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Identification and Application of Plasmids Suitable for Transfer of Foreign DNA to Members of the Genus *Gordonia*

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Gene transfer systems for *Gordonia polyisoprenivorans* strains VH2 and Y2K based on electroporation and conjugation, respectively, were established. Several parameters were optimized, resulting in transformation efficiencies of >4 × 10^5 CFU/μg of plasmid DNA. In contrast to most previously described electroporation protocols, the highest efficiencies were obtained by applying a heat shock after the intrinsic electroporation. Under these conditions, transfer and autonomous replication of plasmid pNC9503 was also demonstrated to proceed in *G. alkanivorans* DSM44187, *G. nitida* DSM44499^T, *G. rubropertincta* DSM43197^T, *G. rubropertincta* DSM46038, and *G. terrae* DSM43249^T. Conjugational plasmid DNA transfer to *G. polyisoprenivorans* resulted in transfer frequencies of up to 5 × 10^{-6} of the recipient cells. Recombinant strains capable of polyhydroxyalkanoate synthesis from alkanes were constructed.

Since reclassification of the gram-positives *Rhodococcus aichiensis* and *Nocardia amarae* to the genus *Gordonia* (13), this taxon is now a well-defined genus among the *Corynebacterium, Mycobacterium*, and *Nocardia* (CMN) group of actinomycetes. Species of *Gordonia* have attracted much interest in recent years due to their unusual and diverse capabilities to catalyze biotransformations and biodegradation of poorly approachable substances (2, 7, 9, 16). Although the number of reports of newly identified species of this genus steadily increases, no suitable genetic transfer systems have yet been described. Molecular analysis of rubber degradation by *G. polyisoprenivorans* and of other interesting pathways of *Gordonia* species is hampered by the lack of suitable and efficient gene transfer systems. Therefore, the present study identified plasmids, which can be transferred to *G. polyisoprenivorans* and other species of this genus by conjugational transfer or electroporation and which are stably maintained.

Identification of vector systems for *G. polyisoprenivorans*. The 6.3-kbp plasmid pNC9503 (Fig. 1b) was recently described as an *E. coli*/*Rhodococcus* shuttle vector (12). It possesses a unique restriction site for *Xba*I and comprises the kanamycin resistance gene from Tn*903* (24) for selection in *E. coli* and *Rhodococcus*/*Gordonia*. In addition, pNC9503 carries a thiostrepton resistance gene from *Streptomyces azureus* for selection in coryneform bacteria. The origin of replication (*oriV*) in actinomycetes is located on a fragment derived from the native *Rhodococcus rhodochrous* plasmid pNC903. A partial sequence revealed that it was 90% similar to the sequence of the *R. rhodochrous* plasmid pRC4 (10), which encodes a RepA and RepB protein. This plasmid, in turn, shares sequence similarity with the *Mycobacterium fortuitum* plasmid pAL5000 (17). Plasmid pNC9501 (Fig. 1a) is a derivative of pNC9503 differing from the latter only in possessing two additional unique restriction sites for *Kpn*I and *Eco*RI.

These vectors were introduced into *G. polyisoprenivorans* strains VH2 and Y2K applying a basic electroporation protocol previously developed for *R. opacus* PD630 (12). Electroporation of strain Kd2 failed. The electroporated cells were plated on media containing 25 μg of thiostrepton or 25 μg of kanamycin/ml for the selection of transformants. Resistant colonies appeared after 4 to 6 days of incubation at 30°C. Plasmid DNA was isolated from each 20 randomly chosen transformants and then analyzed with respect to their restriction patterns. All transformants harbored plasmid DNA, indicating that autonomous replication of both plasmids occurs in *G. polyisoprenivorans.* They were therefore suitable as *E. coli*-*G. polyisoprenivorans* shuttle vectors. However, restriction analysis revealed that ca. 50% of the plasmids recovered from the recombinant clones had undergone identical modifications resulting in truncations of the 5.1-kbp *Eco*RI fragment of plasmid pNC9503 by deletion of ca. 800 bp. By changing the electroporation protocol, these modifications were prevented (see below).

Because first electroporation experiments led to transformation rates of only about 10^3 transformants/μg of plasmid DNA, the electroporation protocols for both strains of *G. polyisoprenivorans* were systematically optimized. For this, one parameter of cultivation or of the electroporation conditions was altered at a time, whereas the others were kept constant. The optimum of the field strength was 10 kV/cm. Transformation efficiencies depended strongly on the cultivation conditions, the medium, and the type and concentration of cell wall-weakening additives. The most suitable basic medium to obtain electrocompetent cells was Luria-Bertani (LB) broth (18); LB broth was twofold more efficient than nutrient broth (ADSA-Micro, Barcelona, Spain) or standard I complex nutrient broth (Merck, Darmstadt, Germany). Highest transformation efficiencies were obtained if cells were used from the early growth phase when the cultures had reached optical densities of 0.5 at 600 nm. Therefore, all subsequent alterations of medium composition and cultivation conditions were done with LB me-

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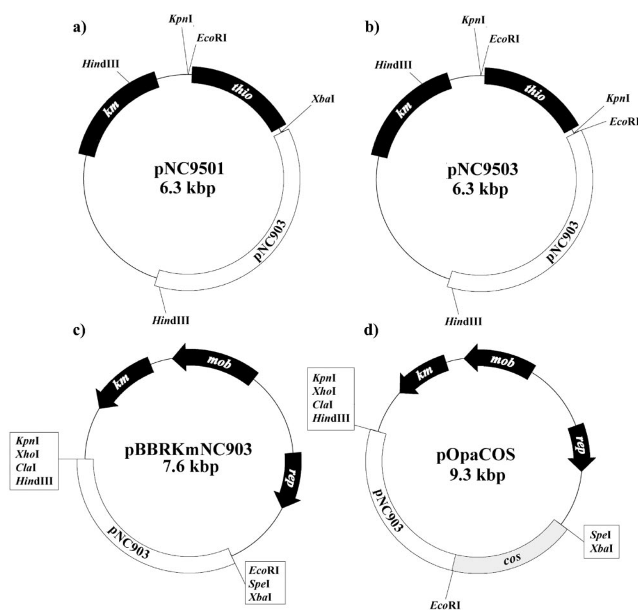

FIG. 1. Molecular organization of the *E. coli*/*Rhodococcus* (*Gordonia*) shuttle vectors used in the present study. pNC9501 (a) and pNC9503 (b) differ in the localization and orientation of restriction sites for *Eco*RI, *Kpn*I, and *Xba*I. (c) Mobilizable plasmid pBBRKmNC903. (d) Mobilizable cosmid pOpaCOS. Relevant structural genes and other elements are indicated: *km*, kanamycin resistance gene; *thio*, thiostrepton resistance gene; pNC903, fragment from pNC903 comprising the *ori* for replication in coryneform actinomycetes; *mob*, required for mobilization; *rep*, required for replication in *E. coli*; *cos*, *cos* site required for lambda packaging.

dium, and the effects of sucrose, glycine, and isonicotinic acid hydrazide on transformation efficiency were investigated in a range previously described for *Rhodococcus* spp. (12). Glycine and sucrose in the medium enhanced the electroporation efficiency most effectively at concentrations of 0.5% (wt/vol) and 1.5% (wt/vol), respectively. Optimal concentration of isonicotinic acid hydrazide was 1.5 μg/ml; its addition increased the efficiency of electroporation about twofold. Plasmid DNA concentrations of ≤0.25 μg/ml resulted in the highest transformation rates. Temperatures and the duration of temperature shifts used for preincubation or incubation after the electroporation pulse also affected transformations. For *G. polyisoprenivorans* highest transformation efficiencies of up to 4×10^5 CFU/μg of plasmid DNA were obtained with cells grown at 30°C, and if they were incubated for 10 min at 0°C before and for 6 min at 46°C after the electroporation pulse. This heat shock also suppressed the 800-bp deletion of transformed plasmid DNA. The optimized electroporation protocol is as follows: DNA was purified from *E. coli* strains and dialyzed against distilled H_2O by using microfilters (pore size of 0.025 μm; Millipore, Eschborn, Germany). For growth of *G. polyisoprenivorans* 50 ml of LB medium supplemented with 0.5% (wt/vol) glycine, 1.5% (wt/vol) sucrose, and 1.5 μg of isonicotinic acid hydrazide/ml in a 250-ml Erlenmeyer flask were inoculated with 1 ml of an overnight preculture in standard I complex nutrient broth medium, and the cells were grown at 30°C to an optical density of 0.5 at 600 nm. Cells were harvested, washed twice, and concentrated 20-fold in cold double-distilled H_2O. Competent cells were either used directly for electroporation or stored at −70°C. Immediately before electroporation, 400 μl of competent cells were mixed with 0.001 to 10 μg of DNA and preincubated 10 min on ice. Electroporation with a model 2510 electroporator was performed in electrocuvettes (Eppendorf-Netheler-Hinz, Hamburg, Germany) with gaps of 2 mm and at the following settings: 10 kV/cm, 600 Ω, and 25 μF. Time constants of 4 to 5 ms were reached. Pulsed cells were immediately diluted with 600 μl of LB, incubated for 6 min at 46°C, regenerated at 30°C for 4 h, and plated on appropriate selective media, and transformants were identified after 4 to 6 days of incubation. In controls, no spontaneous kanamycin-resistant colonies occurred. The survival rate without heat shock was 68% (VH2) and 63% (Y2K) after electroporation and dropped to 44% (VH2) and 36% (Y2K) if heat shock was applied. This protocol was also applied to 16 different strains belonging to 12 different species of the genus *Gordonia* (Table 1). The transformation efficiencies for the other *Gordonia* strains were significantly lower than for *G. polyisoprenivorans* VH2 and Y2K and ranged between 10^2 and 10^4 CFU/μg of plasmid DNA. Autonomous replication of plasmid pNC9503 was shown to occur in *G. alkanivorans* DSM44187, *G. nitida* DSM44499^T, *G. rubropertincta* DSM43197^T, *G. rubropertincta* DSM46038, and *G. terrae* DSM43249^T.

Construction of mobilizable vectors for conjugational transfer. Because efficiencies of plasmid DNA transfer by electroporation decrease with increasing plasmid sizes (23), transfer of vectors by conjugation using *E. coli* S17-1 as a donor for *G. polyisoprenivorans* was also investigated. Two mobilizable vectors were constructed. (i) *oriV*, comprising a 2.4-kbp *Eco*RI/*Hin*dIII fragment of plasmid pNC9503, which mediates stable replication in *G. polyisoprenivorans*, was cloned into *Eco*RI/*Hin*dIII-digested DNA of the gram-negative broad-host-range vector pBBR1MCS-2 (14) (GenBank accession no. U23751), yielding plasmid pBBRKmNC903 (Fig. 1c). (ii) A 1.7-kbp *Bgl*II fragment containing the *cos* sites enabling lambda packaging of large DNA molecules for creating genomic libraries was derived from vector pHC79 (11) (GenBank accession no. L08873). It was treated with mung bean nuclease and subsequently cloned into *Sma*I-digested pBBR1MCS-2 DNA, yielding pBBR1MCS-2cos (data not shown). Afterward, the 2.4-kbp *Eco*RI/*Hin*dIII restriction fragment of plasmid pNC9503 containing the *oriV* of pNC903 was cloned into *Eco*RI/*Hin*dIII-digested pBBR1MCS-2cos DNA, yielding pOpaCOS (Fig. 1d). Applying a protocol described previously (6), recipient transfer frequencies of 6×10^{-7} for vector pBBRKmNC903 and 5×10^{-6} for vector pOpaCOS were obtained.

Recombinant biosynthesis of polyhydroxyalkanoates (PHAs) in *G. polyisoprenivorans*. To analyze the suitability of these plasmids for transfer and heterologous expression of foreign genes in *G. polyisoprenivorans*, recombinant strains of *G. polyisoprenivorans* VH2 and Y2K capable of PHA synthesis were constructed. Substrates of PHA_{MCL} synthase (3-hydroxyacyl-coenzyme A) are available from β-oxidation when the cells grow on *n*-alkanes. Furthermore, PHA biosynthesis was previously reported for various species of the closely related genus *Rhodococcus* (1, 8) and could be established in recombinant strains of *R. opacus*. When pAK71 (12) harboring *phaC1* from *Pseudomonas aeruginosa* was introduced into VH2 and Y2K, the recombinant strains accumulated PHAs, contributing up to

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
Gordonia spp.		
G. alkanivorans MoAcy2	Alkane-degrading wild type	DSM44187
G. alkanivorans HKI 0136	Alkane-degrading wild type	DSM44369^T
G. amarae 9c		DSM43391
G. amarae Se6		DSM43392^T
G. amicalis IEGM	Benzothiophene-desulfurizing wild type	DSM44461^T
G. desulfuricans 213E	Benzothiophene-desulfurizing wild type	DSM44462^T
G. hirsuta K718a		DSM44140^T
G. hydrophobica 1610/1b		DSM44015^T
G. nitida LE31	3-Ethylpyridine- and 3-methylpyridine-degrading wild type	DSM44499^T
G. polyisoprenivorans Kd2	Rubber-degrading wild type	DSM44302^T
G. polyisoprenivorans VH2	Rubber-degrading wild type	DSM44266
G. polyisoprenivorans Y2K	Rubber-degrading wild type	2
G. rhizosphera 141		DSM44383^T
G. rubropertincta N4		DSM43197^T
G. rubropertincta 60017		DSM46038
G. terrae NCTC 10669		DSM43249^T
G. terrae T6		DSM43342
G. westfalica Kb1	Rubber-degrading wild type	DSM44215^T
E. coli		
S17-1	*thi-1 proA hsdR17* (r_K^- m_K^+) *recA1*, *tra* genes of RP4 integrated into the chromosome	22
XL1-Blue	*recA1 endA gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ^- *lac*[F *proAB lacI*^{q} ZΔ M15 Tn*10*(Tc^r)]	4
Plasmids		
pNC9501	*E. coli*/*Rhodococcus* (*Gordonia*) shuttle vector; *km*, *thio*, pNC903 *ori*	H. Saeki, Japan Energy Corporation (12)
pNC9503	*E. coli*/*Rhodococcus* (*Gordonia*) shuttle vector; *km*, *thio*, pNC903 *ori*	H. Saeki, Japan Energy Corporation (12)
pBBRKmNC903	*E. coli*/*Rhodococcus* (*Gordonia*) shuttle vector, mobilizable; *km*, pNC903 *ori*	This study
pOpaCOS	*E. coli*/*Rhodococcus* (*Gordonia*) shuttle vector, mobilizable; *cos*, *km*, pNC903 *ori*	This study
pAK71	*phaC1* from *P. aeruginosa*; *thio*, pNC903 *ori*	12
pHC79	*ap*, *tc*, *cos*	11
pBBR1MCS-2	Gram-negative broad-host-range vector, mobilizable; *km*	14

8.3 or 13.2%, respectively, of the cell dry matter during cultivation on mineral salts medium under conditions of N starvation on long-chain *n*-alkanes (20). Gas chromatography (GC) and GC-mass spectrometry (MS) analysis of accumulated PHAs (3) revealed that copolyesters mainly consisting of odd-numbered 3-hydroxyalkanoates (3HHp, 3HHN, 3HUD, and 3HTD; >88 mol%) were synthesized from pentadecane, whereas PHAs mainly consisting of even-numbered 3-hydroxyalkanoates (3HO, 3HD, and 3HDD; ~75 mol%) were synthesized from hexadecane (Table 2).

Conclusions. The present study succeeded in establishing and optimizing two different gene transfer systems for the rubber-degrading, gram-positive bacterium *G. polyisoprenivorans* strains VH2 and Y2K and several other members of the genus *Gor-*

TABLE 2. PHA accumulation by recombinant strains of *G. polyisoprenivorans* VH2 and Y2K after cultivation in media containing different carbon sources[a]

G. polyisoprenivorans strain	Plasmid	Carbon source	PHA content (% CDW)	PHA composition (mol%)							
				3HHx	3HHp	3HO	3HN	3HD	3HUD	3HDD	3HTD
VH2	pNC9503	Pentadecane	ND	ND	ND	ND	ND	ND	ND	ND	ND
	pAK71	Pentadecane	8.3	ND	19.1	6.0	32.9	5.7	23.0	TR	13.3
	pNC9503	Hexadecane	ND	ND	ND	ND	ND	ND	ND	ND	ND
	pAK71	Hexadecane	7.4	TR	12.2	28.5	10.3	31.3	ND	17.7	ND
Y2K	pNC9503	Pentadecane	ND	ND	ND	ND	ND	ND	ND	ND	ND
	pAK71	Pentadecane	13.2	ND	18.2	6.2	32.0	4.1	24.2	TR	13.1
	pNC9503	Hexadecane	ND	ND	ND	ND	ND	ND	ND	ND	ND
	pAK71	Hexadecane	8.7	TR	13.0	25.7	12.8	30.0	ND	18.5	ND

[a] The cells were cultivated in 50 or 1,000 ml of MSM containing 0.01% (wt/vol) NH_4Cl in the presence of 25 μg of thiostrepton/ml for 96 h at 30°C. Hexadecane and pentadecane were added at concentrations of 0.2% (vol/vol). The content and composition of PHAs were analyzed by GC and coupled GC-MS as described previously (3). ND, not detected; TR, traces (<2.0 mol%); 3HHx, 3-hydroxyhexanoate; 3HHp, 3-hydroxyheptanoate; 3HO, 3-hydroxyoctanoate; 3HN, 3-hydroxynonanoate; 3HD, 3-hydroxydecanoate; 3HUD, 3-hydroxyundecanoate; 3HDD, 3-hydroxydodecanoate; 3HTD, 3-hydroxytridecanoate; CDW, cellular dry weight.

donia based on electroporation. Furthermore, conjugational plasmid transfer with *E. coli* S17-1 as the donor, enabling the transfer of large constructs as required for the phenotypic complementation of mutants, was established. This is the first description of genetic transfer of DNA and maintenance of foreign plasmids for various species of the genus *Gordonia*. It will make these bacteria accessible for genetic engineering, complementation of mutants, and heterologous expression of genes to reveal the molecular and biochemical basis of interesting metabolic pathways of *Gordonia* species. Transformation efficiencies of up to 4×10^5 CFU/μg of plasmid DNA are sufficiently high to comply with the demands of standard genetic techniques and resemble those reported for *R. opacus* (12), *Rhodococcus* sp. strain TE1 (21), *R. fascians* (5), and *Clavibacter michiganensis* subsp. *sepedonicus* (15). The application of a heat shock after electroporation increased transformation efficiencies, as reported for *Corynebacterium glutamicum* (25), and prevented the specific deletion of introduced plasmid DNA. Presumably, both effects were due to the inactivation of a restriction system (19, 25). The newly established electrotransformation protocol was successfully applied to establish a functional active PHA synthase of *P. aeruginosa* in *G. polyisoprenivorans*, resulting in PHA_{MCL} biosynthesis from *n*-alkanes. The *E. coli lacZ* promoter of pAK71 located upstream of *phaC1* was obviously recognized by the *G. polyisoprenivorans* RNA polymerase. Since PHA_{MCL} biosynthesis did not depend on IPTG (isopropyl-β-D-thiogalactopyranoside) addition, *G. polyisoprenivorans* obviously does not produce a *lac* repressor, and *lacZ* promoter dependent genes are constitutively expressed.

REFERENCES

1. **Alvarez, H. M., R. Kalscheuer, and A. Steinbüchel.** 1997. Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effects of inhibitors and polyethylene glycol. Fett/Lipid **99:**239–246.
2. **Arenskötter, M., D. Baumeister, M. M. Berekaa, G. Pötter, R. M. Kroppenstedt, A. Linos, and A. Steinbüchel.** 2001. Taxonomic characterization of two rubber-degrading bacteria belonging to the species *Gordonia polyisoprenivorans* and analysis of hypervariable regions of 16S rDNA sequences. FEMS Microbiol. Lett. **205:**277–282.
3. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
4. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: high efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection. BioTechniques **5:**376–378.
5. **Desomer, J., P. Dhaese, and M. van Montagu.** 1990. Transformation of *Rhodococcus fascians* by high-voltage electroporation and development of *R. fascians* cloning vectors. Appl. Environ. Microbiol. **56:**2818–2825.
6. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147:**198–205.
7. **Gilbert, S. C., J. Morton, S. Buchanan, C. Oldfield, and A. McRoberts.** 1998. Isolation of a unique benzothiophene-desulphurizing bacterium, *Gordona* sp. strain 213E (NCIMB 40816), and characterization of desulphurization pathway. Microbiology **144:**2545–2553.
8. **Haywood, G. W., A. J. Anderson, D. Williams, E. A. Dawes, and D. Ewing.** 1991. Accumulation of a poly(hydroxyalkanoate) copolymer containing primilary 3-hydroxyvalerate from simple carbohydrate substrates by *Rhodococcus ruber* NCIMB 40126. Int. J. Biol. Macromol. **13:**83–88.
9. **Hernandez-Perez, G., F. Fayolle, and J.-P. Vandecasteele.** 2001. Biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE) and *t*-amyl methyl ether (TAME) by *Gordonia terrae*. Appl. Microbiol. Biotechnol. **55:**117–121.
10. **Hirasawa, K., Y. Ishii, M. Kobayashi, K. Koizumi, and K. Maruhashi.** 2001. Improvement of desulfurization activity in *Rhodococcus erythropolis* KA2–5–1 by genetic engineering. Biosci. Biotechnol. Biochem. **65:**239–246.
11. **Hohn, B., and J. Collins.** 1980. A small cosmid for efficient cloning of large DNA fragments. Gene **11:**291–298.
12. **Kalscheuer, R., M. Arenskötter, and A. Steinbüchel.** 1999. Establishment of a gene transfer system for *Rhodococcus opacus* PD630 based on electroporation and its application for recombinant biosynthesis of poly(3-hydroxyalkanoic acids). Appl. Microbiol. Biotechnol. **52:**508–515.
13. **Klatte, S., F. A. Rainey, and R. M. Kroppenstedt.** 1994. Transfer of *Rhodococcus aichiensis* Tsukamurella 1982 and *Nocardia amarae* Lechevalier and Lechevalier 1974 to the genus *Gordona* as *Gordona aichiensis* comb. nov. and *Gordona amarae* comb. nov. Int. J. Syst. Bacteriol. **44:**769–773.
14. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
15. **Laine, M. J., H. Nakhei, J. Dreier, K. Lehtilä, D. Meletzus, R. Eichenlaub, and M. C. Metzler.** 1996. Stable transformation of the gram-positive phytopathogenic bacterium *Clavibacter michiganensis* subsp. *spedonicus* with several cloning vectors. Appl. Environ. Microbiol. **62:**1500–1506.
16. **Linos, A., A. Steinbüchel, C. Spröer, and R. M. Kroppenstedt.** 1999. *Gordonia polyisoprenivorans* sp. nov., a rubber-degrading actinomycete isolated from an automobile tire. Int. J. Syst. Bacteriol. **49:**1785–1791.
17. **Rauzier, J., J. Moniz-Pereira, and B. Gicquel-Sanzey.** 1988. Complete nucleotide sequence of pAL5000, a plasmid from *Mycobacterium fortuitum*. Gene **71:**315–321.
18. **Sambrook, J. E., F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Schäfer, A., J. Kalinowski, and A. Pühler.** 1994. Increased fertility of *Corynebacterium glutamicum* recipients in intergeneric matings with *Escherichia coli* after stress exposure. Appl. Environ. Microbiol. **60:**759–763.
20. **Schlegel, H. G., H. Kaltwasser, and G. Gottschalk.** 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. **38:**209–222.
21. **Shao, Z., W. A. Dick, and R. M. Behki.** 1995. An improved *Escherichia coli-Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* ssp. using electroporation. Lett. Appl. Microbiol. **21:**261–266.
22. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vitro genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–791.
23. **Szostková, M., and D. Horáková.** 1998. The effect of plasmid DNA size and other factors on electrotransformation of *Escherichia coli* JM109. Bioelectrochem. Bioenerg. **47:**319–323.
24. **Takeshita, S., M. Sato, M. Toba, W. Masahashi, and T. Hashimoto-Gotoh.** 1987. High-copy-number and low-copy-number plasmid vectors for *lacZ*α-complementation and chloramphenicol- or kanamycin-resistance selection. Gene **61:**63–74.
25. **van der Rest, M. E., C. Lange, and D. Molenaar.** 1999. A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. Appl. Microbiol. Biotechnol. **52:**541–545.