Conjugative Transfer of Cadmium Resistance Plasmids in *Rhodococcus fascians* Strains

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The presence of a 138-kilobase plasmid (pD188) correlated with increased resistance to cadmium in *Rhodococcus fascians* D188. This plasmid could be transferred by a conjugation-like system in matings between *R. fascians* strains. Transconjugants expressed the cadmium resistance and could be used as donors in subsequent matings. Four other *R. fascians* strains (NCPB 1488, NCPB 1675, NCPB 2551, and ATCC 12974) could also be used as donors for cadmium resistance in matings. Strain NCPB 1675 showed a 100% cotransfer of cadmium and chloramphenicol resistance markers.

Rhodococcus fascians (basonym *Corynebacterium fascians*) (22) is a gram-positive plant-pathogenic bacterium that causes fasciation of dicotyledonous (21) and monocotyledonous plants, such as members of the family *Liliaceae* (9, 15, 23). Previous work has shown that several *R. fascians* strains harbor large plasmids (13, 16, 17). The presence of a 99-kilobase-pair (kb) plasmid in a strain called MW2 and the simultaneous loss of cadmium resistance, hydrocarbon utilization, and virulence correlated with the loss of this plasmid have been reported by Murai (16). On the contrary, Lawson et al. (13) claimed that the single large plasmid (112 kb) they found was present in virulent as well as avirulent strains. Although the relationship between the strains used in the analyses by Murai (16) and Lawson et al. (13) was not clear, it seemed likely to the authors of the latter study that a number of strains in both studies had identical origins, especially the avirulent isolate (CF16) used by Murai (16).

The genetic analysis of these phenotypic traits has been hindered by the lack of an appropriate system for the introduction and exchange of genetic material. Exchange of chromosomal markers has been well documented for the genus *Rhodococcus* (1, 4), but there is no evidence for the transfer of plasmids in this genus.

In this paper, we report the isolation of a 138-kb plasmid (pD188) present in the cadmium-resistant *R. fascians* strain D188, the construction of isogenic strains with and without pD188, and the use of these strains for the assignment of the cadmium resistance gene(s) to the plasmid. Furthermore, we describe the conjugal transfer of plasmid pD188 and other plasmids between different *R. fascians* strains.

Mutant isolates of the cadmium-resistant *R. fascians* strain D188 were obtained in three ways.

First, a cadmium-sensitive mutant, labeled D188-1 (Table 1), was obtained by culturing strain D188 at 37°C, which is the upper growth limit.

Another class of cadmium-sensitive mutants was obtained unexpectedly by selecting mutants resistant to rifampin. These rifampin-resistant mutants (e.g., strain D188-2; Table 1) were obtained at very low frequency (3×10^{-10}) in an unmutagenized culture of *R. fascians* D188. Their colony size was reduced, and they had a longer generation time. The inability of the rifampin-resistant mutant to maintain the 138-kb plasmid is an unsolved question. Curing by rifampin has been reported in *Escherichia coli* and *Staphylococcus*

aureus (3, 12), but in these species, rifampin-resistant mutants retained their plasmids (3), whereas here the acquisition of rifampin resistance seemed to be responsible for the loss of pD188.

In many gram-positive bacterial species, protoplasting and regeneration leads to curing of resident plasmids (8, 19). *R. fascians* D188 was treated with lysozyme (10 mg/ml; Sigma Chemical Co.) in TE buffer (50 mM Tris hydrochloride, 20 mM EDTA [pH 7]) containing 20% polyethylene glycol. The resulting bacteria were not true protoplasts, since they did not round up (as seen under the light microscope) and were not sensitive to osmotic shock. The cell wall, however, was modified, since this treatment renders the bacteria very sensitive to detergents, in contrast to untreated bacteria. Screening of 250 colonies obtained after plating for single colonies yielded 25% cadmium-resistant and 75% cadmium-sensitive (e.g., D188-3; Table 1) colonies. These results and sensitivity toward bacteriophage ϕ CF1 (7) of the different *R. fascians* strains are summarized in Table 1.

The presence of a large plasmid (pD188) in *R. fascians* D188 was demonstrated by combining a lysis method for oral streptococci (6) with a large-plasmid isolation method (11). The size of these covalently closed circular DNA molecules was determined by electron microscopy (14) as 41.9 ± 1.6 μ m, corresponding to 126 ± 4.8 kb. Restriction endonuclease digests (*Bam*HI and *Eco*RI) and subsequent determination of the fragment sizes yielded a slightly larger value of 138 kb.

To examine the correlation between cadmium resistance and the presence of pD188, we used large 32 P-labeled pD188 subclones as probes in hybridization analyses with Southern blots of restricted total DNAs of D188 and its derivative mutants. pJGV20 and pJGV21 were isolated as clones of large pD188 *Hind*III fragments in pBR322 (24 and 40 kb, respectively), whereas pJGV05606 (insert 32 kb) was identified as a subclone of pD188 in a cosmid library prepared with partially *Sau*3A-digested total DNA from a wild-type D188 strain (J. Desomer, unpublished results). Plasmids pJGV20 and pJGV21 hybridized only with total wild-type DNA (Fig. 1, lanes 2A, 3A, and 4C). Plasmid sequences could not be detected in cadmium-sensitive mutant strain D188-1 (Fig. 1, lanes 2B and 3B) or D188-2 (Fig. 1, lanes 4D and 6D). Moreover, when the 250 colonies obtained after treatment with polyethylene glycol and lysozyme were screened in a dot blot analysis for hybridization with one of these probes (pJGV05606), a strict correlation was found between cad-

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TABLE 1. List of bacterial strains and phages used in this study and their characteristics

Strain or phage	<i>R. fascians</i> parent strain(s)	Resistance to ^a :			Other characteristics ^b and reference
		Cadmium	Chloramphenicol	Phage ϕ CF1	
<i>R. fascians</i>					
D188		R	S	S	
D188-1	D188	S	S	S	Obtained by growing at 37°C
D188-2	D188	S	S	S	Rif ^r
D188-3	D188	S	S	S	Obtained by protoplasting and regeneration
D188-4	D188-1	S	S	S	Str ^r
D188-5	D188-4	S	S	S	Str ^r Ery ^r
D188-6	D188-1	S	S	S	Rif ^r
D188-7	D188 $\times$ D188-4	R	S	S	
D188-9	NCPPB 1488 $\times$ D188-4	R	ND	S	
D188-10	ATCC 12974 $\times$ D188-4	R	ND	ND	
D188-11	NCPPB 1675 $\times$ D188-5	R	R	ND	
NCPPB 1488		R	ND	R	
ATCC 12974		R	ND	R	
NCPPB 2551		R	ND	R	
NCPPB 1675		R	R	ND	
<i>E. coli</i>					
MC1061					<i>araD139</i> Δ (<i>ara leu</i>)7697 Δ <i>lacX74 galU galK hsrR hsrM rpsL</i> ; 5
K803					F ⁻ <i>nodR154 (hsrR hsrM) supE44 supF58 lacY1 galK2 galT22 metB1 trpR55</i> ; 24
Phage ϕ CF1					Lysogenic; 7

^a Strains were resistant (R) or sensitive (S) to 27.5 μ g of Cd(NO₃)₂ or 25 μ g of chloramphenicol per ml. ND, Not determined.

^b Ery^r, Erythromycin resistant (100 μ g/ml); Rif^r, rifampin resistant (50 μ g/ml); Str^r, streptomycin resistant (100 μ g/ml).

mium resistance and the presence of the plasmid (Fig. 2). The detection of a 7.6-kb fragment in the lanes with D188-1 (data not shown) or D188-2 total DNA (Fig. 1, lane 6D) hybridized with pJGV05606 is probably due to (partial)

homology of a plasmid fragment with the chromosomal background, since the extra band can also be observed as a doublet in the lane with wild-type total DNA (Fig. 1, lane 6C) and is absent in pJGV05606. This homology could account

FIG. 1. Examples of hybridization of *R. fascians* D188 derivatives with a set of subclones from plasmid pD188. Total DNAs, prepared from the different *R. fascians* D188 derivatives, were digested with enzyme *Bam*HI, run on a 0.8% agarose gel, and blotted to nylon membranes. (1 to 3) The same strip of nylon membrane containing wild-type *R. fascians* D188 DNA (lanes A) and D188-1 DNA (lanes B). (1) The blot was hybridized with pJGV03704, a cosmid clone derived from the chromosome of *R. fascians* D188. This control panel shows the comparable amount of DNA in both lanes. (2 and 3) Autoradiograms from the same blot hybridized with plasmids pJGV20 and pJGV21, respectively. Hybridization can be observed only in the wild-type DNA (lanes 2A and 3A). (4, 5, and 6) Autoradiograms of the blot containing wild-type D188 DNA (lanes C) and D188-2 DNA (lanes D) hybridized against pJGV20, pJGV03612 (another chromosomal cosmid clone from D188), and pJGV05606. Note the 7.6-kb doublet containing pD188 *Bam*HI fragment 6 (lane C) and a remaining, presumably chromosomal, homologous counterpart (lanes C and D).

for the relatively high background in the dot blot experiment (Fig. 2).

From these experiments, we conclude that there is a correlation between the loss of cadmium resistance and the loss of pD188 in strains D188-1, D188-2, and D188-3. These data confirm the observation of Doyle and Hanson (reported in reference 16) that cadmium resistance is plasmid encoded; however, the size of pD188 (138 kb) appears to be larger than the size of the plasmid (99 kb) reported previously (16).

Conclusive evidence that cadmium resistance is located on pD188, however, requires that the resistance phenotype be restored upon reintroduction of this plasmid in a mutant cadmium-sensitive strain. To test this, we marked the cadmium-sensitive acceptor strain D188-1 with resistances towards the antibiotics streptomycin, erythromycin, and rifampin (Table 1). Donor and recipient strains were grown for 2 days in yeast extract buffer (YEB) at 28°C. The resulting cultures were mixed at a ratio of 1:1 (usually 5 ml of each culture) and filtered through a nitrocellulose filter (pore size, 0.45 μ m; diameter, 25 mm; Millipore Corp.). The filters were incubated on YEB agar plates for 24 to 28 h (28°C). The cells were washed from the filter with 5 ml of a buffer containing 10 mM Tris hydrochloride and 10 mM MgSO₄ and then diluted and plated on YEB medium containing the appropriate selective agents.

When the mating mixture from wild-type D188 and strain D188-4 was plated on YEB medium supplemented with streptomycin and Cd(NO₃)₂, we obtained transfer frequencies of cadmium resistance of up to 7.5×10^{-4} transconjugants per recipient cell (Table 2). When either the donor or the acceptor strain was omitted, no streptomycin- or cadmium-resistant colonies could be observed, indicating that the spontaneous rates of mutation toward cadmium resistance or toward the used marker antibiotic were orders of magnitude lower than the observed transfer frequencies. The possibility of transduction was ruled out, since no transconjugants appeared in the control experiment in which only donor culture supernatant was used.

A time course experiment (Fig. 3) in which the conjugations were interrupted (by vortexing in the resuspending buffer described above at different times after the onset of mating) showed a lag phase of about 6 to 7 h during which no transconjugants could be observed, followed by an increase in transfer frequency for up to 24 h of mating. This lag phase is probably the reflection of some biological process, such as induction, that has to take place before conjugation proceeds. It also indicates that this system differs from the other known gram-positive, conjugation-like systems in *Bacillus* spp., *Streptococcus* spp., and *Corynebacterium* spp. (2, 10, 20).

FIG. 2. Dot blot hybridization of polyethylene glycol-lysozyme-treated *R. fascians*. Cadmium-sensitive and cadmium-resistant colonies, isolated after treatment with lysozyme and polyethylene glycol 6000, were lysed. Lysates were heated to 95°C, chilled on ice, and bound to nylon membranes. They were then denatured and neutralized as recommended by Amersham Corp. These filters were hybridized against ³²P-labeled plasmid pJGV05606 DNA. The cadmium-resistant clones, such as those in A3, A12, and B1, hybridized, whereas the cadmium-sensitive clones, such as those in A1, A2, and A4, did not.

Table 2 also shows that both streptomycin- and erythromycin-resistant D188-1 derivatives can be used as acceptors, but it was impossible to obtain transconjugant colonies in a mating between D188 and rifampin-resistant D188-1 (D188-6) by selecting for rifampin and cadmium resistance.

To test the presence of the 138-kb plasmid in the transconjugants from the cross D188 $\times$ D188-4 (Table 2), the same set of plasmid clones as described above was used in hybridizations with total DNAs prepared from the transconjugants. In all cadmium-resistant transconjugants, hybridization with pD188 sequences was observed (Fig. 4, lanes C to G). Moreover, the hybridization pattern observed in the transconjugants was the same as that in the donor strain (Fig. 4, lane A), indicating that no major rearrangements took place during the transfer process. These observations allow us to allocate cadmium resistance to plasmid pD188.

From crosses between transconjugant D188-7 and an appropriately marked acceptor strain (D188-5), we conclude that transconjugant strains can act as efficient donors of the cadmium-resistant plasmid (Table 2).

To determine whether cadmium resistance was transmissible only in strain D188, we used four other cadmium-resistant strains (NCPB 1488, NCPB 1675, NCPB 2551,

TABLE 2. Summary of conjugations between different *R. fascians* strains

Donor	Acceptor	Acceptor (CFU/ml)	No. of transconjugants		Transfer frequency for Cd ^a
			Cd ^r	Cm ^r	
D188	D188-4	2.0×10^8	1.5×10^4		7.5×10^{-4}
D188	D188-6	2.5×10^8	None		$<1 \times 10^{-7}$
D188-7	D188-5	7.0×10^7	2.0×10^6		1.5×10^{-2}
NCPB 2551	D188-4	1.0×10^7	1.0×10^4		1.0×10^{-3}
ATCC 12974	D188-4	2.0×10^7	3.0×10^3		1.5×10^{-4}
NCPB 1488	D188-4	2.0×10^8	2.5×10^4		1.5×10^{-4}
NCPB 1675	D188-5	6.0×10^8	2.0×10^2	2×10^2	3.0×10^{-7}
D188-9	D188-5	2.5×10^8	1.4×10^6		5.6×10^{-3}
D188-10	D188-5	5.0×10^7	1.7×10^4		3.4×10^{-4}

^a Expressed as the number of cadmium-resistant transconjugants divided by the number of recipient cells in the mating mixture.

and ATCC 12974). ATCC 12974 was used since it is considered as the type strain for *R. fascians*.

When probed with the set of subclones from pD188, all four strains contained some homologous sequences. Whereas the hybridization pattern in strain NCPPB 1488 was the same as that in strain D188, the ATCC 12974 and NCPPB 1675 patterns showed considerable differences. Strain NCPPB 2551 harbored a plasmid with a *Bam*HI restriction pattern which was almost identical to that of pD188 except for two fragments (data not shown).

From the data in Table 2 regarding the crosses between these strains and D188-4 or D188-5, we conclude that all four strains are efficient in transferring cadmium resistance to D188-4 or D188-5. In addition, strain NCPPB 1675 transferred its chloramphenicol resistance with an efficiency identical to the transfer efficiency of the cadmium resistance in this cross (NCPPB 1675 $\times$ D188-5; Table 2). Moreover, each chloramphenicol-resistant transconjugant was cadmium resistant, and vice versa. Transconjugants (e.g., D188-11) from this cross harbored the same plasmid as NCPPB 1675 did.

Since NCPPB 1488 is resistant whereas D188-4 is sensitive to phage ϕ CF1 (Table 1), transconjugants from the cross NCPPB 1488 $\times$ D188-4 could be tested for susceptibility towards ϕ CF1. All transconjugants tested were sensitive, indicating that the plasmid is transferred from NCPPB 1488 to D188-4, rather than the chromosomal streptomycin resistance marker being transferred from D188-4 to NCPPB 1488.

In two of the four cases, transconjugants (D188-9 and D188-10) were used as donors in subsequent matings (Table 2).

Heavy-metal resistance plasmids are widespread in gram-positive species such as *S. aureus* (18) and *Corynebacterium flaccumfaciens* subsp. *oortii* (10). At least in the latter case, the plasmid is transmissible between *Corynebacterium* species. To our knowledge, this is the first report of plasmids of this type and conjugative transfer thereof in the genus *Rhodococcus*. The ability to transfer plasmids between *R. fascians* strains and perhaps between this species and other rhodococci will probably be useful in the study of the pathogenicity of *R. fascians* and might enhance the development of a cloning system for other rhodococci.

FIG. 3. Kinetics of conjugation. Filter matings between D188 and D188-4 at 28°C were interrupted at different intervals, and the transfer of pD188 to D188-4 was monitored. Symbols: □, donor cells; ○, acceptor cells; ▽, transconjugants.

FIG. 4. Plasmid detection in transconjugants. Total DNAs from *R. fascians* D188 (A), D188-4 (B), and Cd^r transconjugants from the cross D188 $\times$ D188-4 (C to G) were digested with *Bam*HI restriction enzyme and run on a 0.8% agarose gel, blotted to a nylon membrane, and hybridized with ³²P-labeled pJGV20. The typical hybridization pattern can be seen only in lane A (wild type) and in the lanes with transconjugant DNAs (lanes C, D, E, and G contain partially digested DNA).

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