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Transformation of *Actinomyces* spp. by a Gram-Negative Broad-Host-Range Plasmid

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The gram-negative broad-host-range vector pJRD215 was transferred by electroporation into strains of *Actinomyces viscosus* or *Actinomyces naeslundii* at efficiencies which ranged from 10^2 to 10^7 transformants per μg of plasmid DNA. The *Actinomyces* transformants expressed pJRD215-encoded resistance to kanamycin and streptomycin. Moreover, the transforming plasmid DNA had not undergone any deletions or rearrangements, nor had it integrated into the genomes of these strains.

No genetic transfer system for recombinant DNA methodology has been reported for *Actinomyces* spp. Consequently, it has not been possible to study gene expression in these organisms. Such methodologies require two components: a reproducible procedure for gene transfer, and a cloning vector capable of replicating in these bacteria. A pilot study was undertaken to screen approximately 300 *Actinomyces* strains for the presence of covalently closed circular plasmid DNA by the plasmid enrichment method of Anderson and McKay (2). However, none contained detectable plasmid DNA (29).

This study was initiated to examine several broad-host-range plasmids (Table 1) of gram-positive bacterial origin and several *Streptomyces* and *Corynebacterium* plasmids (10, 14, 20) for their suitability as potential cloning vehicles for *Actinomyces* spp. The *Streptomyces*- and *Corynebacterium*-derived plasmids were of interest since the genomes of these bacteria and those of *Actinomyces* spp. are rich in G+C content (9, 22, 27). The gram-negative broad-host-range vector pJRD215, originally derived from RSF1010 (11, 26), also was included since this vector was transferred to and replicated in *Mycobacterium* spp. (17). The feasibility of transformation by electroporation was investigated in *Actinomyces* spp., since this method proved to be an efficient means of genetic transfer in several gram-positive bacteria such as streptococci (12), *Corynebacterium glutamicum* (16), mycobacteria (6, 19), and others (1, 3, 18, 23, 25). Of all the vectors tested, only pJRD215 was transferred, and its selectable markers were expressed in *Actinomyces* strains. To our knowledge, this is the first report of a transformation system for *Actinomyces* spp.

Bacterial strains and plasmids. The bacterial strains and plasmids used are described in Table 1. Additional plasmids used included several shuttle vectors derived from *Streptomyces-Escherichia coli* (pSKN01 and pSKN02) (20) and *Corynebacterium-E. coli* (pJC1, pEC7, pEK0, and pEKEx2) (10, 14). *Actinomyces* strains were grown in a complex medium (CAM) (8) or *Lactobacillus* carrying medium (LCM) (13).

Preparation of electroporation-competent cells and transformation of *Actinomyces viscosus* by electroporation. Initial efforts to transform *A. viscosus* strains MG1 and T14V with plasmids involved the use of an electroporation protocol designed for *Mycobacterium smegmatis* (19), with modifications. Early-exponential-phase cultures (500 ml, LCM with 20 mM DL-threonine [4], optical density at 600 nm of 0.2) were chilled on ice for 1.5 h and washed extensively first with a large volume of ice-cold sterile distilled water and then with sterile 10% glycerol. The cells were suspended in 10% glycerol to a final volume of 2 ml, and aliquots were stored at −80°C. Bacterial suspensions were thawed on ice, and 100 μl (5×10^8 cells) was mixed with plasmid DNA (CsCl-purified [24] unless stated otherwise) in no more than 5 μl of 10 mM Tris-HCl–0.1 mM EDTA (pH 8.0). Electrotransformation was conducted in a chilled 0.2-cm cuvette (Bio-Rad Laboratories, Richmond, Calif.), using a Bio-Rad Gene Pulser connected to a pulse controller (2.5 kV, 25-μF capacitor, and a resistance of 1,000 Ω in parallel with the sample). The pulsed cells were diluted with 1 ml of CAM and incubated at 37°C for 2 h, and samples were spread onto brain heart infusion agar (Difco Laboratories, Detroit, Mich.) supplemented with various antibiotics (Sigma Chemical Co., St. Louis, Mo.). Antibiotics and concentrations (micrograms per milliliter) for transformant selection were as follows: kanamycin, 40; streptomycin, 50; chloramphenicol, 5; and thiostrepton, 40. Plates were incubated at 37°C for up to 5 days.

A. viscosus MG1 colonies (approximately 10^4/μg of plasmid DNA) resistant to both kanamycin and streptomycin were obtained with pJRD215. No transformants were obtained in the absence of plasmid DNA or electric pulse. No transformants were obtained with plasmids of gram-positive bacterial origin. Similarly, transformants of *A. viscosus* T14V were obtained only with pJRD215, albeit at a lower efficiency (approximately 300 colonies per μg of DNA). Representative transformants were examined for plasmid DNA by the procedure of Anderson and McKay (2), with modifications. Early stationary phase bacteria from a 2-ml culture in CAM were washed in 25 mM sodium phosphate buffer (pH 7.0) and suspended in a buffer containing 6.7% sucrose and 25 mM Tris-HCl (pH 7.0). Washed cells were digested with 250 μg of egg white lysozyme (grade I; Sigma) and 1,250 U of mutanolysin at 37°C for 30 to 60 min and were lysed with 1% sodium dodecyl sulfate at 55°C for 10 min. The lysates were extracted first with NaCl-saturated phenol and then with chloroform-isoamyl alcohol (24:1, vol/vol) and precipitated with absolute alcohol. All of six randomly selected *A. viscosus* MG1 or T14V transformants harbored plasmid DNA of approximately 10 kb (data not shown). Results of restriction and Southern blot analyses (30) revealed that plasmid isolated from *A. viscosus* MY14 and MY19 was pJRD215 and that it had not integrated

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description[a]	Reference(s) or source
Bacterial strains		
A. viscosus		
MG1	Km^s Sm^s	7
MY14	*A. viscosus* MG1 with pJRD215, Km^r Sm^r	This study
T14V[b]	Km^s Sm^r	8, 28
MY19[c]	*A. viscosus* T14V with pJRD215, Km^r Sm^r	This study
T14VSM[b]	Km^s Sm^r	A. L. Delisle
A. naeslundii		
WVU45	Km^s Sm^s	8
MY40	*A. naeslundii* WVU45 with pJRD215, Km^r Sm^r	This study
W1544	Km^s Sm^s	7
Plasmids		
pIJ702	*Streptomyces* plasmid, contains a melanin-producing gene, Tsr^r, 5.8 kb	21
pMC16	pAMβ1-derived plasmid from *Streptococcus gordonii* Challis, Km^r, 6.5 kb	5
pUB110	*Staphylococcus* plasmid, Km^r, 4.5 kb	15
pJRD215	Km^r Sm^r Mob$^+$, 10.2 kb	11

[a] Susceptibility to kanamycin (Km), streptomycin (Sm), or thiostrepton (Tsr) is denoted by the superscript "s," and resistance is denoted by the superscript "r." Mob, mobilization function.

[b] Bacterial strain was made resistant to streptomycin by incubation of the organism in a growth medium containing streptomycin.

[c] Resistance to streptomycin is mediated by the streptomycin resistance gene of pJRD215 and a spontaneous mutation as described in footnote *b*.

into the *Actinomyces* genome. Moreover, no detectable deletions or rearrangements of pJRD215 in *A. viscosus* were observed. Interestingly, some modifications were observed in certain restriction endonuclease recognition sites of pJRD215 propagated in *A. viscosus*. For instance, only two of the three *Acc*I sites were detected in the *A. viscosus*-derived pJRD215.

Development of optimal transformation conditions. Optimal electrotransformation conditions for *Actinomyces* spp. were determined with *A. viscosus* MG1 as the host strain and pJRD215 from *E. coli* as the transforming DNA. High transfer frequencies of 2.9×10^7 and 3.1×10^7 transformants per μg of DNA were obtained with parallel resistances of 200 and 400 Ω, respectively (Table 2). No correlation was observed between transformation efficiency and pulse length under these conditions. Significant decreases were noted in cell survival and transformation efficiency with increasing pulse length at parallel resistances greater than 400 Ω (Table 2). Given these results, subsequent electroporation of actinomycetes in this study was performed with a parallel resistance of 400 Ω. Transformation of *A. viscosus* MG1 with the other plasmids derived from gram-positive bacteria was unsuccessful even under optimized electroporation conditions.

TABLE 2. Effect of pulse length on transformation efficiency of *A. viscosus* MG1 with pJRD215[a]

Resistance (Ω)	Time constant (ms)	No. of surviving cells	No. of transformants/μg of DNA
200	4.5	2.7×10^8	2.9×10^7
400	8.0	4.2×10^8	3.1×10^7
600	10.4	1.7×10^7	4.8×10^6
800	12.5	9.7×10^6	5.5×10^5
1,000	14.3	6.5×10^6	3.2×10^4

[a] *A. viscosus* MG1 (5×10^8 cells) in a 0.2-cm cuvette was transformed with 100 ng of pJRD215 under electroporation conditions with various parallel resistance of the pulse controller. The voltage and capacitance were held constant at 2.5 kV and 25 μF, respectively. Four to eight plates from each electroporation mix were counted. Each experiment was performed at least three times, and all yielded comparable transformation frequencies.

TABLE 3. Relationship between the amount of plasmid DNA added and the total number of transformants obtained

Amt (ng) of plasmid DNA/assay[a]	Time constant (ms)	No. of transformants/assay
0.1 ng	7.8	2.4×10^3
1.0 ng	8.0	1.4×10^4
10.0 ng	7.9	1.0×10^5
20.0 ng	8.4	4.6×10^5
50.0 ng	8.1	1.2×10^6
100.0 ng	8.2	3.7×10^6
500.0 ng	7.8	1.1×10^6
1,000.0 ng	7.6	4.2×10^5

[a] Covalently closed circular plasmid DNA purified by CsCl-ethidium bromide density gradient centrifugation was used in each assay to transform *A. viscosus* MG1 (100 μl containing 5×10^8 cells in 10% glycerol) under the standard electroporation conditions. Transformation efficiency was determined by counting multiple plates from at least three electroporation assays as indicated in the footnote to Table 2.

Characteristics of the *Actinomyces* transformation system. A linear relationship was observed between the total number of transformants obtained and the amount (within the range of 0.1 to 100 ng) of DNA used in the transformation of *A. viscosus* MG1 under the optimal electroporation conditions (Table 3). A constant transformation efficiency (10^7 transformants per μg of plasmid DNA) was obtained over this range. The transformation efficiency decreased at higher DNA concentrations.

Actinomyces naeslundii WVU45 was transformed poorly by pJRD215 (10^2 transformants per μg of plasmid DNA), while the efficiency of DNA transfer in *A. viscosus* T14V and T14VSM was approximately 4 orders of magnitude lower than that obtained with *A. viscosus* MG1. However, a higher (≈100-fold) transformation efficiency was obtained with pJRD215 isolated from its homologous host for *A. viscosus* T14V or *A. naeslundii* WVU45 (Table 4). A slight increase (≈50%) in transfer frequency was observed in *A. viscosus* MG1 with pJRD215 from *A. viscosus* MY14 (Table 4). The transformation efficiencies with pJRD215 propagated in *A. viscosus* MY14 in the other *Actinomyces* strains were comparable to those obtained with pJRD215 from *E. coli* (Table 4). No *Actinomyces* transformants were obtained with pJRD215 linearized by digestion at the single *Eco*RI site (11). Unlike the case for several other species of gram-positive bacteria (12, 16, 18), transformation of *Actinomyces* strains was not influenced by the presence of glycine or threonine in the growth medium.

Stability of pJRD215 in *Actinomyces* spp. After growth in an antibiotic-free CAM medium for at least 25 generations, high percentages (96, 93, and 94%) of CFU of *A. viscosus* (strains MG1, T14V, and T14VSM, respectively) retained the plasmid, as determined by their ability to grow in the presence of streptomycin and kanamycin. In contrast, only 74 and 49% of CFU of *A. naeslundii* WVU45 and W1544, respectively, maintained the plasmid under similar conditions. Total genomic DNA from representative antibiotic-susceptible colonies was isolated, and results of Southern blot hybridizations with the pJRD215 DNA probe showed the absence of detectable plasmid (data not shown).

TABLE 4. Transformation efficiency of *Actinomyces* spp.[a] with pJRD215 isolated from *E. coli* or *Actinomyces* strains

Strain	No. of transformants/μg of plasmid DNA derived from:		
	E. coli DH5α	*A. viscosus* MY14	Homologous host strain
A. viscosus MG1	4.1×10^7	6.5×10^7	6.5×10^7
A. viscosus T14V	1.8×10^3	2.2×10^3	2.3×10^5
A. viscosus T14VSM	1.7×10^3	2.6×10^3	ND[b]
A. naeslundii W1544	3.7×10^5	4.0×10^5	ND
A. naeslundii WVU45	3.3×10^2	2.1×10^2	4.9×10^4

[a] Competent cells of *A. viscosus* MG1, T14V, and T14VSM and *A. naeslundii* WVU45 were prepared in LCM containing 20 mM DL-threonine. *A. naeslundii* W1544 did not grow in this medium, and competent cells were prepared in CAM supplemented with 1% glycine, which inhibited 40% cell growth of this strain. One hundred nanograms of purified plasmid DNA was used in each transformation under the standard electroporation conditions described in the text. *A. viscosus* and *A. naeslundii* cultures were incubated aerobically and anaerobically in an anaerobic GasPak system (BBL Microbiology System, Cockeysville, Md.), respectively. Transformation efficiency was determined by counting multiple plates obtained from at least three electroporation assays as described in the footnote to Table 2.

[b] ND, not determined.

Concluding remarks. A reproducible and high-frequency transfer of plasmid DNA to *Actinomyces* spp. was obtained by electrotransformation. Both antibiotic resistance genes encoded by the transforming plasmid, of gram-negative bacterial origin, were expressed in *Actinomyces* spp. Taken together, the results of this study provide the essential genetic tools for the development of recombinant DNA methodologies in this gram-positive genus.

The kanamycin resistance gene of pJRD215 was originally derived from Tn*5*, while the streptomycin resistance gene was placed under the control of a pBR322-derived *tet* promoter (11). Therefore, the expression of both antibiotic resistance genes in *Actinomyces* spp. implied that the promoters of these genes were recognized and functional in these gram-positive bacteria. Although transfer and expression of pJRD215 in mycobacteria was demonstrated previously by Hermans et al. (17), the transformation efficiencies of pJRD215 in *Mycobacterium aurum* and *M. smegmatis* (2×10^2 to 5×10^2 transformants per μg of plasmid DNA, respectively) were relatively low, and molecular rearrangements of the plasmid were noted in both strains. In contrast, a reasonably high frequency of transfer of pJRD215 was obtained in a majority of *Actinomyces* strains, apparent deletions or rearrangements of pJRD215 in the *Actinomyces* transformants were not detected, and once transferred, the plasmid was maintained stably in these bacteria. These findings, combined with the presence of a polylinker (11), offer the hope that pJRD215 will prove to be a suitable cloning vehicle for members of the genus *Actinomyces*.

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