequently, isolation of a generalized transducing phage would significantly increase the spectrum of genetic tools available

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Abbreviations: *Eca*, *Erwinia carotovora* subsp. *atroseptica*; *Ecc*, *Erwinia carotovora* subsp. *carotovora*; *Echr*, *Erwinia chrysanthemi*.

for *Eca*. Unfortunately, although generalized transducing phages have been described before for a few specific *Echr* and *Ecc* strains (Chatterjee & Brown, 1981; Resibois *et al.*, 1984; Mukvich *et al.*, 1987; Toth *et al.*, 1993), none of these phages infected *Eca* strain SCRI1043. Indeed, to date, there have been no published reports of any generalized transducing phages for *Eca*. In this paper we describe the isolation and characterization of the first generalized transducing phage for *Eca* strain SCRI1043. We also show that this phage (ϕ M1) infects a large spectrum of *Eca* strains and so could be useful for the general genetic analysis of many *Eca* isolates.

METHODS

Bacterial strains. *Erwinia* strains used are listed in Table 1. They were grown at 27 °C unless otherwise stated.

Media. Luria broth (LB) and phage buffer (PB) were as described by Sambrook *et al.* (1989). Minimal medium (MM) was as described by Chatterjee *et al.* (1985). Ampicillin, for the selection of pHCP2, and kanamycin, for the selection of strains carrying Tn5 insertions, were used at 50 µg ml⁻¹ final concentration.

Isolation and characterization of auxotrophs. Some auxotrophs of *Eca* strain HA100 [SCRI1043(pHCP2)] had been isolated previously (Hinton *et al.*, 1989). Further auxotrophs (Table 2) of HA100 were generated by λ ::Tn5 mutagenesis as

described previously (Hinton *et al.*, 1989; Salmond *et al.*, 1986). The auxotrophic requirements were determined using a pool plate method and, in each case, the auxotrophy was shown to be due to a single Tn5 insertion by standard reversion analysis (Salmond *et al.*, 1986; Hinton *et al.*, 1989).

Isolation of bacteriophage ϕ M1. Mixed sewage (1.5 litre), consisting of untreated raw sewage, activated sludge and final effluent (Finham sewage works, Coventry, Warwickshire, UK) was pooled and centrifuged at 9000 r.p.m. for 5 min at 4 °C. The supernatant was collected and the dry constituents of LB added. Bacterial cells were killed by the addition of 0.5 ml chloroform, and the centrifugation step was repeated. The supernatant (250 ml) was transferred to a 2 litre flask and inoculated with a 5 ml overnight culture of SCRI1043 in LB. The culture was incubated at 25 °C for 24–48 h in a shaking waterbath at 250 r.p.m. until the culture had reached stationary phase. Chloroform was again added to kill bacterial cells, and after another round of centrifugation (at 9000 r.p.m. for 15 min at 4 °C), the supernatant (lysate) was stored over chloroform. The lysate was serially diluted, added to 300 µl of an exponential-phase SCRI1043 culture, allowing adsorption for 15 min at 25 °C, and a 0.7% (w/v) overlay (3 ml molten agar) made on an LB agar plate. Plates were incubated overnight at 25 °C and then scored for plaque formation.

Plaque purification and preparation of high-titre lysates. From the overnight lawns of the indicator SCRI1043, plaques were removed by puncturing with a Pasteur pipette, then resuspended in 200 µl sterile PB containing 10 µl chloroform. Phages were eluted from the top agar plugs at 4 °C for 4–6 h and then titrated on SCRI1043 by serial dilution. High-titre,

Table 1. *Erwinia* strains used in this study

Strains shown in bold and underlined indicate those susceptible to ϕ M1. In addition to the strains listed, the following were tested: *Echr* (5 strains), *E. uredoovora* (3), *E. rubrifaciens* (5), *E. rhapontici* (4), *E. amylovora* (3), *E. herbicola* (6), *E. quercina* (2) and *E. nigrifluens* (1). None of these were sensitive to ϕ M1 (see Results).

Organism	Strains	Source*	Reference
<i>Eca</i>			
SCRI culture collection strains	<u>1</u> , 8, 13, <u>28</u> , <u>31</u> , 32, 34, 37, 39, 45, 46, 48, 52, 58, 68, 83, <u>84</u> , 93, 94, 96, <u>98</u> , 191, 1034, 1035, 1036, 1037, 1039, 1040, <u>1043</u> , <u>1044</u> , 1045, 1046, <u>1049</u> , <u>1050</u> , 1052, 1053	SCRI*	SCRI1043 only, Hinton <i>et al.</i> (1989)
Other <i>Eca</i> strains	<u>BS44</u> , BS131, BS209, BS227, C301, C361, <u>C465</u>	SCRI*	
<i>Ecc</i>			
SCRI culture collection strains	101, 102, 103, 105, 106, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 129, 130, 132, 135, 139, 143, 144, 149, 152, 155, 166, 169, 171, 172, 174, 178, 182, 192, 193, 198, 205, 208, 211, 224, 247	SCRI*	SCRI193 only, Salmond <i>et al.</i> (1986)
Other <i>Ecc</i> strains	ATCC 39048	ATCC	Reeves <i>et al.</i> (1994)

* Although these strains came from the Scottish Crop Research Institute (SCRI) culture collection, their original sources were from multiple countries and diverse geographical locations.

Table 2. Transduction of selected markers via ϕ M1 grown on strain HA100 [SCRI1043(pHCP2)]

Recipient strain	Strain characteristics	Transduced marker	Reversion frequency (per p.f.u.)	Transduction frequency (per p.f.u.)	Strain reference
SCRI1043	Prototrophic	Ap ^r (pHCP2)	< 1.0 × 10 ⁻⁸ *	5.3 × 10 ⁻⁶	Hinton <i>et al.</i> (1989)
HA(7.40)	SCRI1043(pHCP2) <i>ade</i> ::Tn5	<i>ade</i> ⁺	3.0 × 10 ⁻⁸	9.7 × 10 ⁻⁶	Hinton <i>et al.</i> (1989)
HA150	SCRI1043(pHCP2) <i>nad</i> ::Tn5	<i>nad</i> ⁺	3.0 × 10 ⁻⁸	1.7 × 10 ⁻⁶	Hinton <i>et al.</i> (1989)
TA100	SCRI1043(pHCP2) <i>ura</i> ::Tn5	<i>ura</i> ⁺	6.8 × 10 ⁻⁷	8.4 × 10 ⁻⁶	This study
TA200	SCRI1043(pHCP2) <i>arg</i> ::Tn5	<i>arg</i> ⁺	1.0 × 10 ⁻⁸	5.9 × 10 ⁻⁶	This study
TA300	SCRI1043(pHCP2) <i>cysB</i> ::Tn5	<i>cysB</i> ⁺	1.0 × 10 ⁻⁸	2.6 × 10 ⁻⁶	This study

* In the case of pHCP2, the frequency of spontaneous ampicillin resistance (Ap^r) is given.

plaque-purified lysates for use in the assay of chromosomal marker transduction were prepared on wild-type SCRI1043 and lysates used for transduction of plasmid pHCP2 were prepared on HA100 (Table 2).

Screening for transduction. A ϕ M1 lysate, made on SCRI1043, was used to assess the ability of ϕ M1 to transduce a *cysB*⁺ marker into the auxotrophic mutant TA300 (*cysB*::Tn5). An overnight culture of TA300 (10 ml) was pelleted after centrifugation at 5000 r.p.m. for 10 min at room temperature, then re-suspended in 1 ml LB. After the addition of ϕ M1 at an m.o.i. of 1 (or after the addition of an equal volume of PB in the control sample for spontaneous mutations), the suspension was incubated at 25 °C for 30 min. The culture was centrifuged again and the pellet washed and re-suspended in 10 ml LB. After incubation at 27 °C for 30 min, the suspension was re-centrifuged, re-suspended in 1 ml MM and spread onto MM agar plates in 100 μ l aliquots. In plasmid transduction experiments, final suspensions were plated onto media containing ampicillin. In each experiment 100 ml samples of neat lysates were spread onto the appropriate media to confirm lysate sterility. All experiments were carried out in triplicate and the mean transduction frequency taken.

UV irradiation. A 10-fold dilution of a high-titre phage ϕ M1 lysate in PB was exposed to short-wave UV irradiation (800 μ W cm⁻²) and at various times 200 μ l lysate was dispensed into 1.5 ml Eppendorf tubes held on ice. Each sample (100 μ l) was then titrated on SCRI1043 to assess the survival of plaque-forming units.

Electron microscopy. Samples were prepared on copper grids (3.09 mm) and negatively stained using 2% (w/v) uranyl acetate. Grids were examined using a JEOL electron microscope at 60 kV.

Restriction analysis. The DNA of ϕ M1 was extracted by the rapid small-scale isolation method (liquid culture) of Sambrook *et al.* (1989). Once purified the DNA was submitted to digestion with restriction endonuclease *Eco*RI and electrophoresed through a 0.7% (w/v) agarose gel (Sambrook *et al.*, 1989). Restriction fragment sizes were determined by comparison with fragments of a 1 kb ladder (Gibco-BRL).

Test for lysogenization. Bacteria from the centre of several turbid plaques were streaked to single colonies on LB agar plates. One hundred colonies were then re-streaked to single colonies. Mini-cultures (LB) made from all one hundred of these colonies were tested for the spontaneous release of phage by spotting them (10 μ l) onto a seeded top agar lawn of SCRI1043, alongside a ϕ M1 control. The colonies were simultaneously tested for immunity to ϕ M1 by spotting 10 μ l of a ϕ M1 lysate onto seeded top agar lawns made from each mini-culture. Plates were incubated overnight then scored for plaque formation. In addition, 100 re-streaked colonies were Southern-blotted onto Hybond-C nitrocellulose membrane (Amersham) and probed with ³²P-labelled ϕ M1 genomic DNA according to the manufacturer's instructions.

Host range. A high-titre lysate of ϕ M1 was spotted onto seeded top agar lawns of various soft-rotting and non-soft-rotting *Erwinia* strains (Perombelon & Salmond, 1995). These included *Eca* (43 strains), *Ecc* (49), *Echr* (5), *E. uredo*vora (3), *E. rubrifaciens* (5), *E. rhapontici* (4), *E. amylovora* (3), *E. herbicola* (6), *E. quercina* (2) and *E. nigri*fluens (1) (Table 1). On signs of retarded cell growth or cell lysis, caused by the action of phages, the original phage lysates were serially diluted to obtain individual plaques, to confirm true virus replication rather than bacteriocinogeny, or lysis from without.

Phage adsorption. A high-titre lysate of ϕ M1 (1 × 10⁹ p.f.u. in 100 μ l PB) was added to an overnight culture of SCRI1043 (1 × 10⁹ c.f.u. in 10 ml LB) at 25 °C. At various times up to 60 min incubation, 100 μ l samples were removed and centrifuged at 3000 r.p.m. for 5 min at 4 °C to remove bacterial cells and adsorbed phage; the supernatant was then serially diluted and the phage titrated.

RESULTS AND DISCUSSION

Isolation and characterization of ϕ M1

Phage ϕ M1 was isolated after sewage enrichment on the bacterial strain *Eca* SCRI1043. The phage belonged to group C of the Bradley classification system (Bradley,

Fig. 1. Electron micrograph of phage ϕ M1. Bar, 100 nm.

Fig. 2. Gel electrophoresis of ϕ M1 DNA digested with restriction endonuclease *Eco*RI. Lanes: 1, 1 kb ladder (Gibco-BRL); 2, ϕ M1 *Eco*RI digest. Band sizes are given in kb.

1967), had a head of 55 nm diameter and a short non-contractile tail of 5–10 nm (Fig. 1). The DNA of ϕ M1 was digested with restriction endonuclease *Eco*RI and from the sum of restriction fragment sizes the genome size was estimated to be approximately 43 kb (Fig. 2). Although ϕ M1 produced slightly turbid plaques, from 100 SCRI1043 colonies surviving phage infection tested for phage release and by DNA hybridization, none showed signs of lysogeny. Although the frequency of lysogeny might be extremely low, we think it more likely that ϕ M1 is a virulent phage. The virulent nature of ϕ M1 was further supported by the ability of the phage to

infect transductants for re-transduction of alternative markers (data not shown).

ϕ M1 was able to form plaques on 11 of the 121 *Erwinia* strains tested. All of the sensitive strains were *Eca* (11/43) and so, although this phage is very subspecies-specific, it does form plaques on a high proportion of these *Eca* isolates (Table 1). From these susceptible *Eca* strains, SCRI1043 was tested for generalized transduction.

Screening for transduction

Preliminary screening for transduction of the *cysB* auxotroph TA100 to prototrophy suggested that ϕ M1 was capable of transduction. Attempts were made to optimize the transduction system by altering the m.o.i., temperature, adsorption time and exposure to UV irradiation.

Adsorption of ϕ M1 to SCRI1043 at an m.o.i. of 1 occurred very slowly, with only 43% of the phage population adsorbing to the host after 2 h incubation at 25 °C (data not shown). After 30 min, 34% of the phage population had adsorbed and this was chosen as the standard time for the assay, as a compromise between reducing the level of host killing by wild-type phage while allowing sufficient adsorption of the phage to generate transductants.

The e.o.p. of ϕ M1 at 18 °C, 25 °C and 30 °C was studied. The number of plaques produced at 18 °C (1.7×10^{12}) was slightly higher than at 25 °C (9.0×10^{11}). At 30 °C, however, no plaques were produced, even after adsorption at 25 °C. The failure of ϕ M1 to form plaques at 30 °C, even using high-titre lysates, suggested that ϕ M1 was unable to replicate in SCRI1043 at this temperature. Thus, ϕ M1 appears to be naturally thermosensitive for replication. Because of the lack of phage replication at 30 °C, this temperature was chosen as the optimum for selection of transductants, since it would prevent superinfection killing by wild-type phage.

Transduction frequencies at m.o.i.s of 10, 1 and 0.1 were studied. An m.o.i. of 1 produced the highest transduction frequencies (2.0×10^{-6} , compared to 1.8×10^{-8} and 4.2×10^{-6} for m.o.i.s of 10 and 0.1, respectively). An m.o.i. of 1 was therefore chosen for further transduction assays.

A number of phages require divalent cations for efficient adsorption, for example λ requires the presence of magnesium ions (Arber, 1983). In this study the presence of magnesium ions (10 mM magnesium sulphate) did not increase the rate of adsorption of ϕ M1 to SCRI1043. No other adsorption factors were tested.

Generalized nature of transduction

To show evidence of generalized transduction, a series of Tn5-induced auxotrophic derivatives of SCRI1043 (Table 2) were used as recipients in transduction assays. ϕ M1 lysates were prepared on either SCRI1043 or its

Fig. 3. Effect of UV irradiation on the transduction of *ura*⁺ via ϕ M1. ■, Number of *ura*⁺ transductants of TA100; *, ϕ M1 titre.

derivative, strain HA100. HA100 carries the Ap^r LamB⁺ plasmid, pHCP2 and so, in addition to multiple chromosomal markers, transduction of the plasmid could also be tested. Frequencies of transduction, spontaneous ampicillin resistance and reversion to prototrophy are given in Table 2. The chromosomal markers and plasmid pHCP2 were transduced at similar frequencies ranging from 9.7×10^{-6} for *ade*⁺ to 1.7×10^{-6} for *nad*⁺. These results showed that ϕ M1 is a generalized transducing phage. Co-transduction of markers Ap^r and LamB⁺, identified by the adsorption of phage λ to Ap^r transductants and the presence of plasmid DNA on gels after electrophoresis of total DNA from these transductants, suggests that the complete pHCP2 plasmid was transduced (data not shown). This is not surprising since pHCP2 is considerably smaller than the packaging capacity of the phage (43 kb) and, therefore, the approximate length of DNA capable of being transduced by the phage (Clement *et al.*, 1982). Transduction frequencies for ϕ M1 are generally slightly higher than those of other *Erwinia* transducing phages, for example phage Erch-12 (7.0×10^{-9} – 1.0×10^{-7} per p.f.u.), ϕ EC2 (1.0×10^{-8} – 1.0×10^{-6} per p.f.u.), ϕ 59 (1.1×10^{-8} – 1.8×10^{-6} per p.f.u.) and ϕ KP (2.1×10^{-7} – 1.0×10^{-6} per p.f.u.) (Chatterjee & Brown, 1981; Resibois *et al.*, 1984; Mukvich *et al.*, 1987; Toth *et al.*, 1993).

Using this phage, we have recently transduced multiple transposon insertions, causing aberrant motility phenotypes and exoenzyme virulence factor regulation defects, into 'clean' SCRI1043 genetic backgrounds (unpublished). We have also used ϕ M1 to successfully transduce a single transposon insertion (*flhD*::Tn5) that causes

pleiotropic defects in virulence gene expression and motility between two *Eca* strains (SCRI1043 and SCRI1044) (unpublished). These recent results confirm the generalized nature of ϕ M1-mediated transduction and exemplify the use of this new phage for strain construction and manipulation.

The effect of UV irradiation on transduction frequency

The ability of UV irradiation to increase the frequency of transduction has been observed in a number of phages, including the coliphage P1 (Masters *et al.*, 1984; Newman & Masters, 1980) and the *Ecc* transducing phage ϕ KP (Toth *et al.*, 1993). The effect of UV irradiation on transduction of the *ura*⁺ marker was investigated (Fig. 3). UV irradiation leading to a 2 log drop in p.f.u. stimulated transduction of the *ura*⁺ marker more than threefold. No other markers were tested.

Host range

The host range of ϕ M1 was tested on a number of *Eca*, *Ecc* and other *Erwinia* strains (Table 1). Of these strains ϕ M1 produced plaques on 11 of 43 *Eca* strains, while none of the other *Erwinia* strains showed phage action. E.o.p.s of ϕ M1, carried out in triplicate on the 11 susceptible strains, varied in most cases between 2.5 and 50% compared to plating on SCRI1043. However, in the case of SCRI1044 and SCRI98, e.o.p.s were 0.25 and 0.1%, respectively. Although transduction between strains with differing e.o.p.s may have resulted in reduced transduction frequencies, transduction between derivatives of the same strain would be unaffected if using a ϕ M1 lysate produced on that strain. In this study transduction was tested in strain SCRI1043 only. Phage ϕ M1 appears to have a very narrow host range. However, although it is very specific to *Eca*, it does infect many (>25%) of the *Eca* isolates in this particular SCRI *Erwinia* collection (strains which were isolated over the course of several years and originating from multiple countries). In contrast, the *Ecc* generalized transducing phage ϕ KP was limited to only 2 of the 49 *Ecc* strains tested (Toth *et al.*, 1993). The promiscuous nature of ϕ M1 within a collection of *Eca* isolates suggests that this phage will be useful for the general genetic analysis of many strains of the potato blackleg pathogen. In addition, ϕ M1, because of its apparent *Eca* specificity, might have some value within a phage typing programme in conjunction with other *Eca*-specific phages.

Conclusion

In *Eca* strain SCRI1043, ϕ M1 acts as a generalized transducer capable of transducing both chromosomal auxotrophy markers and plasmid DNA at high frequency. Although generalized transducing phages have previously been described for both *Ecc* (ϕ KP, ϕ 59) and *Echr* (ϕ EC2, Erch-12) this is the first report of a generalized transducing phage for *Eca* (Chatterjee & Brown, 1981; Mukvich *et al.*, 1987; Resibois *et al.*,

1984; Toth *et al.*, 1993). We had previously made *Eca* strain SCRI1043 susceptible to a wide range of genetic techniques, including transformation, and transposon mutagenesis via suicide plasmids and λ (Hinton, 1985; Salmond *et al.*, 1986; Hinton *et al.*, 1989). The isolation of ϕ M1 and its use in generalized transduction now complete the spectrum of classical genetic tools required to enable molecular genetic analysis of bacterial virulence in the blackleg pathogen.

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