A Highly Efficient Transposon Mutagenesis System for the Tomato Pathogen *Clavibacter michiganensis* subsp. *michiganensis*

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A transposon mutagenesis system for *Clavibacter michiganensis* subsp. *michiganensis* was developed based on antibiotic resistance transposons that were derived from the insertion element IS1409 from *Arthrobacter* sp. strain TM1 NCIB12013. As a prerequisite, the electroporation efficiency was optimized by using unmethylated DNA and treatment of the cells with glycine such that about 5×10^6 transformants per μg of DNA were generally obtained. Electroporation of *C. michiganensis* subsp. *michiganensis* with a suicide vector carrying transposon Tn1409C resulted in approximately 1×10^3 transposon mutants per μg of DNA and thus is suitable for saturation mutagenesis. Analysis of Tn1409C insertion sites suggests a random mode of transposition. Transposition of Tn1409C was also demonstrated for other subspecies of *C. michiganensis*.

Clavibacter is a genus of plant-pathogenic actinomycetes that belongs to the family *Microbacteriaceae*, a sister-group of the family *Micrococcaceae* (Stackebrandt et al. 1997). Currently, the genus *Clavibacter* is only represented by one species (i.e., *C. michiganensis*) with several subspecies. *C. michiganensis* subsp. *michiganensis* causes bacterial wilt and canker of tomato (*Lycopersicon esculentum* Mill.) (Davis et al. 1984; Strider 1969), *C. michiganensis* subsp. *sepedonicus* induces ring rot of potato (*Solanum tuberosum*) (Manzer and Genereux 1981), *C. michiganensis* subsp. *nebraskensis* causes wilt and blight of maize (*Zea mays*) (Schuster 1975), and *C. michiganensis* subsp. *insidiosus* is responsible for bacterial wilt and stunting of alfalfa (*Medicago sativa*) (McCulloch 1925).

In *C. michiganensis* subsp. *michiganensis* NCPPB382, two essential determinants for virulence are carried by the native plasmids pCM1 and pCM2 (Meletzus et al. 1993). These genes, encoding an endocellulase, CelA (Jahr et al. 2000), and a protein of unknown function, Pat-1 (Dreier et al. 1997), have been cloned and characterized. To be able to identify other pathogenicity-related genes that are located on the chromosome, an efficient system for mutagenesis would be bene-

ficial. Transposon mutagenesis offers the advantage that mutants can immediately be selected by the antibiotic resistance encoded by the transposon. Furthermore, mutations occur in random loci and mutated DNA sequences are physically marked by the transposon insertion, facilitating subsequent cloning and identification of the mutated gene.

Recently, we described the IS (insertion sequence) element IS1409 from the actinomycete *Arthrobacter* sp. strain TM1 NCIB12013 and the construction of a family of artificial transposons (Gartemann and Eichenlaub 2001). Transposon Tn1409 was shown to transpose randomly in *Arthrobacter* spp.; therefore, it seemed obvious to test if this was also the case in the related genus *Clavibacter*.

As the transposon is delivered on a suicide vector, the rate of transposition events directly depends on the transformation rate. A transformation rate of approximately 10^3 transformants per μg of vector DNA as obtained with a previously published procedure (Meletzus et al. 2000) yielded only between 0 and 5 transposon mutants per μg of vector DNA, which requires a tremendous effort to obtain a specific mutant. Therefore, the protocol for transformation had to be optimized to achieve a significant increase in the transformation rate. After systematic variation of all parameters that we considered to be important, we now can present an optimized method for transformation and transposon mutagenesis of *C. michiganensis* subsp. *michiganensis* using Tn1409C.

RESULTS

Optimization of the electroporation protocol.

In order to increase the rate for the introduction of heterologous DNA into *C. michiganensis* subsp. *michiganensis* by electroporation, several parameters of the standard protocol as published by Meletzus and colleagues (2000) were varied. These parameters were growth phase (early, middle, and late log phase, and stationary phase), amount of cells per transformation assay (1×10^8 to 1×10^9), amount of DNA (0.01 to 5 μg), and methylation status of DNA (isolated either from the *dam*⁻/*dcm*⁻ *Escherichia coli* strain WA337 or the *dam*⁺/*dcm*⁺ *E. coli* strain JM109). Also, the effect of electrical parameters (parallel resistances 200 to 1,000 Ω) and treatment of competent cells with cell wall-damaging reagents during growth (glycine, lysozyme, and penicillin G) or directly before elec-

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troporation (glycine) were tested. Furthermore, after electroporation, cells were treated with a heat shock, and regeneration time and the composition of the regeneration medium were altered. Optimization was done in a stepwise fashion with the *E. coli*/Clavibacter shuttle vector pDM302 (Meletzus et al. 1993) as test plasmid.

Cells harvested during early log phase (optical density [OD]₅₈₀ 0.5 to 0.7) gave a significantly higher transformation rate than cells harvested during later growth phases. The number of transformants was best with 2×10^8 cells per experiment and 1 µg of covalently closed circular (CCC) DNA per experiment. Increasing the number of cells and DNA concentration did not result in more transformants. The highest efficiency was obtained with a parallel resistance of 600 Ω. Treatment of cells with a heat shock (32 to 38°C for 6 min) directly after the pulse reduced survival and consecutively reduced transformation efficiency. A longer regeneration time (2 to 4 h) had no effect on survival or on the amount of transformants. Methylation of DNA had a significant effect on transformation efficiency. Using unmethylated DNA isolated from *E. coli* WA337 (*dam*⁻/*dcm*⁻), the number of transformants was increased fourfold in contrast to DNA from *E. coli* JM109 (*dam*⁺/*dcm*⁺) (Fig. 1).

Cell wall-damaging agents can greatly increase transformation efficiency in Gram-positive bacteria (Buckley et al. 1999; Haynes and Britz 1989, 1990). When early log phase *C. michiganensis* subsp. *michiganensis* cells were incubated for 1 h in medium with 2.5% glycine, a dramatic increase of the electroporation efficiency was observed, which yielded approximately 5×10^6 transformants per µg of unmethylated DNA (Fig. 1). Such transformation rates, which are higher than with the standard protocol by a factor 1,000, could regularly be achieved.

The final electroporation protocol for *C. michiganensis* subsp. *michiganensis* is as follows: 250 ml of tryptone broth with yeast (TBY) medium in a 1-liter Erlenmeyer flask is inoculated with cells from a fresh plate and incubated overnight at 25°C on a rotary shaker at 120 rpm. This preculture is diluted in 218 ml of TBY medium to give an OD₅₈₀ of 0.3 and incubated at the same conditions to an OD₅₈₀ of 0.6 (approximately 2 h). Afterward, 32 ml of a 20% (wt/vol) glycine stock solution is added to a final concentration of 2.5% (wt/vol) and the culture is incubated for an additional 1 h under the same conditions as before. The cells are then harvested by centrifugation at 4°C and $8,000 \times g$ for 10 min and washed with 40 ml of cold distilled water. All washing steps are performed at 0°C. After two additional washing steps with 20 ml of distilled water, the cells are washed twice with 10% (vol/vol) glycerol (in distilled water) and resuspended in 1 ml of 15% (vol/vol) glycerol (in distilled water).

For electroporation, 100 µl of these cells are mixed with 0.01 to 5 µg of CCC plasmid DNA from a *dam*⁻/*dcm*⁻ *E. coli* strain. Electroporation is performed in 0.2-cm cuvettes (Peqlab Biotechnologie, Erlangen, Germany) with 25 µF, 12.5 kV/cm, and 600 Ω, resulting in a time constant of 12 to 16 ms. Immediately after the pulse, the cells are mixed with 0.4 ml of sorbitol broth (SB) medium and incubated for 3 h at 25°C. Finally, they are plated on SB media plates containing appropriate antibiotics and incubated for 4 to 6 days at 25°C.

Protocol for transposon mutagenesis with Tn1409C.

To demonstrate transposition of the artificial *Arthrobacter* transposon Tn1409 (Gartemann and Eichenlaub 2001) in *C. michiganensis* subsp. *michiganensis*, plasmid pKGT452C was used, consisting of vector pUC13 carrying Tn1409C with the chloramphenicol exporter gene *cmx* from *Corynebacterium striatum* (Tauch et al. 1998) (Fig. 2A). Since pUC13 cannot replicate in the genus *Clavibacter*, pKGT452C comprises a suicide vector in *C. michiganensis* subsp. *michiganensis*. Two derivatives of pKGT452C (α and β), which differ in the orientation of the *cmx* gene in relation to the *mpA* gene, were tested. Plasmid DNA isolated from *E. coli* WA337 (*dam*⁻/*dcm*⁻) was electroporated into *C. michiganensis* subsp. *michiganensis* strains NCPPB382 and CMM100. After plating on SB medium containing chloramphenicol, between 500 and 1,000 resistant clones per electroporation were generally obtained when using pKGT452Cβ, whereas yields with pKGT452Cα were approximately 40 times lower.

Analysis of transposition sites.

To be a suitable tool for mutagenesis, a transposon should transpose in a random fashion. In order to gain information on the sites into which transposition of Tn1409C had occurred, Southern hybridization experiments with 75 randomly selected chloramphenicol resistant clones were performed. Total DNA was isolated from the clones and digested with *Bam*HI for hybridization against the *cmx* (Fig. 2B) and pUC13 probes. Results of these hybridizations with the *cmx* probe are exemplified in Figure 3. In all clones tested, hybridization

Fig. 1. Transformation efficiency using methylated and unmethylated heterologous DNA electroporated into *Clavibacter michiganensis* subsp. *michiganensis* after incubation with differently concentrated glycine solutions. *C. michiganensis* subsp. *michiganensis* CMM100 was electroporated using the optimized protocol with pDM302 ccc-plasmid DNA isolated from *Escherichia coli* JM109 (*dam*⁺/*dcm*⁺) or *E. coli* WA337 (*dam*⁻/*dcm*⁻) after glycine treatment. For the first two sets of entries, the absolute values are provided.

signals were obtained with fragments of different sizes, but larger than the minimal size of 1,988 bp (Fig. 2B I.), indicating a random insertion of Tn1409C. Occurrence of two hybridizing bands, as in the clone displayed in lane 23 of Figure 3, indicates a cointegrate, as the expected 4,664-bp fragment also appears in hybridizations with a pUC13 probe (data not shown). Cointegrate formation was observed only twice among the 75 clones tested. Another two clones produced two different bands with the *cmx* probe, but no band with the pUC13 probe, possibly indicating a double transposition event.

To gain information on the transposition locus and the sequence of the target duplication, 10 mutants were chosen randomly. Total DNA of these mutants was digested with *Bam*HI to clone the chromosomal region adjacent to the left inverted repeat (IRL) or digested with *Sph*I to clone the region adjacent to the right inverted repeat (IRR). The fragments were ligated into *Bam*HI- or *Sph*I-digested pUC18, and *E. coli* JM109 was transformed with the corresponding ligation mixtures. Clones that carry a hybrid pUC18 with a DNA fragment consisting of *C. michiganensis* subsp. *michiganensis* DNA and part of the transposon with the chloramphenicol resistance gene were selected on TBV plates containing ampicillin and chloramphenicol. Sequencing was done using primer ISC2 or ISN2 binding inside of the inverted repeats IRL and IRR, respectively (Gartemann and Eichenlaub 2001). The duplication of 8 bp as typical for IS1409 was confirmed in the 10 transposition mutants, as shown in Table 1. No apparent consensus sequence was found, indicating a random mode of transposition of Tn1409.

Besides integration of the transposon into housekeeping genes, one integration occurred in a protease gene. The other integration sites were located in open reading frames for hypothetical proteins.

In some rare cases, integration of the delivery vector pKGT452C by recombination occurred. This could be mediated by a 300-bp DNA region from *Arthrobacter* spp. located downstream of the *tnpA* gene outside of Tn1409 on the trans-

poson delivery vector pKGT452C β (Fig. 2A), which could not be avoided during the construction of the vector.

To test the stability of transposon mutants, one mutant was grown on a TBV plate without antibiotics over a period of 6 months with transfer to a fresh plate every 2 weeks. After 6 months, total DNA was isolated from 10 randomly chosen colonies, digested with *Bgl*II, and probed with the *tnpA* probe. No change in the band pattern compared with the original was observed after continuous growth for 6 months (data not shown), indicating that the transposon mutants obtained with Tn1409C are stable.

Mutagenesis of *C. michiganensis* subsp. *insidiosus*, subsp. *nebraskensis*, and subsp. *sepedonicus*.

Mutagenesis with Tn1409 was shown to be an effective genetic tool for *C. michiganensis* subsp. *michiganensis*; therefore, tests were performed to determine whether or not this transposon could also be used in other agroeconomically important species of *C. michiganensis* (i.e., *C. michiganensis* subsp. *insidiosus*, subsp. *nebraskensis*, and subsp. *sepedonicus*). As previously shown, a hybridization of total DNA of various *Clavibacter* strains against the internal 1-kb *Sst*I fragment of IS1409 did not give a signal (Gartemann and Eichenlaub 2001). This indicates that these strains do not carry an IS1409-like insertion element and reduces the possibility for homologous recombination with endogenous IS elements.

After electroporation with pKGT452C β DNA and selection on chloramphenicol-containing medium, resistant clones were obtained for each subspecies of *C. michiganensis* with a frequency of 2 to 16 CFU/ μ g of unmethylated DNA. Hybridization of *Nco*I-digested total DNA of randomly chosen clones of *C. michiganensis* subsp. *nebraskensis*, subsp. *sepedonicus*, and subsp. *insidiosus* against the *tnpA* probe (Fig. 2B II.) is shown in Figure 4. The pattern obtained shows that transposition took place, but the number of clones analyzed is too low to allow a statement on random or nonrandom transposition. In one case

Fig. 2. A, Physical map of the transposon delivery vector pKGT452C β . B, Physical map of a transposon insertion site in a random *Clavibacter michiganensis* mutant. I, Hybridizing bands after *Nco*I digestion of total *C. michiganensis* subsp. *michiganensis* DNA with the *tnpA* probe. II, Hybridizing bands after *Bam*HI digestion of total *C. michiganensis* subsp. *michiganensis* DNA with the *cmx* probe. Hybridizing fragments are shown in scale as lines with the corresponding size in base pairs. Probes are indicated by bars with the corresponding gene shown as an arrow; *cmx* = chloramphenicol exporter gene, *tnpA* = transposase gene, *bla* = β -lactamase gene, IRL = left inverted repeat, and IRR = right inverted repeat.

(Fig. 4, lane 5), two transposition events seem to have occurred. The experiment indicates that transposon Tn1409C should also be a valuable tool in other subspecies of *C. michiganensis*, provided that the transformation rate can be improved.

DISCUSSION

Here we describe for the first time a highly efficient system for transposon mutagenesis, based on a new, improved electroporation method, in the plant-pathogenic actinomycete genus *Clavibacter*. After the optimization of the electroporation protocol, a transformation rate of approximately 5×10^6 transformants per μg of DNA was obtained, which was the basis for high-yield transposon mutagenesis in *C. michiganensis* subsp. *michiganensis*.

Currently, only two *Clavibacter* insertion sequences are known, IS1121 of *C. michiganensis* subsp. *sepedonicus* (Gen-

Fig. 3. Hybridization of total DNA of 25 randomly chosen *Clavibacter michiganensis* subsp. *michiganensis* transposon mutants against the *cmx* probe. Total DNA of *C. michiganensis* subsp. *michiganensis* transposon mutants was digested with *Bam*HI, prepared for Southern hybridization, and hybridized against the *cmx* probe. Lane 1, negative control *C. michiganensis* subsp. *michiganensis* 382; lanes 2 to 26, randomly chosen transposon mutants; lane 27, digoxigenin-11-dUTP-labeled *Eco*RI/*Hind*III-digested lambda marker. All mutants show a single hybridization fragment greater than the expected 1,988 bp, indicating random transposition, except mutant in lane 23, which shows two hybridizing bands, indicating a double transposition event.

Bank accession no. AF079817) and IS1122 of *C. michiganensis* subsp. *insidiosus* (GenBank accession no. AF079818). IS1121 has been used for diagnostic polymerase chain reaction (Samac et al. 1998), but nothing is known whether they are functional or not. IS1237 (Laine et al. 1994) of *Leifsonia (Clavibacter) xyli* subsp. *cynodontis*, no longer a member of the genus *Clavibacter* (Evtushenko et al. 2000; Suzuki et al. 1999), belongs to the IS5 group of insertion sequences and is not functional due to frame shifts in the transposase gene (Laine et al. 1994). IS1409 from *Arthrobacter* sp. strain TM1 NCIB12013 (Gartemann and Eichenlaub 2001) was chosen for this study because of its high transposition rate, its apparent random mode of transposition, and the close relationship between genera *Arthrobacter* and *Clavibacter*.

Using the optimized transformation protocol and non-methylated DNA for electroporation, transposition events were generally obtained with a frequency of 0.5 to 1×10^3 CFU/ μg of DNA. The importance of DNA methylation for transformation efficiency was also shown for *Corynebacterium glutamicum*, in which the involvement of a restriction-modification system (Schäfer et al. 1997; Tauch et al. 1994) and the *mcr* system (Vertès et al. 1993a, 1993b) was proven.

The highest rate of transposition was achieved with the transposon construct in which the resistance gene and the transposase gene are transcribed in the same direction, which may allow a readthrough into the transposase gene from the promoter of the resistance gene.

An initial screening of a first set of mutants of *C. michiganensis* subsp. *michiganensis* already displayed several phenotypes that might be interesting for further characterization, e.g., mutants with changed surface structures (such as super smooth or rough) or changes in pigmentation (white or beige instead of yellow). Meanwhile, we have established a bank represented by 8,000 mutants that is currently analyzed with the goal to identify genes relevant for the plant-pathogen interaction.

We could also show that transposon Tn1409C can be used for mutagenesis in other subspecies of *C. michiganensis* (i.e., subsp. *nebraskensis*, subsp. *sepedonicus*, and subsp. *insidiosus*); however, an optimization of the transformation protocol for each subspecies is necessary to provide higher transposition rates.

It is worthwhile to mention that the optimized transformation protocol is positively affecting all methods that depend on the transformation rate. Especially, replacing a wild-type gene with an insertionally inactivated one as a method of test-

Table 1. Target duplication and homologies of interrupted DNA region of 10 randomly chosen *Clavibacter michiganensis* subsp. *michiganensis* NCPPB382 transposon mutants

Mutant	8-bp Target duplication	Putative function or ORF ^a	GC content (%) ^b	Identities/positives (%)	Acc. no. ^c
23-39	GGTTTCAG	?	61	...	...
34-41	GGTTGTAG	Oxidoreductase	72	44/63	NP_104615
75-11	CCGAATCG	ABC-transporter, ATP-binding protein	74	45/59	AAF12012
94-17	GGTAATCA	?	57	...	...
116-27	ACTTTACC	β -Xylo-glucosidase	65	45/61	CAA91219
119-38	ACAAAATG	?	74	...	...
137-40	CGTCAACC	Phosphate ABC-transporter, ATP-binding protein	53	69/87	CAB88472
303-3 ^d	AATTTAGT	?	56	...	...
C4 ^d	AGTTTAGA	?	57	...	...
C6 ^d	AGTAAACC	Hypothetical protein (<i>Xylella fastidiosa</i>)	56	50/68	AAF83341

^a ORF = open reading frame and ? = no significant homologies.

^b GC content of interrupted gene/DNA region.

^c Accession number of the best homology.

^d Only one side sequenced.

ing the phenotype of a hypothetical gene now becomes possible in *C. michiganensis* subsp. *michiganensis*. This is one of the prerequisites for a functional analysis of genes, for example, identified in a genome project.

MATERIALS AND METHODS

Media and growth conditions.

Bacterial strains and plasmids used and constructed in this study are listed in Table 2. *E. coli* was grown on TBV (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 5 g/liter,

pH 7.5) at 37°C. *Clavibacter* strains were grown at 25°C on the rich media TBV or nutrient broth yeast extract (NBVE; nutrient broth at 8 g/liter, yeast extract at 5 g/liter, pH 7.5).

For growth of recombinant *E. coli* strains bearing pKGT452C α and β , media contained ampicillin (150 μ g/ml) and chloramphenicol (10 μ g/ml). After electroporation of pKGT452C derivatives into *Clavibacter* strains, they were grown on SB medium (tryptone at 10 g/liter, yeast extract at 5 g/liter, NaCl at 4 g/liter, 0.5 M sorbitol, 20 mM MgCl₂, 20 mM CaCl₂, pH 7.5) with addition of chloramphenicol (10 μ g/ml) and restreaked on TBV plates containing chloramphenicol (10 μ g/ml).

DNA isolation, DNA manipulation, and DNA transfer.

Plasmid DNA used for sequencing and electroporation was isolated and purified by Qiagen columns as specified by the manufacturer (Qiagen, Hilden, Germany). Preparation of total DNA for hybridization experiments was done according to Hopwood and colleagues (1985).

DNA restriction, ligation, and transformation were carried out following standard procedures (Sambrook et al. 1989), and electroporation into *E. coli* following the Gene Pulser (Bio-Rad Laboratories, Krefeld, Germany) manual.

Electroporation.

Based on the electroporation procedure of Meletzus and colleagues (2000), an optimized protocol was developed.

Southern blot analysis.

In Southern hybridizations, the *cmx* gene (Tauch et al. 1998) specifying the chloramphenicol exporter from transposon Tn1409 and the *tnpA* gene encoding the transposase were used as probes. A 2.2-kb *SphI/BglII* fragment of pKGT452C β , carrying the complete *cmx* gene, and two *BamHI* fragments of 864 and 883 bp, carrying the complete *tnpA* gene, were digoxigenin-11-dUTP labeled with the Random Primed DNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany).

pUC13 was labeled with digoxigenin-11-dUTP by nick translation (Sambrook et al. 1989) and used as a probe to detect integration of the whole suicide vector. Digested chromosomal and plasmid DNA samples were separated on 0.7 to

Fig. 4. Hybridization of total DNA of transposon mutants of several *Clavibacter michiganensis* subspecies against the *tnpA* probe. Total DNA of *C. michiganensis* subsp. *insidiosus* (lanes 3 to 6), *C. michiganensis* subsp. *nebraskensis* (lanes 7 to 10), *C. michiganensis* subsp. *sepedonicus* Cs7 (lanes 11 to 14), *C. michiganensis* subsp. *sepedonicus* Cs4 (lanes 15 and 16), and *C. michiganensis* subsp. *michiganensis* (negative control, without transposon, lane 2) was digested with *NcoI* and hybridized against the *tnpA* probe. Lanes 1 and 17, digoxigenin-11-dUTP-labeled *EcoRI/HindIII*-digested lambda marker. The expected band pattern with a constant hybridizing fragment of 1,540 bp (marked with an arrow) and one variable fragment larger than 625 bp was achieved for *C. michiganensis* subsp. *insidiosus*, subsp. *nebraskensis*, and subsp. *sepedonicus*. The band pattern in lane 5 (*C. michiganensis* subsp. *insidiosus*) may indicate that two transposition events occurred in this mutant.

Table 2. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype ^a	Reference or source
Strains		
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> NCPPB382	pCM1, pCM2	NCPPB ^b
<i>C. michiganensis</i> subsp. <i>michiganensis</i> CMM100	Curing derivative of <i>C. michiganensis</i> subsp. <i>michiganensis</i> NCPPB382, plasmid free	Meletzus et al. 1991
<i>C. michiganensis</i> subsp. <i>insidiosus</i> NCPPB1109	...	NCPPB
<i>C. michiganensis</i> subsp. <i>nebraskensis</i> NCPPB2597	...	NCPPB
<i>C. michiganensis</i> subsp. <i>sepedonicus</i> Cs4	...	Laine et al. 1996
<i>C. michiganensis</i> subsp. <i>sepedonicus</i> Cs7	...	Laine et al. 1996
<i>Escherichia coli</i> JM109	F <i>traD</i> 36 <i>lacF</i> , $\Delta(lacZ)$ M15, <i>proAB/recA1 recA1 endA1</i> <i>gyrA96</i> (Nal ^r) <i>thi hsdR17</i> (r_K^+ m_K^+) <i>supE44 relA1</i> $\Delta(lac-proAB)$	Yanisch-Perron et al. 1985
<i>E. coli</i> WA337	F ⁻ <i>dcm-6 dam-3 met</i> β 1 <i>galK2</i> , <i>galT22</i> , <i>lacY1</i> , <i>tsx-78</i> , <i>supE44</i>	W. Wackernagel, University of Oldenburg, Oldenburg, Germany
Plasmids		
pUC13	Ap ^r ; cloning vector	Vieira et al. 1982
pUC18	Ap ^r ; cloning vector	Yanisch-Perron et al. 1985
pDM302	<i>E. coli/Clavibacter</i> shuttle vector, Cm ^r , Nm ^r	Meletzus et al. 1993
pKGT452C α	Tn1409C α ; <i>tnpA</i> and <i>cmx</i> in different orientations	Gartemann et al. 2001
pKGT452C β	Tn1409C β ; <i>tnpA</i> and <i>cmx</i> in the same orientation	Gartemann et al. 2001

^a Ap^r = ampicillin resistance, Cm^r = chloramphenicol resistance, and Nm^r = neomycin resistance.

^b NCPPB = National Collection of Plant Pathogenic Bacteria, U.K.

1.0% agarose gels and transferred to a nylon membrane (SS Nytrans 0.2; Schleicher & Schuell, Dassel, Germany) using a vacuum blot apparatus (Vacugene; Pharmacia, Freiburg, Germany). Hybridizations were carried out at 68°C overnight in a buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.02% sodium dodecyl sulfate (SDS), 0.1% Na-laurylsarcosyl, and 2% blocking reagent (Boehringer Mannheim). The nylon membrane was washed twice in 0.1× SSC, 0.1% SDS at 68°C for 20 min each time. Detection was carried out as recommended by the manufacturer (Boehringer Mannheim).

DNA sequencing.

Sequencing was done by the IIT-Biotech GmbH (Bielefeld, Germany) using an ABI prism 377 apparatus (Applied Biosystems, Foster City, CA, U.S.A.).

Clones for sequencing were prepared by subcloning. The synthetic oligonucleotide primers ISC2 and ISN2 (Gartemann and Eichenlaub 2001) were used to determine the insertion sites of Tn1409 and the target duplication. The sequence of interrupted genes was determined by primer walking using synthetic oligonucleotide primers obtained from Sigma-ARK (Darmstadt, Germany). DNA and derived protein sequences were compared with database entries using the BLAST programs (Altschul et al. 1997) at the server of the National Center of Biotechnology Information.

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