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Transformation of *Rhodococcus fascians* by High-Voltage Electroporation and Development of *R. fascians* Cloning Vectors

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The analysis of the virulence determinants of phytopathogenic *Rhodococcus fascians* has been hampered by the lack of a system for introducing exogenous DNA. We investigated the possibility of genetic transformation of *R. fascians* by high-voltage electroporation of intact bacterial cells in the presence of plasmid DNA. Electrotransformation in *R. fascians* D188 resulted in transformation frequencies ranging from 10^5/μg of DNA to 10^7/μg of DNA, depending on the DNA concentration. The effects of different electrical parameters and composition of electroporation medium on transformation efficiency are presented. By this transformation method, a cloning vector (pRF28) for *R. fascians* based on an indigenous 160-kilobase (chloramphenicol and cadmium resistance-encoding) plasmid pRF2 from strain NCPPB 1675 was developed. The origin of replication and the chloramphenicol resistance gene on pRF28 were used to construct cloning vectors that are capable of replication in *R. fascians* and *Escherichia coli*. The electroporation method presented was efficient enough to allow detection of the rare integration of replication-deficient pRF28 derivatives in the *R. fascians* D188 genome via either homologous or illegitimate recombination.

Rhodococcus fascians (27) is a gram-positive actinomycete that causes witches'-broom or leafy galls on dicotyledonous plants (26) and malformations of the bulbs of monocotyledonous plants (e.g., members of the family *Liliaceae*) (10, 19, 28).

Large plasmids such as pRF1 (70 kilobases [kb]), pRF2 (160 kb), pD188 (138 kb), and pRF3 (140 kb) were detected in *R. fascians* pD20, NCPPB 1675, D188, and NCPPB 1488, respectively (J. Desomer, unpublished results). Heavy metal (cadmium) or antibiotic (chloramphenicol) resistance, or both, could be assigned to some of these plasmids by curing and conjugation experiments (8). However, no correlation was observed between the presence of these plasmids and virulence towards *Nicotiana tabacum* (7).

In order to isolate and study further the genes determining the disease symptoms caused by *R. fascians*, suitable cloning vectors and a system for introducing exogenous DNA into this bacterium had to be developed.

Two analogous systems have been described for the genus *Rhodococcus*. The first is based on polyethylene glycol (PEG)-mediated transfection of *Rhodococcus erythropolis* protoplasts by DNA of φEC, a lysogenic actinophage (2). The second system uses *Escherichia coli-Rhodococcus* shuttle vectors based on a cryptic *Rhodococcus* sp. plasmid (pMSV3000), pBR322, and the thiostrepton resistance gene of *Streptomyces azureus*. The method of transformation is also PEG-mediated DNA uptake into *Rhodococcus* sp. protoplasts (29).

Although this procedure can be applied successfully to different *Rhodococcus* species, *R. fascians* remains refractory to this approach of transformation in our laboratory. We chose to develop our own vectors based on an indigenous 160-kb plasmid, pRF2, from *R. fascians* NCPPB 1675 encoding chloramphenicol resistance (Cm^r). High-voltage electroporation, a quick and reproducible alternative that has been employed for many other bacterial species (for a review, see reference 5), was used as the transformation method. After the conditions for electrotransformation were optimized, bifunctional vectors that could replicate in both *R. fascians* and *E. coli* were constructed and the Cm^r gene and the origin of replication on these vectors were located.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2, respectively. *E. coli* strains were grown at 37°C in Luria broth (LB) (21), while *R. fascians* strains were cultured at 28°C in either LB or yeast extract broth (YEB) (21).

Selective antibiotic concentrations for *R. fascians* strains were as follows: streptomycin, 100 μg/ml; erythromycin, 100 μg/ml; chloramphenicol, 25 μg/ml; phleomycin, 1.5 μg/ml; and cadmium nitrate, 27.5 μg/ml (0.001% Cd). For *E. coli*, 200 μg of triacillin per ml was used.

Plasmid isolation. Small-scale plasmid preparations from *E. coli* were performed as described by Birnboim and Doly (1). Large-scale preparations from *E. coli* were also done by the alkaline lysis method, as modified by Maniatis et al. (17).

For large-scale plasmid preparations from *R. fascians*, 2 liters of LB was inoculated and incubated with shaking for 40 h at 28°C. The cells were pelleted at 9,000 × *g*, washed with 100 ml of TE buffer (50 mM Tris hydrochloride, 20 mM EDTA [pH 7]), and resuspended in 400 ml of 20% PEG solution in TE buffer. One hundred milligrams of lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was incubated at 37°C for 2 to 4 h. The cells were pelleted at 9,000 × *g*, resuspended in 10 ml of TE buffer, and treated according to the alkaline lysis method described for *E. coli* (17).

Electroporation conditions. The following optimized protocol was devised from different experiments described in Results. *R. fascians* cells to be electroporated were grown in 0.5 liter of LB at 28°C on a gyratory shaker (150 rpm), harvested in mid-log phase (9,000 × *g*), washed once with demineralized water, and concentrated 50-fold in a 30% PEG 1000 solution in demineralized water. These cells were either used directly or stored in small aliquots at −80°C.

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TABLE 1. Bacterial strains used in this study

Species and strain	Characteristics	Reference
R. fascians		
D188	Cd^r, contains plasmid pD188 (138 kb)	8
D188-1	Cd^s, plasmid-free mutant	8
D188-5	Cd^s Str^r Ery^r, plasmid-free mutant	8
NCPPB 1675	Cd^r Cm^r, contains plasmid pRF2 (160 kb)	8
E. coli		
MC1061	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK hsrR hsrM rpsL*	3
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA relA1*	13

Four hundred microliters of ice-cold cells was mixed with the DNA of interest in a 2-mm-gapped electrocuvette (Bio-Rad Laboratories, Richmond, Calif.) and subjected to a 2.5-kV electric pulse from a Gene Pulser (Bio-Rad) connected to a pulse controller (25-μF capacitor; external resistance, 400 Ω). Pulsed cells were diluted immediately with 0.6 ml of YEB and incubated for 4 h at 28°C, after which they were spread on YEB plates containing appropriate antibiotics. Survival ratios were estimated from serial dilutions plated on media without antibiotics. Transformants could be scored after 5 to 7 days. Each set of experiments contained controls in which either the transforming DNA or the electric pulse was omitted.

Hybridization conditions. DNA fragments were transferred from agarose gels to nylon membranes as described by Southern (25). Hybridization conditions with ^{32}P-labeled probes, prepared by nick translation (24) or by the multiprime reaction (11, 12), were as recommended by Amersham Corp., Arlington Heights, Ill.

Restriction endonuclease reactions and ligations. DNA was digested with restriction enzymes according to the recommendations of the supplier (Boehringer GmbH, Mannheim, Federal Republic of Germany; New England BioLabs, Inc., Beverly, Mass.; or Bethesda Research Laboratories, Gaithersburg, Md.) or the method of O'Farrell et al. (23). Ligation of DNA fragments was performed as described elsewhere (17).

RESULTS

Identification of an in vivo-deleted pRF2 derivative. The first part of the construction of shuttle vectors consisted of reducing the 160-kb plasmid pRF2 from *R. fascians* NCPPB 1675, coding for Cm^r and cadmium resistance (Cd^r) (8), to a suitably sized, autonomously replicating plasmid still encoding at least one of these types of resistance. Initial attempts to transform *R. fascians* D188 cells by electroporation (at 2.5 kV in 4-mm-gapped cuvettes) in the presence of pRF2 DNA yielded four Cm^r transformants. Three harbored the original pRF2 plasmid, as demonstrated by small-scale plasmid preparation followed by restriction enzyme digestion. The fourth transformant, however, contained a new 50-kb plasmid (designated pRF21). The resident plasmid, pD188, was not found in any of the four transformants. Southern hybridizations of pRF21, with labeled plasmid pRF2 DNA as a probe, revealed that all pRF21 sequences hybridized with pRF2, whereas most restriction fragments of pRF21 were common to both plasmids. These data suggested that pRF21 was an in vivo deletion mutant of pRF2. When pRF21 was used in subsequent electrotransformation of D188-5 cells (a plasmid-free D188 derivative), Cm^r transformants were tested for cadmium resistance. All of these colonies were cadmium susceptible (Cd^s), indicating that the deletion generating pRF21 from pRF2 removed the cadmium resistance genes.

In vitro deletion of pRF21. The plasmid pRF21 was reduced in size by two consecutive partial digestions followed by religation and electrotransformation. As shown in Fig. 1A, *Xba*I generated only four fragments in pRF21. Therefore, this enzyme was used to partially digest the plasmid, followed by religation of the DNA. *R. fascians* D188-5 cells were transformed by the ligation mixture, and Cm^r transformants that contained deletion mutants pRF22 and pRF23, which lacked *Xba*I fragment 2 and fragment 4, respectively, were identified (Fig. 1A).

Although pRF22 (35 kb) was substantially smaller than pRF21, it was still too large to be an efficient vector. To reduce the size of pRF22 further, this plasmid was partially digested with *Bam*HI, religated, and used to transform D188-5 cells. Five different deleted plasmids could be distinguished (pRF24, pRF25, pRF26, pRF27, and pRF28 [Fig. 1B]). Since pRF28 was the smallest of all the isolated plasmids, it was chosen to be further characterized in order to establish a vector that could replicate in both *E. coli* and *R. fascians*.

Foreign sequences were inserted into unique restriction sites as a first approach to determine which ones could be used for cloning without disturbing either the replication or the chloramphenicol resistance.

When *Sma*I-linearized pUC13 was inserted into the unique *Stu*I site of pRF28 (generating pRF29) or when *Xba*I-linearized pUC13 was inserted into the unique *Xba*I site (resulting in pRF30 and pRF31) (Fig. 2), chimeric plasmids in *E. coli* that could transform *R. fascians* D188-5 cells at high frequency to Cm^r (Table 3) were obtained. Plasmid analysis of these transformants showed intact autonomously replicating pRF29, pRF30, or pRF31 that could retransform *E. coli* MC1061 to ampicillin resistance (Ap^r).

These plasmids can be readily obtained from *E. coli* in large amounts, and therefore one (pRF29) was used to optimize the conditions of electrotransformation of *R. fascians*.

Optimization of electrotransformation efficiencies. Figure 3 shows the effects of different experimental variables on transformation efficiency with pRF29 as transforming DNA. Transformation efficiencies increased with increasing voltages of the applied pulses, with the highest transformation being obtained at 12 kV/cm regardless of the electroporation buffer used. Adding 30% PEG 1000 to the cells resulted in three- to fivefold higher transformation efficiencies compared with those obtained by using water as the electroporation medium (Fig. 3A). Moreover, cell survival at 12 kV/cm increased from 50% in water to 100% in 30% PEG 1000 (Fig. 3B). Cells suspended in 30% PEG 1000 could be frozen at −70°C for several weeks and used for electrotransformation after thawing. No striking effect on transformation efficiencies was observed when the different external resistances of the pulse controller were used, except between 100 and 200 Ω (Fig. 3C).

In contrast, the transformation efficiencies were greatly influenced by the DNA concentration. When all electrical parameters were maintained at optimized settings and the DNA concentration varied between 0.001 and 10 μg/ml, the number of transformants increased in the DNA concentra-

TABLE 2. Plasmids used in this study

Plasmid(s)	Size (kb)	Antibiotic resistance	Description of isolation	Reference
pGR-1	5.7	Ap^r (*E. coli*)	pGEM2 containing the 3-kb bleomycin resistance-encoding *Bam*HI fragment from *S. verticillus*	G. De la Riva (unpublished data)
pJGV4	8.1	Ap^r Km^r (*E. coli*); Km^r (*Bacillus subtilis*)	pBR322 and pUB110 joined at their *Bam*HI sites, followed by removal of one *Bam*HI site and insertion of *cos* site of λ	J. Desomer (unpublished data)
pJGV121	10.5	Ap^r Km^r (*E. coli*)	Deletion mutant of pJGV12106, leaving a 2.5-kb sequence homologous to the *R. fascians* D188 genome inserted in pJGV4	This work
pJGV12106	38	Ap^r Km^r (*E. coli*)	Clone selected from a *R. fascians* D188 (partially *Sau*3A) cosmid library cloned in pJGV4	J. Desomer (unpublished data)
pMSA-1	11.7	Bleomycin resistance (*Streptomyces lividans*)	pIJ702 containing a 5.9-kb bleomycin resistance-encoding *Sst*I fragment from *S. verticillus*	J. Davies and M. Sugiyama (unpublished data)
pRF2	160	Cm^r Cd^r (*R. fascians*)	Plasmid isolated from strain NCPPB 1675	8
pRF21	50	Cm^r (*R. fascians*)	Deletion mutant of pRF2 obtained after electrotransformation of D188	This work
pRF22	35	Cm^r (*R. fascians*)	In vitro deletion of *Xba*I fragment 2 of pRF21	This work
pRF23	48.3	Cm^r (*R. fascians*)	In vitro deletion of *Xba*I fragment 4 of pRF21	This work
pRF24	17.5	Cm^r (*R. fascians*)	In vitro deletion of *Bam*HI fragments 2, 4, 5, and 7 of pRF22	This work
pRF25	14.5	Cm^r (*R. fascians*)	In vitro deletion of *Bam*HI fragments 2, 4, 5, 6, and 7 of pRF22	This work
pRF26	15.5	Cm^r (*R. fascians*)	In vitro deletion of *Bam*HI fragments 2, 4, 5, 7, and 9 of pRF22	This work
pRF27	18	Cm^r (*R. fascians*)	In vitro deletion of *Bam*HI fragments 2, 4, 6, and 7 of pRF22	This work
pRF28	10.5	Cm^r (*R. fascians*)	In vitro deletion of *Bam*HI fragments 2, 3, 4, 5, 7, 8, and 9 of pRF22	This work
pRF29	13.2	Ap^r (*E. coli*); Cm^r (*R. fascians*)	Cointegrate of pRF28 (*Stu*I linearized) and pUC13 (*Sma*I linearized)	This work
pRF30, pRF31	13.2	Ap^r (*E. coli*); Cm^r (*R. fascians*)	Cointegrates of pRF28 (*Xba*I linearized) and pUC13 (*Xba*I linearized) (both orientations)	This work
pRF32, pRF33	13.2	Ap^r (*E. coli*); Cm^r (*R. fascians*)	Cointegrates of pRF28 (*Bgl*II linearized) and pUC18 (*Bam*HI linearized) (both orientations)	This work
pRF34, pRF35	13.2	Ap^r (*E. coli*); Cm^r (*R. fascians*)	Cointegrates of pRF28 (*Hin*dIII linearized) and pUC18 (*Hin*dIII linearized) (both orientations)	This work
pRF36	16.2	Ap^r (*E. coli*); Cm^r bleomycin resistance (*R. fascians*)	Cointegrate of *Xba*I-linearized pRF28 and *Xba*I-linearized pGR-1	This work
pRF37	10.8	Ap^r (*E. coli*); bleomycin resistance (*R. fascians*)	pUC13 containing the 5.1-kb *Bam*HI-*Xba*I fragment of pRF28 and the 3-kb bleomycin resistance-encoding *Bam*HI fragment from *S. verticillus*	This work
pRF38	11.1	Ap^r (*E. coli*); bleomycin resistance (*R. fascians*)	pUC13 containing the 5.4-kb *Bam*HI-*Stu*I fragment of pRF28 and the 3-kb bleomycin resistance-encoding *Bam*HI fragment from *S. verticillus*	This work
pRF39	10.3	Ap^r (*E. coli*)	Deletion of 0.8-kb *Pst*I fragment of pRF38	This work
pRF40	21	Ap^r Km^r (*E. coli*); Cm^r (*R. fascians*)	Cointegrate of *Bgl*II-linearized pRF28 and *Bam*HI-linearized pJGV121	This work
pRF41	6.1	Ap^r (*E. coli*)	pUC18 containing the 3.4-kb *Xba*I-*Xho*I fragment of pRF28	This work
pRF42	8.5	Ap^r (*E. coli*)	pUC9 containing *Bam*HI fragment 1 of pRF28	This work
pRF43	8.4	Ap^r (*E. coli*)	pUC13 containing the 5.4-kb *Bam*HI-*Stu*I fragment and the adjacent *Bam*HI fragment 4 of pRF28	This work
pRF44	13.3	Ap^r (*E. coli*); Cm^r (*R. fascians*)	pRF41 containing a 7.2-kb *Xba*I fragment homologous to the *R. fascians* D188 genome	This work
pRF45	13	Ap^r (*E. coli*)	pJGV121 containing the 2.4-kb *Hin*dIII fragment of pRF41 (second *Hin*dIII site in polylinker)	This work
pUC9, pUC13, pUC18	2.7	Ap^r *lacZ*		30

tion range of 0.001 to 0.1 μg/ml and remained practically constant in the range of 0.1 to 10 μg/ml. The transformation efficiency, expressed as the number of CFU normalized to 1 μg of DNA, however, dropped from 2.5×10^7 to 4.3×10^4 CFU/μg (Fig. 3D).

Localization of the origin of replication in pRF28. As mentioned above, inserting pUC13 at either the *Xba*I or the *Stu*I restriction site of pRF28 did not abolish its replication in *R. fascians*.

Cloning of pUC18 in the unique *Bgl*II site (pRF32 and

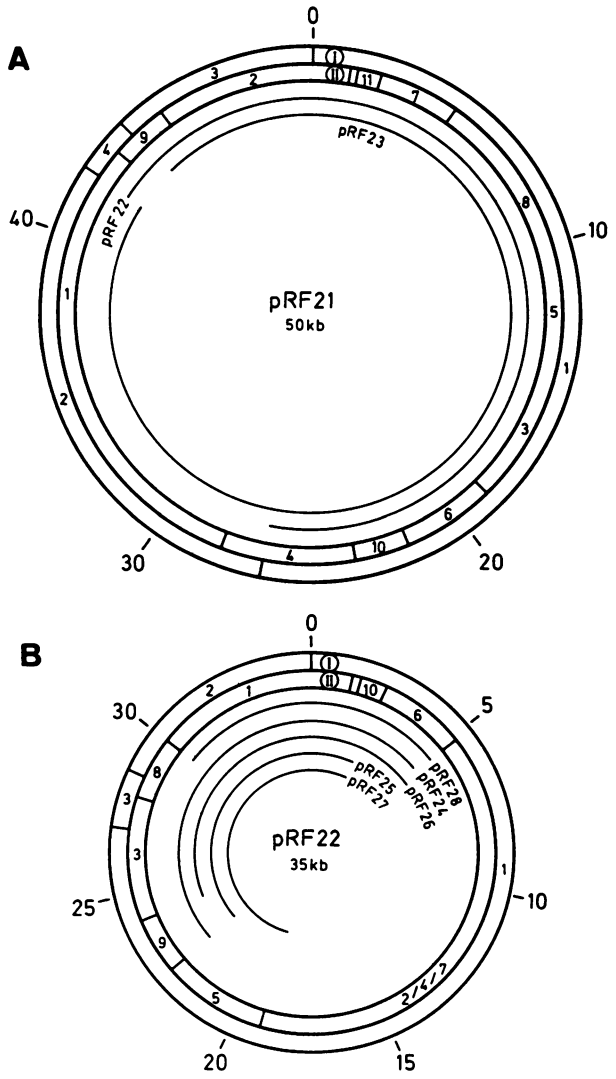

FIG. 1. Circular restriction fragment maps of pRF21 (A), pRF22 (B), and their derivatives. I, *Xba*I restriction fragment map; II, *Bam*HI restriction fragment map. The arcs represent the portions of the plasmids pRF21 and pRF22 still present in the various deleted plasmids. The numbers outside the circles indicate the scale in kilobases; the ones inside the circles are the *Xba*I or *Bam*HI fragment numbers ordered by descending molecular weight.

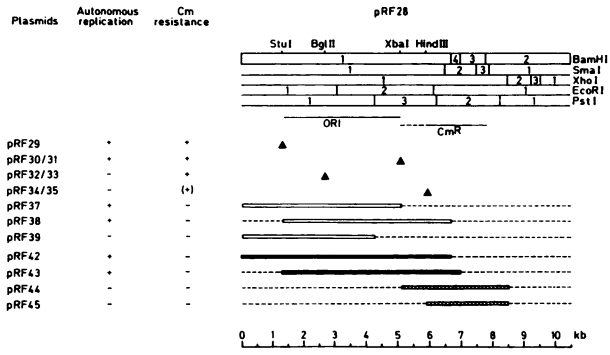

FIG. 2. Linear restriction fragment map of pRF28 and localization of the chloramphenicol resistance gene and origin of replication. The restriction enzymes indicated at the top of the map have unique cleavage sites in pRF28. Restriction fragments are numbered by descending molecular weight. The lines under the map represent the smallest portions sufficient for the origin of replication (ORI) or for chloramphenicol resistance (Cm^r). The triangles represent the different sites where pUC13 or pUC18 was inserted, and the bars represent the regions of pRF28 cloned in pUC9 (■), in nonreplicating vectors with homology to the *R. fascians* genome (▨), or in pUC13 containing the bleomycin resistance gene (□). More details of the construction of the pRF28 derivatives can be found in Table 2 and the text. (+), Low Cm^r transforming capacity.

pRF33) or in the *Hin*dIII site (pRF34 and pRF35) of pRF28, however, generated recombinant plasmids in *E. coli* that allowed transformation of D188-5 to Cm^r only at low frequencies (30 transformants per μg of DNA for pRF32; a single isolate for pRF34) (Fig. 2; Table 3). Moreover, these colonies typically appeared 2 to 3 days later than Cm^r transformants obtained by electroporation of D188-5 in the presence of pRF29 DNA.

These Cm^r transformants were analyzed for their plasmid content, but none of them contained autonomously replicating plasmids. Southern hybridizations of *Bgl*II-digested total DNA of D188-5 Cm^r transformants with pRF32 to ^{32}P-labeled pRF28 revealed single high-molecular-size bands (>13 kb) that differed between transformants after autoradiography (Fig. 4). Since pRF32 contains no *Bgl*II recognition site, this result indicated that pRF32 (or part of it) had integrated at different locations in the *R. fascians* genome in different transformants. No obvious homology between D188-5 chromosomal sequences and pRF28 or either of its derivatives could be detected by Southern hybridization (Fig. 4). Therefore, we presumed that integration occurred through short stretches of homology or by illegitimate recombination.

For further localization of the origin of replication, a second antibiotic resistance gene had to be introduced, allowing us to distinguish deletions that removed replication sequences from those that deleted the chloramphenicol resistance gene. It seemed most appropriate to test whether *Streptomyces* antibiotic resistance genes functioned when introduced on pRF28 into *R. fascians*, since the genus *Rhodococcus* belongs to the class *Actinomycetes*. Therefore, a cointegrate plasmid (pRF36) was constructed in *E. coli* between pRF28 and pGR1 at their respective *Xba*I sites. pGR1 contains a 3,000-base-pair *Bam*HI fragment of *Streptomyces verticillus* conferring resistance to the antibiotics bleomycin and phleomycin (M. Sugiyama and J. Davies, unpublished results). Plasmid pRF36 transformed D188-5 cells to either chloramphenicol or phleomycin resistance, whereas the control plasmid, pRF30, conferred resistance only to chloramphenicol. The bleomycin resistance gene could thus be used as a second marker gene in the localization of the replication origin.

The *Bam*HI fragment encoding bleomycin resistance was subsequently cloned in pUC13 containing the 5.1-kb *Bam*HI-*Xba*I fragment of pRF28 (spanning the *Bgl*II site), yielding pRF37. The same fragment was also cloned in pUC13 containing the 5.4-kb *Stu*I-*Bam*I fragment of pRF28 (also spanning the *Bgl*II site), yielding pRF38.

Both pRF37 and pRF38, prepared from *E. coli*, could be used to electrotransform strain D188-5 to phleomycin resistance (Table 3). Plasmid analysis of these transformants showed autonomously replicating plasmids in both cases, demonstrating that the common 3.8-kb *Stu*I-*Xba*I fragment was sufficient for autonomous replication of pRF28 and its different constructed derivatives. Removal of the 800 base pairs up to the *Pst*I site located at the left of the *Xba*I site

TABLE 3. Electroporation of *R. fascians*: transformation efficiencies with different plasmids or *R. fascians* strains[a]

Strain	Transforming DNA		Cell density (CFU/ml)	Time constant (ms)	No. of transformants (CFU/μg of DNA)[b]
	Plasmid	Concn (μg/ml)			
D188-5	pMSA-1	1	2.5×10^9	4.5	<10
D188	pRF29	1	5.7×10^9	4.0	6.6×10^4
D188-5	pRF29	1	1.5×10^9	4.1	1.7×10^5
D188-5	pRF30	2.5	2.5×10^9	4.3	5.0×10^3
D188-5	pRF32	2.5	2.5×10^9	4.1	3.0×10^1
D188-5	pRF34	2.5	1.5×10^9	4.0	Single isolate
D188-5	pRF37	1	2.5×10^9	4.2	0.5×10^3
D188-5	pRF38	1	2.5×10^9	4.2	1.0×10^3
D188	pRF44	2.5	2.5×10^9	4.7	2.0×10^2

[a] Electroporation buffer was 30% PEG 1000. Electrical parameters were as follows: E_0, 12,000 V/cm; resistance, 400 Ω; and capacitance, 25 μF.
[b] Transformants were selected on YEB plates containing chloramphenicol (25 μg/ml), except when transforming DNA was pMSA-1, pRF37, or pRF38 (selected on YEB plates containing 1.5 μg of phleomycin per ml).

(orientation as in Fig. 2) in pRF37 yielded a plasmid (pRF39) that could no longer transform *R. fascians* to phleomycin resistance. These results were in agreement with the insertional inactivation of the replication at the *Bgl*II site described above. As yet, these results provide no explanation for the very low frequency of transformation by a pRF28 derivative having an insertion at the *Hin*dIII site, but this is discussed further below.

In the next experiment, pMSA-1 (containing the bleomycin resistance gene cloned as an *Sst*I fragment in pIJ702) was used in a electrotransformation of D188-5 to test whether a *Streptomyces* plasmid (pIJ702) could replicate in *R. fascians*. No phleomycin resistance transformants were obtained (Table 3), although the selectable marker functioned in *R. fascians*. These data indicated that pIJ702 was unable to replicate in *R. fascians*.

Incompatibility between pRF28 and pD188. *R. fascians* D188 cells were electrotransformed by pRF28 or its derivatives 4 to 10 times less efficiently than D188-5 cells (Table 3). Also, the Cm^r transformants obtained in the initial electroporations of D188 cells in the presence of the original plasmid, pRF2, appeared to have lost their resident plasmid, pD188. When D188(pRF28) was tested for Cd^r (encoded by pD188), all colonies were found to be Cd^s, indicating that pD188 was also lost in this case. In a control mock electroporation of D188 cells, 25% of the surviving cells were still Cd^r. These data suggested that electroporation as such seemed to have a curing effect on pD188, but the remaining difference must be accounted for by the introduction of pRF28 (or pRF2). A region of high homology, shared by pD188 and pRF28 (or pRF2) as revealed by Southern hybridization (data not shown), might contain a common origin of replication. Therefore, we concluded that pD188 and pRF28 are incompatible plasmids and that the presence of pD188 in electrotransformed cells hindered the establishment of the incoming plasmids.

Stabilization of nonreplicating vectors in *R. fascians* by homologous recombination. By using the electroporation protocol described above, the possibility of stabilizing nonreplicating constructs containing DNA sequences homologous to the *R. fascians* genome by reciprocal recombination was investigated. For these experiments, pRF40 was constructed in *E. coli* by ligation of pJGV121 (a vector containing a 2.5-kb fragment from the *R. fascians* genome) to pRF28 linearized at the *Bgl*II site. Because of the insertion at the *Bgl*II site of pRF28, pRF40 did not replicate in *R. fascians*. Consequently, the Cm^r gene of pRF28 could be maintained in *R. fascians* only by integration into the chromosome, either via single homologous or nonhomologous recombination (see above). Surprisingly, the analysis of Cm^r transformants by Southern hybridization revealed only the patterns expected for single homologous recombination (Fig. 5B).

Localization of the chloramphenicol resistance gene in pRF28. The existence of Cm^r-encoding pRF2 derivatives, such as pRF25 (Fig. 1B), that lacked *Bam*HI fragment 6 of pRF22 (corresponding to *Bam*HI fragment 2 of pRF28 [Fig. 2]), indicated that this fragment is not necessary for Cm^r. Vectors containing different parts of pRF28 were constructed in *E. coli* (pRF42, pRF43, pRF44, and pRF45) and introduced into *R. fascians* (Fig. 2). Stabilization in *R. fascians* of these constructs was either by replication (pRF42 and pRF43) or by integration in the genome by homologous recombination (pRF44 and pRF45) (see above). Only the 3.4-kb *Xba*I-*Xho*I fragment of pRF28 conferred Cm^r to the transformants (pRF44). Deletion of the 800 base pairs from the *Xba*I site to the *Hin*dIII site of this fragment and ligation of this fragment to pJGV121 yielded pRF45, which no longer transformed *R. fascians* cells to Cm^r upon electroporation. Nevertheless, insertion of pUC18 at the *Hin*dIII site of pRF28 (pRF34) did not completely abolish the Cm^r-transforming capacity (see above).

From these data, we concluded that all structural information for the Cm^r gene was located between the *Hin*dIII site and the right *Bam*HI site of *Bam*HI fragment 3 (orientation as in Fig. 2). The adjacent *Xba*I-*Hin*dIII fragment, however, was necessary for expression of the Cm^r gene and might contain the promoter.

The few Cm^r transformants to which pRF34 gave rise were possibly the result of fortuitous integration, which resulted in the Cm^r gene coming under the control of an adjacent chromosomal promoter. Insertion of pUC18 at the *Hin*dIII site of pRF28 (pRF34) did not abolish the replication ability of pRF28, but because no replicating plasmids could be found in the Cm^r transformants obtained after electrotransformation by this plasmid, the data supported the idea that the Cm^r gene in pRF34 could be activated only upon integration in the vicinity of an exogenous promoter.

DISCUSSION

Electroporation of intact cells has been reported as an efficient method for the transformation of several bacterial species. In organisms such as *E. coli* (9), *Agrobacterium tumefaciens* (18), *Lactobacillus* spp. (4, 15), and *Bacillus thuringiensis* (16), electrotransformation has proved simpler, more reproducible, and more efficient than classical methods

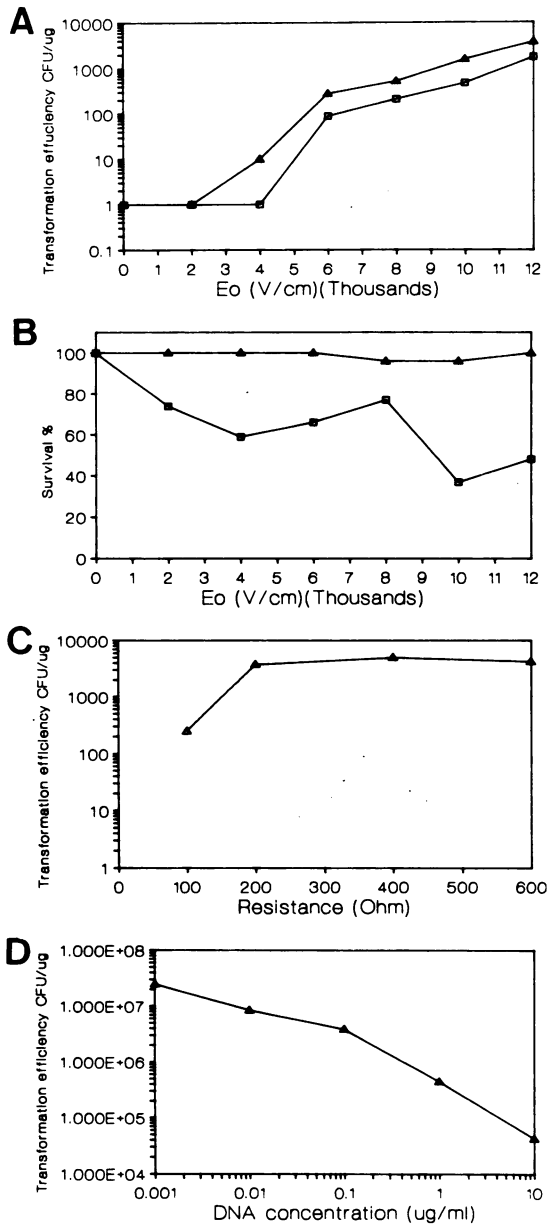

FIG. 3. Effect of different electroporation parameters on transformation and survival of *R. fascians* D188-5. Electroporations were carried out at 12 kV/cm, 25 μF, and 200 Ω in the presence of 10 μg of pRF29 per ml except for the specific parameter that was varied in each experiment. (A) Effect of the initial voltage of the applied pulse (E_0) and of composition of the electroporation medium on transformation efficiency; (B) effect of the initial voltage of the applied pulse (E_0) and the composition of the electroporation medium on survival of the electroporated cells; (C) effect of the external resistance coupled with the electroporating chamber on transformation efficiency; (D) effect of DNA concentration on transformation efficiency. The *y* axis is logarithmic in panels A, C, and D. Symbols: △, electroporation medium used was 30% PEG in water; □, electroporation medium used was water.

of transformation (competence and PEG-mediated DNA uptake in protoplasts). For other species (e.g., *Campylobacter jejuni* [20]), it is at present the only successful method for introduction of DNA.

Transformation systems for the genus *Rhodococcus* have been described (2, 29), but none of them worked in our

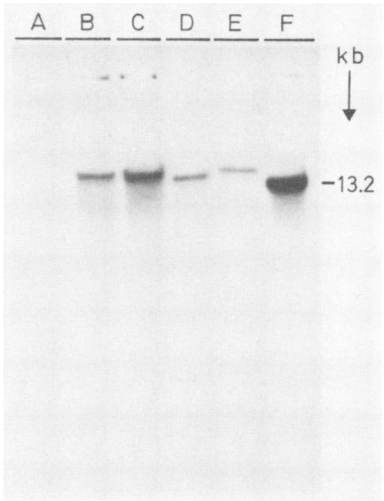

FIG. 4. Southern hybridization of ^{32}P-labeled pRF28 with *Bgl*II-digested total DNA of different Cm^r isolates (lanes B to E) electrotransformed with pRF32. A single high-molecular-weight band differing among the transformants was observed in each lane. Two reference lanes containing *Bgl*II-digested total DNA of D188-5 (lane A) and *Stu*I-linearized pRF32 (lane F) were also included on the same Tris–acetate–0.8% agarose gel.

laboratory to transform *R. fascians*. Large plasmids encoding heavy metal resistances, antibiotic resistance, or both have been isolated from several *R. fascians* strains (8). The reports on bacterial electrotransformation and the availability of a commercial apparatus that allowed delivery of high-voltage pulses led us to test this method by using the 160-kb plasmid pRF2 from *R. fascians* NCPPB 1675 coding for Cm^r and Cd^r. The first successful attempt and the concomitant isolation of a deleted Cm^r-encoding replicon (pRF21) permitted both the optimization of electrotransformation and the development of cloning vectors for *R. fascians*.

The optimal initial field strength of the discharge pulse (E_0) described in the different reports on bacterial electrotransformation varies between 7 kV/cm (*B. thuringiensis* [16]) and 12 kV/cm (*C. jejuni* [20]) and is a crucial parameter in adapting the electrotransformation method for a given bacterial species. Our data indicated that, within the range examined, a higher field strength resulted in more transformants per microgram of DNA, with the highest transformation efficiency being obtained at 12 kV/cm. The threshold value below which no transformants were detected depended on the electroporation buffer used (4 kV/cm for demineralized water and 2 kV/cm for a 30% PEG 1000 solution).

The electroporation medium also influenced the transformation efficiency. Whereas the use of H_2O as the electroporation medium yielded 10^3 to 10^4 CFU/μg of DNA at 12 kV/cm, the addition of 30% PEG 1000 (as described in references 15 and 16) resulted in a 3- to 10-fold increase in transformation efficiency; this increase, however, differed considerably among several batches of PEG 1000 (data not shown).

Although the effect of the addition of PEG has been attributed to volume exclusion (15), interactions with cell membranes, and an increased time constant, the most important factor may be the increased survival of the electroporated cells. Survival dropped to 50% (compared with an unpulsed control) as transformation efficiency increased

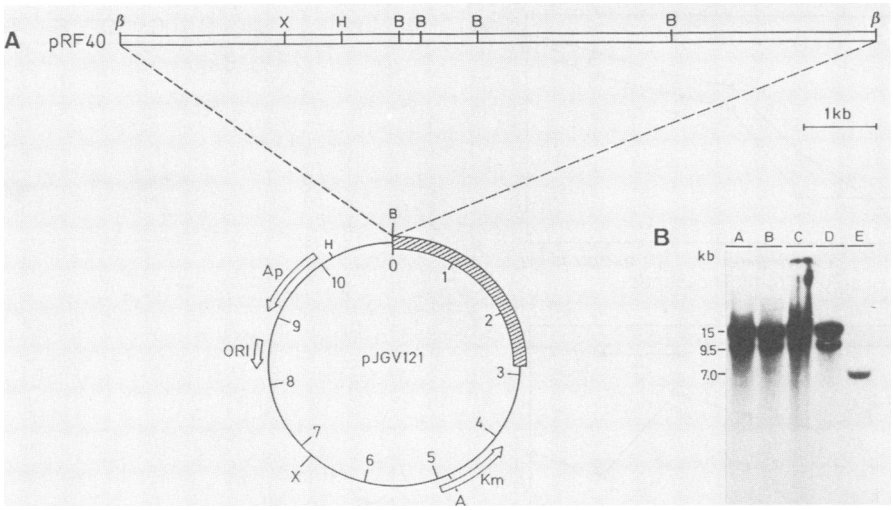

FIG. 5. (A) Circular restriction map of pJGV121, a vector unable to replicate in *R. fascians* and containing homology to the strain D188 genome (striped portion). The straight bar at the top represents the pRF28 insertion in pJGV121, which yields pRF40. The digits inside the circle indicate the scale in kilobases. Ap, Ampicillin resistance of pBR322; Km, kanamycin resistance of pUB110; ORI, origin of replication of pBR322; H, *Hin*dIII; A, *Apa*I; X, *Xba*I; B, *Bam*HI; β, *Bgl*II. (B) Southern analysis of Cm^r transformants obtained by electroporation of *R. fascians* D188 in the presence of pRF40. Total DNA was *Bam*HI digested and run on a Tris–acetate–0.8% agarose gel, transferred to Hybond nylon membranes, and hybridized against ^{32}P-labeled pJGV121. Lanes A to D contain DNA of Cm^r transformants, while lane E contains wild-type D188 DNA. The autoradiogram reveals the 7-kb *Bam*HI fragment homologous to pJGV121 in D188 (lane E). In lanes A to D this fragment has disappeared, whereas two new composite fragments of 15 and 9.5 kb appear, originating by single homologous recombination of this fragment with pRF40.

when demineralized H_2O was used as the electroporation medium. This correlation between increased lethality and increased transformation has also been reported for most other bacterial electrotransformations (9, 16; G. De la Riva, personal communication). The addition of 30% PEG to the electroporation medium maintained the survival rate at about 100%. These data indicated that the death of a significant proportion of the cell population was not a prerequisite for efficient transformation, as has also been reported for *C. jejuni* (20).

The duration of the electrical discharge as expressed by the time constant has been reported to be another important factor in electroporation (20) but did not significantly alter the transformation efficiency in this system.

Varying the DNA concentration over a range of 1 ng/ml to 10 μg/ml caused a drop in transformation efficiency from 10^7 CFU/μg at 1 ng/ml to 10^4 CFU/μg at 10 μg/ml. In the low DNA concentration range (1 ng to 0.1 μg) the actual number of transformants increased but then stabilized as the DNA concentration was further increased. A possible interpretation for this result is the existence of an "electrocompetent" subpopulation, as has also been proposed for *Lactobacillus casei* (5). Different growth conditions might influence the size of this subpopulation; however, no influence of the growth phase of the *R. fascians* electroporated cells on transformation efficiency was observed (data not shown).

The efficient curing of resident plasmids by electroporation could be an alternative to the methods described earlier (8). The availability of electroporation as an efficient means of introducing DNA into *R. fascians* allowed the development of several cloning vectors for this species. Starting from the fortuitously isolated in vivo deletion mutant of pRF2 (pRF21), a cloning vector for *R. fascians* (pRF28) was constructed by two consecutive deletions. pRF28 is a 10.5-kb plasmid that encodes resistance to chloramphenicol and has unique restriction sites for *Stu*I, *Xba*I, *Bgl*II, and *Hin*dIII, of which the first two can be used as cloning sites without interfering with the ability of the plasmid to replicate or confer Cm^r. The small *Xho*I fragments located in *Bam*HI fragment 2 can also be substituted by foreign DNA.

The origin of replication of pRF28 was located on a 3.8-kb *Stu*I-*Xba*I fragment, whereas the 2.7-kb fragment located between the *Xba*I site and the right *Bam*HI site of *Bam*HI fragment 3 was proven sufficient for encoding Cm^r by the insertion of pUC13 or pUC18 at each unique restriction site and subcloning of portions of pRF28. This information will allow us to design appropriate cloning vectors of specific needs for *R. fascians*.

During the analysis of pRF28, a bleomycin resistance gene of *S. verticillus* was expressed from its own promoter (pRF36) in *R. fascians*. As the heterologous expression of *Streptomyces* genes has also been described for other *Rhodococcus* species (29), the marker genes of *Streptomyces* species might be good candidates for use in the further development of vectors for rhodococci.

Another important feature observed during this work was the ability of *R. fascians* to rescue marker genes by homologous or illegitimate recombination when electrotransformed by nonreplicative plasmids. Vectors that did not contain a functional origin of replication for *R. fascians* but carried DNA sequences homologous to the *R. fascians* D188 genome could integrate via homologous recombination. This method can be used to specifically alter *R. fascians* DNA sequences of interest by gene replacement.

A cointegrate (pRF32) of pUC18 and pRF28, in which the origin of replication was disrupted by the insertion of the pUC18, still gave rise to Cm^r transformants, albeit at a lower frequency, when introduced in *R. fascians* D188-5 via electroporation. pRF28 sequences appeared integrated at different locations in the chromosome of different transformants. We presumed that integration occurred via either illegitimate recombination or short stretches of homology, as no detectable homology between D188-5 total DNA and pRF28 could be found.

Some plasmid constructions made in the course of the analysis of pRF28, such as pRF30 and pRF37, can be used as cloning vectors that replicate in both *E. coli* and *R. fascians*. pRF37 has unique restriction sites for *Hin*dIII, *Xba*I, *Stu*I, and *Kpn*I that can be used for cloning without disturbing any plasmid function. It codes for ampicillin resistance (Ap^r) in *E. coli* and confers bleomycin resistance upon introduction in *R. fascians*. pRF30, which codes for Cm^r in *R. fascians* and Ap^r in *E. coli*, contains a unique restriction site for *Stu*I, whereas the small *Xho*I fragments can be substituted.

Transformation of *R. fascians* cells via high-voltage electroporation has allowed the development of cloning vectors for this species. In addition, preliminary observations of integration via either homologous or illegitimate recombination offer good prospects for the development of random insertion mutagenesis and a gene replacement system. These combined tools will certainly enhance the genetic and molecular analysis of interesting phenotypes of *R. fascians* such as plant pathogenicity, surface-active compound production (6), and metabolic features (nomilin catabolism [14] and hydrocarbon utilization [22]) and might also be useful with other species of the genus *Rhodococcus*.

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