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Rapid generation of directed and unmarked deletions in *Xanthomonas*

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Summary

We have devised a rapid four-step procedure for the generation of directed and unmarked chromosomal deletions in bacteria, based on the use of a novel cloning vector containing the *Bacillus subtilis sacB* gene that encodes levansucrase and confers sucrose sensitivity, which can be used for counter-selection. Using this technique, we describe the construction of a 6.5 kb directed and unmarked deletion in a phytopathogenicity region of the chromosome in *Xanthomonas campestris*. This procedure allows rapid and easy transfer of a wide variety of mutant allelic DNA to the bacterial chromosome, and should be adaptable to various bacteria besides *Xanthomonas* spp.

Introduction

The widespread use of recombinant DNA techniques allows numerous manipulations of cloned bacterial genes including the generation of point mutations, insertional disruptions, and deletions. The ideal conditions for analysis of the phenotypes resulting from these different manipulations involve transfer of the engineered fragment to the bacterial chromosome. Such an exchange can be achieved several ways via single- or double-homologous recombination between the manipulated DNA segment and the chromosome, providing that there is a marker gene available for selection (Gutterson *et al.*, 1983; Hamilton *et al.*, 1989; Kulakauskas *et al.*, 1991; Ried and Collmer, 1987; 1988; Russel and Dahlquist, 1989; Ruvkun and Ausubel, 1981; Shevell *et al.*, 1988).

The presence of antibiotic-resistance markers can result in some limitations in complementation analysis or in the construction of strains with multiple mutations (Ried and Collmer, 1987). In order to circumvent this problem, a marker-exchange-eviction mutagenesis method based on the two double cross-over events between the chromosome and the manipulated allele has been described and successfully used in the enterobacteria *Erwinia chrysanthemi* and *Escherichia coli* to yield chromosomal unmarked deletion mutants (Ried and Collmer, 1987; 1988; Russel and Dahlquist, 1989; Blomfield *et al.*, 1991). This method, however, remains relatively tedious since it requires the replacement of the deletion by a specific DNA cassette (containing both a dominant conditional lethal gene for counter-selection and an antibiotic-resistance gene), and also involves two low-frequency independent marker-exchange events between the plasmid and the chromosome (Ried and Collmer, 1987; Ried and Collmer, 1988; Russel and Dahlquist, 1989; Blomfield *et al.*, 1991). In this study we describe a new rapid four-step procedure for the construction of unmarked and directed chromosomal deletions based on the use of *sacB*, a counter-selection gene encoding *Bacillus subtilis* levansucrase and conferring sucrose sensitivity upon several Gram-negative bacteria (Gay *et al.*, 1985; 1983). This method was applied to the construction of a 6.5 kb unmarked deletion in a phytopathogenic *Xanthomonas campestris* strain.

Results

Mutagenesis and mapping of a phytopathogenicity gene using integrative plasmids

Plasmids with fragments internal to a transcriptional unit insertionally disrupt the target gene by integration through a single, 'Campbell-type' homologous recombination (Campbell, 1962). The plant-inducible *hrpXc* locus of *Xanthomonas campestris* pv. campestris controls both pathogenicity on crucifers and hypersensitivity on non-host plants (Kamoun and Kado, 1990a; Kamoun *et al.*, 1991). To construct an HrpXc mutant rapidly in *X. campestris* pv. campestris, strain 2D520 was electroporated with *c.* 1 μg of DNA of pUCD3161 (containing a 338 bp *hrpXc* internal fragment) to obtain the kanamycin-resistant integrative derivative JS61. Southern blot hybridization analysis confirmed that JS61 contains a single copy of pUCD3161 integrated into the *hrpXc* gene (data not shown). Moreover, unlike its wild-type counterpart, JS61 was unable to cause black rot on crucifers or

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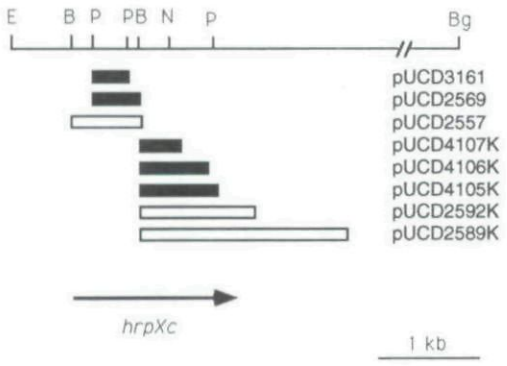

Fig. 1. Mapping of *hrpXc* using integrative plasmids. The restriction map of the *hrpXc* region is shown for the following restriction enzymes: *Eco*RI (E), *Bam*HI (B), *Pst*I (P), *Nhe*I (N), and *Bgl*II (Bg). The different subclones constructed are shown as boxes that represent the size and location of the insert. Filled boxes represent fragments internal to *hrpXc*; open boxes represent fragments that have one border external to *hrpXc*. The deduced size and orientation of *hrpXc* is given by the arrow.

induce a hypersensitive reaction on non-host plants, suggesting that the *hrpXc* gene is indeed disrupted by the integrated plasmid.

Since only plasmids bearing internal fragments would disrupt the target gene, the boundaries of the *hrpXc* locus can be determined using a collection of plasmids containing overlapping insert fragments. Thus, eight plasmids containing various insert fragments of the *hrpXc* region (Fig. 1) were electroporated into 2D520 to generate a collection of integration mutants. Plasmids pUCD3161, pUCD2596, pUCD4107K, pUCD4106K, and pUCD4105K, which together span a 1.05 kb region of *X. campestris* pv. campestris DNA, all generated Hrp mutants through single homologous recombination in 2D520, as determined by Southern blot hybridization (data not shown). On the other hand, plasmids pUCD2557, pUCD2592K, and pUCD2589K did not affect the pathogenicity of 2D520 after integration, suggesting that they are not internal to *hrpXc*. We can conclude, therefore, that the size of the *hrpXc* locus is between 1.05 kb and 1.6 kb, as shown in Fig. 1.

Frequencies of plasmid integration and excision in Xanthomonas

To determine the frequency of transformation by electroporation, the replicative plasmid pUFR034 (DeFeyter *et al.*, 1990) was electroporated into *X. campestris* pv. campestris 2D520, 2D540R, and HasibH-MR. A high electrotransformation frequency of 10^6–10^7 transformants per µg of DNA was observed consistently. The integrative plasmid pUCD3161, however, yielded transformants at *c.* 2.0×10^2, which sets the relative recombination frequency of this plasmid at about 10^{-5}–10^{-6} in *X. campestris* pv. campestris (Table 1).

To determine the relationship between the integration frequency and the size of recombinogenic DNA, the relative recombination frequencies of pUCD2583 ExoIII deletion derivative plasmids pUCD4107K, pUCD4106K, pUCD4105K, pUCD2589K, and pUCD2592K were estimated. An exponential correlation between the insert size and the relative recombination frequency of each plasmid was observed, ranging from 1.5×10^{-5} for pUCD4107K (274 bp insert) to 3.2×10^{-3} for pUCD2589 (2.1 kb insert) (Table 1, Fig. 2).

Our experience with integration mutants suggested that the excision of integrated plasmids does not occur frequently, since all mutants were stable, whether in culture medium or *in planta*. To determine the frequency of excision of the integrated plasmids, four replica cultures of mutant JS61 were subcultured every 12 generations in 4 ml of medium 523 devoid of antibiotics (ampicillin). After 10 days (*c.* 120 generations), the bacteria were plated and a total of 200 colonies were screened for ampicillin resistance: all were resistant, suggesting that excision revertants formed less than 0.5% of the population. Thus the frequency of excision of pUCD3161 in JS61 is lower than 10^{-4}, since the frequency of reversion per generation, F, is $1-\sqrt[n]{(1-N)}$ (N being the frequency of revertants observed in the population after growing n generations without selection) (Vagner and Ehrlich, 1988; Young and Ehrlich, 1989). Such a relatively low level of excision makes these mutants stable enough to be suitable for most phenotypic analyses, especially pathogenicity assays.

Table 1. Electrotransformation and relative recombination frequencies (RRF) in *X. campestris* of various plasmids used in this study.

Strain/Plasmid	Transformants per µg of DNA[a]	RRF[b]
2D520/pUFR034	6.1×10^6	NA[c]
2D520/pUCD4107K	72	1.5×10^{-5}
2D520/pUCD3161	2.0×10^2	2.2×10^{-5}
2D520/pUCD4106K	1.7×10^2	3.6×10^{-5}
2D520/pUCD4105K	2.8×10^2	6.0×10^{-5}
2D520/pUCD2592K	2.8×10^3	6.2×10^{-4}
2D520/pUCD2589K	1.3×10^4	3.2×10^{-3}
2D540R/pUFR034	7.5×10^6	NA
2D540R/pUCD3161	2.2×10^2	2.0×10^{-5}
HasibH-MR/pUFR034	5.0×10^7	NA
HasibH-MR/pUCD3161	1.2×10^2	1.6×10^{-6}

a. Average of two independent electroporations.
b. RRF is the ratio of the frequency of electrotransformation of integrative plasmids to the replicative plasmid pUFR034, after correction for the plasmid size.
c. NA, not available.

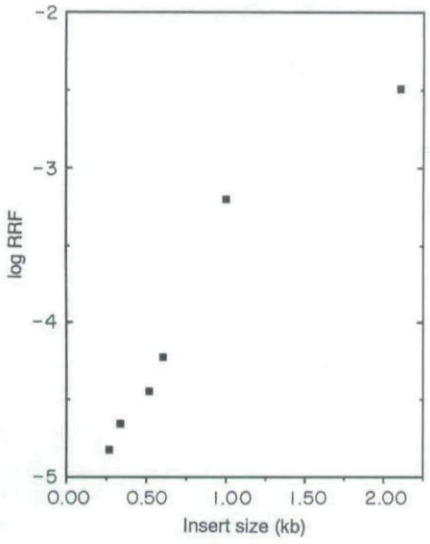

Fig. 2. Correlation between the size of recombinogenic DNA in the integrative plasmids and the relative recombination frequency of the various plasmids. The data were obtained from Table 1. All points are averages of two independent electroporations.

Rationale for unmarked deletion construction

To generate unmarked deletions rapidly in *Xanthomonas*, we have developed the following mutagenesis procedure. Four steps are required. (i) Construction and cloning of the unmarked deletion into pUCD4121 (Table 1, Fig. 1), a derivative plasmid of pUC18 that contains an antibiotic resistance gene (*cat*), and the levansucrase gene (*sacB*) that confers sucrose sensitivity. (ii) Introduction and integration by single homologous recombination of the suicide plasmid into the recipient bacteria; this is easily achieved by electrotransformation and selection for chloramphenicol resistance, as previously described for integrative plasmids. (iii) Resolution of the plasmid through a second homologous cross-over; because the low excision frequency (see above) makes it impractical and tedious to screen for double-recombination derivatives, resolved strains can be directly obtained by selection for sucrose resistance (loss or inactivation of *sacB*) and screening for chloramphenicol sensitivity (loss of the plasmid). (iv) Distinction between the two possible types of double recombinants: (a) strains that restored the original complete wild-type fragment, in which the two cross-overs occurred in the same flanking region of the deletion; (b) unmarked deletion mutants resulting from two cross-overs in both sides of the deletion. Phenotypic screening, polymerase chain reaction (PCR) amplification, or Southern blot hybridization can be performed to identify the strains that transferred the deletion to the chromosome.

To confirm the usefulness of this strategy, we used it to construct an unmarked 6.5 kb chromosomal deletion in *X. campestris* pv. campestris, starting at the 3' end of the *hrpXc* gene.

Construction of an unmarked chromosomal deletion in Xanthomonas

The *hrpXc* locus of *X. campestris* pv. campestris 2D520 spans a 1.5 kb DNA fragment contained in an 11.0 kb *Eco*RI fragment (Kamoun and Kado, 1990a) (Fig. 1). Plasmid pUCD2550 contains this 11.0 kb fragment cloned in the *Eco*RI site of pTZ18R (the various steps of plasmid constructions along with the maps of the relevant plasmids are shown in Fig. 3). Restriction enzyme analysis showed that pUCD2550 contains two *Nhe*I sites, 6.5 kb apart. As determined earlier, one of these *Nhe*I sites is internal to *hrpXc* (Fig. 1). *Nhe*I digestion and self ligation of pUCD2550 yielded pUCD2572 that contains an unmarked deletion of the 6.5 kb *Nhe*I fragment. Subsequently, the 3.0 kb *Sal*I fragment of pUCD2572, which contains about 1.5 kb of DNA flanking the deletion, was subcloned into the *Sal*I site of pUCD4121, yielding the mutagenic plasmid pUCD4150.

To transfer the deletion to the chromosome of *X. campestris* pv. campestris, strain 2D520 was electroporated with *c.* 1 μg of DNA of pUCD4150 to generate the chloramphenicol-resistant and sucrose-sensitive plasmid integrate, JS50 (Fig. 4). In a second step, positive selection for sucrose resistance was performed by plating JS50 on medium 523 (which contains 0.1% sucrose) supplemented with rifampin (50 μg ml^{-1}). Sucrose-resistant revertants of JS50 arose at a frequency of approximately 10^{-3}, yielding 97% of chloramphenicol-sensitive colonies, which probably result from the excision of the plasmid by double-homologous recombination (Fig. 4). To distinguish between the two types of double recombinants (cross-overs 1+2 or cross-overs 1+3), 54 independent sucrose-resistant and chloramphenicol-sensitive derivatives of JS50 were inoculated on Tokyo Hybrid Turnip and tested for black rot induction; of these, 43% (23) appeared to be non-pathogenic, suggesting that they probably transferred the 6.5 kb deletion to their chromosome (cross-overs 1+3). To confirm the genotype of these strains, Southern blot hybridization analysis was performed on four representative strains: JS50-3 and JS50-15 (non-pathogenic), and JS50-32 and JS50-39 (pathogenic). Total chromosomal DNA from these strains was isolated, digested with *Eco*RI, and probed with a 0.34 kb *Pst*I fragment internal to *hrpXc* and adjacent to the 6.5 kb *Nhe*I fragment (Fig. 1). All three non-pathogenic strains appeared to bear a 4.5 kb *Eco*RI fragment of similar size to the *Eco*RI fragment of pUCD2572, suggesting that the unmarked deletion was indeed generated in the chromosome (Fig. 5). In strains JS50-32 and JS50-39, which are pathogenic, the 0.34 kb probe

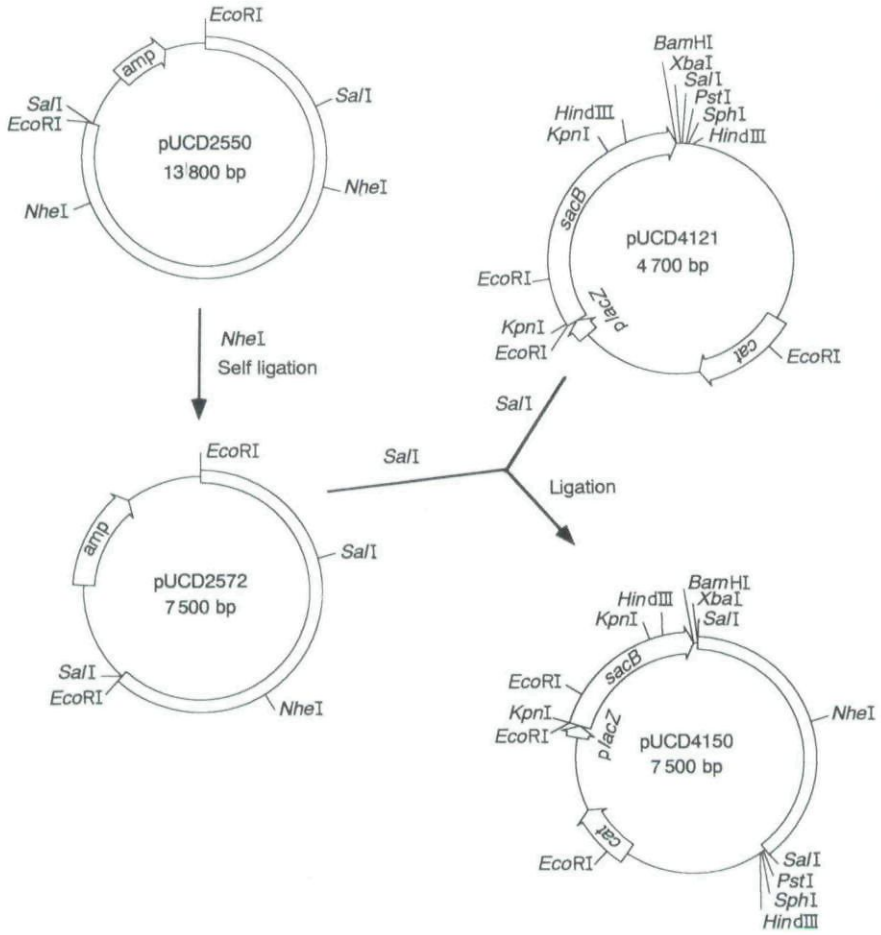

Fig. 3. Construction of deletion plasmids. Plasmids are not drawn to scale. P*lacZ* refers to the promoter of the β-galactosidase gene of pUC18. *amp* refers to the gene encoding β-lactamase that confers ampicillin resistance. *cat* refers to the gene encoding chloramphenicol acetyltransferase that confers chloramphenicol resistance. *sacB* refers to the gene encoding levansucrase that confers sucrose sensitivity.

hybridized to an 11 kb *Eco*RI fragment (Fig. 5), suggesting that the wild-type sequence was reconstituted as illustrated in Fig. 4. Deletion mutant JS50-3 was selected for further analysis and renamed JS50ET.

Analysis of the deletion mutant

Mutant JS50ET appeared to be similar to the wild-type strain in several respects, including *ex planta* growth rate, exopolysaccharide production, and colony morphology. However, JS50ET displayed a typical *hrp* phenotype, i.e. it was non-pathogenic on all tested crucifer cultivars, and non-hypersensitive on non-host plants (*Datura stramonium* and *Capsicum frutescens* Pepper California Wonder). In order to investigate whether the 6.5 kb fragment contains pathogenicity genes in addition to *hrpXc*, genetic complementation analysis of JS50ET was performed with the two cosmids, pUCD652 and pUCD660 (Table 2). Cosmid pUCD652 encompasses the entire 6.5 kb deletion, whereas pUCD660 contains an insert that overlaps about 1.5 kb with the *Nhe*I fragment and contains an entire copy of *hrpXc*. Both transformed strains JS50ET(pUCD652) and JS50ET(pUCD660) were able to induce black rot on cabbage (Early Jersey Wakefield) and hypersensitivity on *Datura*, suggesting that no other pathogenicity gene is present in the deleted region.

Discussion

We have developed a rapid four-step procedure for the introduction of directed and unmarked deletions in the bacterial chromosome and applied it to the construction of a 6.5 kb chromosomal deletion in *X. campestris*. A different and more widely used technique for the generation of unmarked deletions is the marker-exchange-eviction procedure (Ried and Collmer, 1987; 1988; Russel and Dahlquist, 1989; Blomfield *et al.*, 1991). This technique consists first of introduction in the chromosome of a cassette (usually *sacB-Neo*^{R}) by marker exchange (double cross-over) to yield an intermediate strain. Next, the manipulated (deleted) DNA fragment is also exchanged with the chromosomal cassette (by counter-selection for

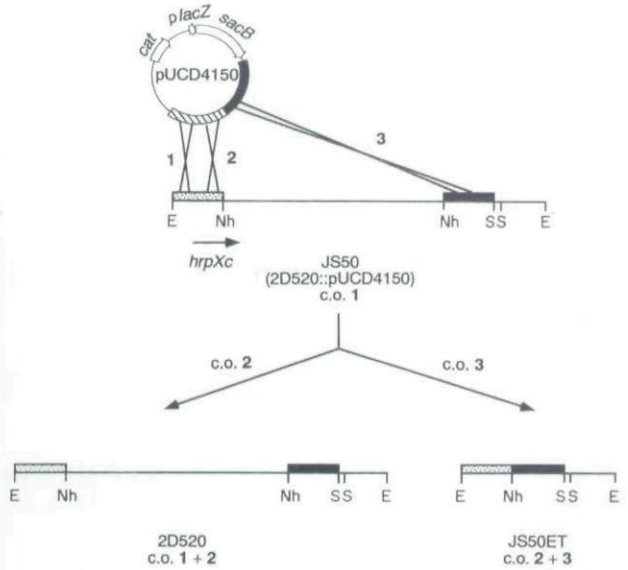

Fig. 4. Schematic representation of the integration of pUCD4150 into *X. campestris* pv. campestris and the generation of an unmarked deletion derivative. C.O. followed by a number refers to a hypothetical crossing-over. Restriction enzymes are *Eco*RI (E), *Nhe*I (N), and *Sal*I (S).

sucrose sensitivity) by means of two additional cross-overs. This multi-step marker-exchange-eviction procedure is particularly slowed down by the necessity to construct a specific intermediate strain (and thus plasmids) for each particular target gene. The procedure we describe here requires only a single plasmid construct for each mutant and is considerably faster to perform than marker-exchange-eviction, in particular because only two cross-overs are needed to obtain the unmarked deletion. However, because the success of this method relies on the two cross-overs occurring on opposite sides of the target allele, the flanking fragments in the mutagenic plasmid should be of similar lengths to avoid highly unequal recombination frequencies that would result in the two cross-overs occurring preferentially on the same side.

For years bacterial conjugation has been the only available method for introducing foreign DNA into *X. campestris* pv. campestris at a reasonable frequency (Atkins *et al.*, 1987; DeFeyter *et al.*, 1990). Therefore the electrotransformation method has been a critical factor in the development of new genetic approaches to the study of xanthomonads. As shown in Table 1, electrotransformation frequencies of the order of 10^6–10^7 transformants per μg of DNA are routinely achieved in several *X. campestris* pv. campestris strains, making it possible to select for genetic events that occur at a low frequency. We have found that 'suicidal' plasmids with inserts as small as 270 bp can be readily integrated into the *X. campestris* pv. campestris chromosome after electrotransformation and selection for the appropriate antibiotic marker of the plasmid (Table 1).

We predict that construction of unmarked deletions by this procedure, using the counter-selection vector pUCD4121 or similar constructs, can be adapted for a wide range of bacteria for the following reasons. (i) *sacB*-encoded sucrose sensitivity is expressed in a number of Gram-negative bacteria (Gay *et al.*, 1985). (ii) High-frequency chemical or electrotransformation protocols are now available for numerous bacteria (Maniatis *et al.*, 1989). (iii) pUC-derived plasmids cannot replicate extra-chromosomally in numerous bacteria, and in some widely available *E. coli* and *Salmonella typhirium polA* mutants (Chater and Hopwood, 1989; Kushner, 1987).

Although we only report here the construction of a 6.5 kb unmarked deletion, this procedure can be applied to transfer into the bacterial chromosome of any kind of manipulated mutant allele. Specifically, alleles containing single base deletions or single base mutations can be successfully transferred to the chromosome using this protocol, yielding mutant strains differing only by a unique base from the wild type (S. Kamoun and C. I. Kado, unpublished). Screening and confirmation of such mutants can be easily performed using PCR amplification technology and DNA sequencing (Erlich *et al.*, 1991). Thus, starting from a unique pUCD4121-based clone it is possible to perform a wide range of genetic manipulations and then transfer them easily to the chromosome.

Experimental procedures

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this work are described in Table 2. Plasmid pUCD4121 contains a 1.6 kb promoterless *sacB* cassette (Steinmetz *et al.*, 1985) under the transcriptional control of the *lacZ* promoter of pTZ18RC.

E. coli and *X. campestris* pv. campestris were routinely grown in LB medium or LB 1.5% agar plates (Miller, 1972) at

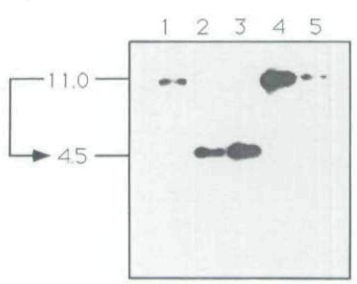

Fig. 5. Chromosomal Southern blot of wild-type and JS50 derivative strains of *X. campestris* pv. campestris. *Eco*RI-digested chromosomal DNA of 2D520 (1), JS50-3 (JS50ET) (2), JS50-15 (3), JS50-32 (4), and JS50-39 (5) were probed at high stringency with the 0.34 kb *Pst*I fragment of pUCD4109. Sizes are in kb.

Table 2. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Relevant characteristics[a]	Source/Reference
Strain		
E. coli		
JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* Δ(*lac–proAB*), [F' *traD36, proAB, lacI*^{q}ZΔM15]	Stratagene[b]
XL1-Blue	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac,* [F' *proAB, lacI*^{q}ZΔM15, Tn*10* (tet^R)]	Stratagene[b]
CC118	*araD139,* Δ(*ara,leu*)7697, Δ*lacX74, galA, phoA*Δ20, *thi, rpsE, rpoB, argE*(am), *recA1*	Manoil and Beckwith (1985)
CC202	CC118, [F42, *lacI3,* zzf-2::Tn*phoA*]	Manoil and Beckwith (1985)
X. campestris pv. campestris		
2D520	Wild-type, Rm^R	Shaw *et al.* (1988)
JS61	2D520::pUCD3161, *hrpXc,* Km(Nm)^R	This study
JS50	2D520::pUCD4150, Rm^R,Cm^R, Suc^S	This study
JS50#	Cm^S, Suc^R derivatives of JS50	This study
JS50ET	2D520 Δ6.5 kb, *hrpXc* , Rm^R	This study
2D540R	Wild-type, Rm^R	Kamoun and Kado (1990b)
HasibH-MR	Wild-type,Rm^R	Hunter *et al.* (1987)
Plasmid		
pUFR034	*ori*pSa, Km(Nm)^R	DeFeyter *et al.* (1990)
pTZ18R, pTZ19R	*ori*pUC, Ap^R	Mead *et al.* (1985)
pUCD2550	pTZ18R::*hrpXc* 11.0 kb; *Eco*RI fragment	Kamoun and Kado (1990a)
pUCD2569	pTZ19R::pUCD2550 1.5 kb; *Eco*RI–*Nhe*I fragment	This study
pBGS18	*ori*pUC, Km(Nm)^R	Spratt *et al.* (1986)
pUCD3161	pBGS18 derivative containing pUCD2569 0.34 kb *Pst*I fragment	This study
pUCD2557	pTZ18R::pUCD2550 0.64 kb; *Bam*HI fragment	This study
pUCD2583	pTZ18R::pUCD2550 3.5 kb; *Bam*HI–*Bgl*II fragment	This study
pUCD2589	pUCD2583 2.1 kb; *Bam*HI–exoIIIΔ	This study
pUCD2589K	pUCD2589::Tn*phoA*, Km(Nm)^R, Ap^S	This study
pUCD2592	pUCD2583 1.0 kb; *Bam*HI–exoIIIΔ	This study
pUCD2592K	pUCD2592::Tn*phoA*, Km(Nm)^R, Ap^S	This study
pUCD4105	pUCD2583 0.6 kb; *Bam*HI–exoIIIΔ	This study
pUCD4105K	pUCD4105::Tn*phoA*, Km(Nm)^R, Ap^S	This study
pUCD4106	pUCD2583 0.5 kb; *Bam*HI–exoIIIΔ	This study
pUCD4106K	pUCD4106::Tn*phoA*, Km(Nm)^R, Ap^S	This study
pUCD4107	pUCD2583 0.27 kb; *Bam*HI–exoIIIΔ	This study
pUCD4107K	pUCD4107::Tn*phoA*, Km(Nm)^R, Ap^S	This study
pUCD2572	pUCD2550 6.5 kb *Nhe*I deletion	This study
pTZ18RC	*ori*pUC, pTZ18R derivative, Cm^R	Brett Tyler[c]
pUC18	*ori*pUC, Ap^R	Vieira and Messing (1987)
pSAC-1	pUC18::*sacB* 1.6 kb, Ap^R, Suc^S	C. I. Kado[c]
pUCD4121	*ori*pUC, pTZ18RC::*sacB Kpn*I–*Bam*HI, Cm^R, Suc^S	This study
pUCD4150	pUCD4121::pUCD2572 3.0 kb *Sal*I fragment	This study
pUCD615	*ori*pSa, Km(Gm)^R, Ap^R	Rogowsky *et al.* (1987)
pUCD652	pUCD615-based cosmid from 2D520 gene bank	Shaw *et al.* (1988)
pUCD660	pUCD615-based cosmid from 2D520 gene bank	Shaw *et al.* (1988)

a. Km(Gm)^R designates a kanamycin resistance gene that also encodes resistance to gentamicin; Rm^R, Cm^R, and Ap^R designate rifampin-, chloramphenicol-, and ampicillin-resistance genes, respectively; Suc^S refers to the sucrose-sensitivity phenotype; *ori* and a plasmid name indicate the source of the origin of replication.

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37°C; *X. campestris* pv. campestris strains were also grown in medium 523 broth or 523 1.5% agar plates (which contains 0.1% sucrose) (Kado *et al.*, 1972). The antibiotics used for *E. coli* were kanamycin (20 μg ml^{-1}), and ampicillin (100 μg ml^{-1}); the antibiotics used for *X. campestris* pv. campestris were rifampin (50 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), ampicillin (100 μg ml^{-1}), kanamycin (30 μg ml^{-1}), and neomycin (30 μg ml^{-1}).

Enzymes and chemicals

Enzymes were obtained from Boehringer Mannheim Biochemicals or Stratagene and used as recommended by the manufacturers. All chemicals were purchased from Sigma Chemical Co.

DNA manipulations

DNA manipulations were performed as described elsewhere (Ausubel *et al.*, 1987; Maniatis *et al.*, 1989). DNA fragments were isolated from agarose gels, using a GeneClean kit (Bio101). Total genomic DNA was isolated from liquid-grown cultures using the CTAB method (Ausubel *et al.*, 1987). Alkaline transfer to Nytran Hybond N+ (Amersham) was performed as described elsewhere (Ausubel *et al.*, 1987). Hybridizations were performed at 65°C in a PR800 hybridization chamber (Hoefer Scientific Instruments) as recommended by the manufacturer.

*Tn*pho*A mutagenesis*

X. campestris pv. campestris 2D520 is naturally ampicillin resistant and kanamycin sensitive. To rapidly convert the ampicillin-resistant pUC118-, PTZ18R-, or pTZ19R- (Mead et *al.*, 1985; Vieira and Messing, 1987) based plasmids into kanamycin-resistant plasmids, the selected plasmids were mutagenized with Tn*phoA* as described elsewhere (Manoil and Beckwith, 1985). Active Tn*phoA* insertions into the β-lactamase gene were first identified as ampicillin-sensitive, kanamycin-resistant, and blue colonies on X-P supplemented LB plates. Restriction enzyme analyses confirmed the presence of the transposon in the β-lactamase gene. Since Tn*5* derivatives transpose at very low frequencies in *X. campestris* pv. campestris (Turner *et al.*, 1984), Tn*phoA* did not interfere with integration experiments.

Electroporation

Electroporation of *E. coli* and *X. campestris* pv. campestris was performed essentialy as we first described it (Kamoun and Kado, 1990a), except that the cells were grown in liquid media to an OD_{600} of 1.2, and washed twice with an ice-cold 10% glycerol solution. Electroporation was performed at 2.5 kV cm^{-1} and 200 ohms, using a Gene-Pulser (BioRad Laboratories). The cuvettes used for electroporation were routinely sterilized and reused as described elsewhere (Cooley *et al.*, 1991).

Plant assays

Plant pathogenicity and hypersensitivity assays of *X. campestris* pv. campestris were conducted as described previously (Kamoun and Kado, 1990a; Shaw and Kado, 1988).

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