Appl Microbiol Biotechnol (2008) 77:1317–1327
DOI 10.1007/s00253-007-1262-8

Transfer of megaplasmid pKB1 from the rubber-degrading bacterium *Gordonia westfalica* strain Kb1 to related bacteria and its modification

Daniel Bröker · Matthias Arenskötter · Alexander Steinbüchel

Received: 24 September 2007 / Revised: 17 October 2007 / Accepted: 22 October 2007 / Published online: 22 November 2007

Abstract Because engineering of the 101.016-bp megaplasmid pKB1 of *Gordonia westfalica* Kb1 failed due to the absence of an effective transfer system, pKB1 was transferred by conjugation from *G. westfalica* Kb1 to a kanamycin-resistant mutant of *Rhodococcus opacus* PD630 at a frequency of about 6.2×10^{-8} events per recipient cell. Furthermore, pKB1 was transferred to *G. polyisoprenivorans* strains VH2 and Y2K and to *Mycobacterium smegmatis* by electroporation at frequencies of 5.5×10^{3}, 1.9×10^{3}, and 8.3×10^{2} transformants per microgram plasmid DNA. The pKB1-encoded cadmium resistance gene *cadA* was used for selection in these experiments. Recombinant pKB1-containing *G. polyisoprenivorans* VH2 and *M. smegmatis* were then used to engineer pKB1. A kanamycin resistance cassette was inserted into the pKB1-encoded *cadA* gene, ligated to suicide plasmid pBBR1MCS-5, and the resulting plasmid was electroporated into plasmid-harboring strains. Homologous recombination between *cadA* on suicide plasmid and the respective sequence in pKB1 led to its integration into pKB1. Thus, two selection markers were accommodated in pKB1 to monitor plasmid transfer into *Gordonia* and related taxa for analysis of genes essential for rubber degradation and others. In this study, two transfer methods for large plasmids and strategies for engineering of pKB1 were successfully applied, thereby, extending the tool box for *Gordonia*.

Keywords *Gordonia* · Megaplasmid · Conjugational transfer · Electroporation · Heavy metal resistance · Cadmium · Genetic manipulation · Suicide plasmid

D. Bröker · M. Arenskötter · A. Steinbüchel (✉)
Institut für Molekulare Mikrobiologie und Biotechnologie,
Westfälische Wilhelms-Universität Münster,
Corrensstrasse 3,
48149 Münster, Germany
e-mail: steinbu@uni-muenster.de

Introduction

Gordonia sp. are Gram-positive bacteria belonging to the so-called CMN group (*Corynebacterium*, *Mycobacterium*, and *Nocardia*) of actinomycetes that possess a variety of interesting metabolic capabilities (Arenskötter et al. 2004). Several members of the genus *Gordonia* exhibit special degradation activities such as degradation of poly(*cis*-1,4-isoprene) by *G. westfalica* strain Kb1 (Linos and Steinbüchel 1998; Linos et al. 1999, 2002) and *G. polyisoprenivorans* (Linos and Steinbüchel 1998; Arenskötter et al. 2001); desulfurization of benzothiophene by *G. desulfuricans* (Gilbert et al. 1998; Kim et al. 1999) or dibenzothiophene by *G. amicalis* (Kim et al. 2000); biodegradation of ethyl *t*-butyl ether (ETBE), methyl *t*-butyl ether (MTBE), and *t*-amyl methyl ether (TAME) by *G. terrae* (Hernandez-Perez et al. 2001); degradation of 3-ethylpyridine and 3-methylpyridine by *G. nitida* (Yoon et al. 2000); and degradation of propane by *Gordonia* sp. strain TY-5 (Kotani et al. 2003, 2007).

There is still a lack of suitable genetic tools to apply genetic engineering and recombinant DNA techniques to members of this interesting group of bacteria. Recently, *Escherichia coli*/*Rhodococcus* shuttle vectors suitable for transfer of foreign DNA to members of the genus *Gordonia* were described (Arenskötter et al. 2003). The origin of replication of a circular 101,016-bp megaplasmid pKB1 from the poly(*cis*-1,4-isoprene)-degrading bacterium *G. westfalica* strain Kb1 was identified and used for construction of *E. coli*/*Gordonia* shuttle vectors suitable for gene cloning and expression in several *Gordonia* species and members of related taxa (Bröker et al. 2004). One of these constructed *E. coli*/*Gordonia* shuttle vectors and an *E. coli*/*Rhodococcus* shuttle vector were suitable for transformation of *G. jacobae* by electroporation (Veiga-Crespo et al. 2006). Little is also known about vectors appropriate for transposon mutagenesis

or for generation of knock-out mutants in *Gordonia* sp. Stable Tn*5096*-based transposon mutagenesis in *G. polyisoprenivorans* strain VH2 was only recently established (Banh et al. 2005). Although the thermosensitive plasmid pCG79 could be transferred to *G. polyisoprenivorans* strain VH2, indicating stable propagation of the plasmid, Tn*611*-based transposon mutagensis in the same strain yielded unstable mutants reverting to a wild-type phenotype (Banh et al. 2005). Others described the insertion of a plasmid by homologous recombination between a cloned sequence within the plasmid and the corresponding sequence in the chromosomes of *G. amarae* (Dogan et al. 2006) and *G. polyisoprenivorans* strain VH2 (Arenskötter et al., unpublished data).

Because evidence was obtained that genes essential for rubber degradation are encoded by pKB1 (Bröker et al. 2004), the need for manipulation of this plasmid arose. Several attempts to engineer pKB1 in *G. westfalica* strain Kb1 failed because of the absence of an effective gene transfer system for this strain. To engineer plasmid pKB1, it has to be transferred to strains that are genetically approachable. To monitor successful transfer of pKB1 into related strains, a pKB1-specific selection marker had to be identified. Attention was drawn to the pKB1-encoded gene *cadA* (ORF34). Its translational product was related to a putative Cd^{2+}/Zn^{2+}-transporting P-type ATPase, and its heterologous expression conferred cadmium but not zinc resistance to *E. coli* strain RW3110 (Bröker et al. 2004). In this study, it was investigated whether *cadA* is a useful selection marker for transfer of pKB1 into cadmium-sensitive strains of *Gordonia* and related taxa by electroporation and conjugation. The various translational products of a large region comprising putative conjugative transfer genes located on pKB1 should enable conjugative transfer of pKB1 from the natural host *G. westfalica* strain Kb1 as donor to related bacteria in which this megaplasmid is replicated. For example, ORF67 presumably encodes a TraA-like protein with 31% identity to TraA encoded by megaplasmid pREA400 of *R. erythropolis* strain AN12; the latter was recently demonstrated to be essential for conjugation (Yang et al. 2007). DNA relaxases encoded by *traA* genes are the main components for initiation of conjugative plasmid transfer because they catalyze the cleavage of one plasmid strand at the *nic* site within the *oriT* via a transesterification reaction. Transfer of the nicked plasmid to the recipient cell occurs unidirectional in the form of a single-stranded DNA intermediate (for reviews, see references Pansegrau and Lanka 1996, Byrd and Matson 1997, and Grohmann et al. 2003).

In this study, we demonstrated the transfer of megaplasmid pKB1 to genetically approachable cadmium-sensitive strains of *Gordonia* and related taxa by conjugation and also by electroporation using *cadA* as selection marker. Furthermore, genetic manipulation of pKB1 in the resulting recombinant strains of *G. polyisoprenivorans* strain VH2 and *M. smegmatis* strain mc^2155 by integration of a suicide vector into plasmid pKB1 was successfully demonstrated. This yielded a recombinant plasmid pKB1 with two useful additional antibiotic selection markers (kanamycin and gentamycin resistance) to monitor transfer of this plasmid into other cadmium-resistant strains for determination of new plasmid-encoded features.

Materials and methods

Bacterial strains, plasmids, and cultivation conditions

Bacteria and plasmids used in this study are listed in Table 1. All strains of the genus *Gordonia* and *Rhodococcus opacus* strain PD630 were grown at 30°C on standard I complex nutrient broth (St-I, Merck, Darmstadt, Germany) or mineral salts medium (MSM; Schlegel et al. 1961). *Mycobacterium smegmatis* strain mc^2155 was grown at 30°C on Luria Bertani (LB) broth (Sambrook et al. 1989) containing 0.05% (*w*/*v*) Tween 80 or mineral salts medium (MSM; Schlegel et al. 1961). Carbon sources were added to liquid MSM as indicated in the text. Cells of *E. coli* strains were cultivated at 37°C in LB broth. Antibiotics were applied according to Sambrook et al. (1989) and as indicated in the text. Liquid cultures were done in Erlenmeyer flasks and incubated on a horizontal rotary shaker at 180 rpm. Solid media were prepared by addition of agar-agar (15 g/l).

Kanamycin-resistant mutant of *R. opacus* strain PD630

For selection of a spontaneous kanamycin resistant mutant of *R. opacus* strain PD630, 50 ml St-I medium in 300-ml Erlenmeyer flasks were inoculated with cells of this strain and incubated at 30°C. After 2 days incubation, 50 µl cell suspensions were used to inoculate Erlenmeyer flasks with fresh St-I medium containing a slightly increased kanamycin concentration. After successive increase of the kanamycin concentration from 0 to 100 µg/ml, cells deriving from a culture containing the highest kanamycin concentration were diluted and spread on St-I agar plates containing 100 µg/ml kanamycin. This procedure yielded the spontaneous kanamycin-resistant mutant PD630Km100, which was used as recipient for conjugative transfer of megaplasmid pKB1.

Determination of metal tolerance

For the recombinant, pKB1-harboring Gram-positive bacteria of the genera *Gordonia*, *Rhodococcus*, and *Mycobacterium*, St-I agar plates containing 100 to 800 µM $CdCl_2$ were prepared using a 0.1-M stock solution of $CdCl_2 \cdot H_2O$ (Merck), which was sterilized by filtration through a 0.22-µm

Table 1 Bacterial strains and plasmids used in this study

Bacteria and plasmids	Relevant markers	Reference and source
Strains		
Gordonia species		
G. alkanivorans strain 44369	Hexadecane-degrading wild type	Kummer et al. 1999
G. polyisoprenivorans strain VH2	Poly(*cis*-1,4-isoprene)-degrading wild type	Arenskötter et al. 2001
G. polyisoprenivorans strain Y2K	Poly(*cis*-1,4-isoprene)-degrading wild type	Arenskötter et al. 2001
G. westfalica strain Kb1	Poly(*cis*-1,4-isoprene)-degrading wild type	Linos et al. 2002
Mycobacterium species		
M. smegmatis strain mc^{2}155	Transformation efficient mutant	Snapper et al. 1990
Rhodococcus species		
R. opacus strain PD630	Wild type, TAG$^+$	DSM 44193
R. opacus strain PD630Km100	Spontaneous kanamycin resistant mutant of *R. opacus* PD630	This study
E. coli strains		
E. coli strain XL1-Blue	*rec*A1, *end*A1, *gyr*A96, *thi*1, *hsd*R17 (r_k^-, m_k^+), *sup*E44, *rel*A1, λ$^-$, *lac* [F′, *pro*AB, *lacI*^{q}, *lacZ*ΔM15, Tn*10*(Tc^r)]	Bullock et al. 1987
E. coli strain DH5α	F$^-$, ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1, *endA*1, *hsd*R17 (r_k^-, m_k^+), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Roche Applied Science, Penzberg, Germany
Plasmids		
pKB1	Megaplasmid pKB1 of *G. westfalica* Kb1, *cadA*	Bröker et al. 2004
pNC9503	*aph*, *tsr*, *oriV* pNC9503	Kalscheuer et al. 1999
pBBRKmNC903	*aph*, *tsr*, *mob*, *oriV* pNC9503	Arenskötter et al. 2003
pDBMCS-2	*aph*, *lacPOZ'*, *mob*, *oriV* pKB1	Bröker et al. 2004
pDBMCS-5	*aacC1*, *lacPOZ'*, *mob*, *oriV* pKB1	Bröker et al. 2004
pCG76	*aadA*, temperature-sensitive derivative of pAL5000	Guilhot et al. 1994
pBluescript SK$^-$	*bla*, *lacPOZ'*	Stratagene
pBBR1MCS-5	*aacC1*, *lacPOZ'*, *mob*	Kovach et al. 1995
pBBRORF34::*aph*	*aacC1*, *lacPOZ'*, *mob*, *cadA* of pKB1 disrupted by *aph*	This study
pKB1pBBRORF34::*aph*	Megaplasmid pKB1 with insertion of pBBRORF34::*aph* (*aacC1*, *aph*)	This study

membrane filter (Roth, Karlsruhe, Germany). The plates were inoculated, and tolerance for cadmium was evaluated after 3 days incubation at 30°C.

Isolation, analysis, and modification of DNA

Plasmid DNA was prepared from crude lysates by the alkaline extraction method (Birnboim and Doly 1979). Before lysis, cells of *Gordonia*, *Rhodococcus*, and *Mycobacterium* were incubated in the presence of lysozyme (2 mg/ml) for 2 h at 37°C. DNA was restricted with restriction endonucleases (Gibco/BRL, Gaithersburg, USA) at the conditions recommended by the manufacturer. All other genetic procedures and modifications of DNA were conducted as described by Sambrook et al. (1989).

Transfer of DNA by electroporation

Plasmids pNC9503, pBBRKmNC903, pDBMCS-2, pDBMCS-5, pCG76, pCGredORF6, pCGredORF42, pCGORF6::*aph*, and pCGORF6::*aacC1* were transferred to *G. westfalica* strain Kb1 by electroporation using a model 2510 electroporator (Eppendorf-Netheler-Hinz, Hamburg, Germany) according to a previously described method (Arenskötter et al. 2003). Transfer of the isolated megaplasmid pKB1 from *G. westfalica* strain Kb1 and of the recombinant plasmid pKB1pBBRORF34::*aph* to species of the genera *Gordonia* and to *M. smegmatis* strain mc^{2}155 was performed by electroporation according to the same method. Also, purified and desalted plasmid pBBRORF34::*aph* was transferred to the pKB1-containing recombinant *G. polyisoprenivorans* strain VH2 and *M. smegmatis* strain mc^{2}155 by electroporation. Preparation of electrocompetent cells and execution of electroporation were done as described recently (Kalscheuer et al. 1999; Arenskötter et al. 2003). Plasmid pKB1pBBRORF34::*aph* was transferred to *E. coli* strain DH5α by electroporation. Preparation of electrocompetent cells of *E. coli* strain DH5α and execution of electroporation were performed according to a protocol described by Sheng et al. (1995).

Transfer of DNA by conjugation

Transfer of plasmids pBBRKmNC903, pDBMCS-2, and pDBMCS-5 (Table 1) was performed by conjugation, applying a previously described protocol (Friedrich et al. 1981), using *E. coli* S17–1 as donor and *G. westfalica* strain Kb1 as recipient. Conjugative transfer of plasmid

pKB1 was carried out by applying the same protocol (Friedrich et al. 1981) using *G. westfalica* strain Kb1 as donor and mutant strain PD630Km100 of *R. opacus* as recipient at a 1:1 cell ratio. Plasmid mobilization frequencies were determined by plating appropriate serial dilutions of the mating cell resuspensions onto selective media containing 500 μM $CdCl_2$ and by plating in parallel dilutions of the control recipient culture to obtain recipient viable counts on non-selective media. Mating efficiencies were calculated as transconjugant colony forming units (CFU) per recipient viable cell counts.

Detection of pKB1-harboring transformants

Single colonies of transformed cells were transferred into 5 ml of selective medium (500 μM $CdCl_2$) with toothpicks. The presence of pKB1 in $CdCl_2$-resistant transformants of strains of the genus *Gordonia*, *R. opacus* strain PD630Km100, and *M. smegmatis* strain mc^2155 was verified by plasmid isolation. Plasmid DNA was extracted by a modified procedure of the alkaline extraction method (Birnboim and Doly 1979), and the identity of pKB1 was demonstrated by restriction with appropriate endonucleases (Gibco/BRL; Fig. 2).

Gel permeation chromatography

Cleavage of poly(*cis*-1,4-isoprene) (#182141, Sigma-Aldrich, Steinheim, Germany) by recombinant pKB1-harboring strains of *R. opacus* PD630Km100 and of *M. smegmatis* mc^2155 was investigated by gel permeation chromatography (GPC). After a cultivation period of 8 weeks, samples were prepared in chloroform and analyzed as previously described (Ibrahim et al. 2006) employing a Waters–GPC system (Waters, Milford, CT, USA), consisting of a 515-HPLC pump, a 410 differential refractometer, a 717plus autosampler, and four in-series-connected Styragel columns (HR3, HR4, HR5, and HR6 with pore sizes of 10^3, 10^4, 10^5, or 10^6 Å, respectively). Molecular weights of poly(*cis*-1,4-isoprene) were calculated from retention times of defined poly(*cis*-1,4-isoprene) standards (PSS Polymer Standards Service GmbH, Mainz, Germany).

Plasmid constructions

Plasmids pCGredORF6 and pCGredORF42 were constructed to test their ability to integrate into plasmid pKB1 in its natural host *G. westfalica* strain Kb1. Using the two sets of oligonucleotides, P4226f*Bam*HI plus P4863b*Xba*I and P39530f*Bam*HI plus P40671b*Xba*I (Table 2), and Pfx DNA polymerase (Gibco BRL) according to the manufacturer's instructions, 637- or 1,141-bp sequences of ORF6 or ORF42 were amplified by PCR, respectively. The PCR products were cloned into *Sma*I-linerarized plasmid pBluescript SK^- (Stratagene, San Diego, USA), excised by restriction with *Bam*HI and *Xba*I, and ligated to *Bam*HI–*Xba*I linearized plasmid pCG76, yielding plasmids pCGredORF6 and pCGredORF42, which were transferred to *G. westfalica* strain Kb1 by electroporation.

To obtain stable mutants of *G. westfalica* strain Kb1, a gentamycin or kanamycin resistance gene should be integrated into pKB1 via homologous recombination. Therefore, two 1,469- and 1,430-bp pKB1-specific fragments were amplified by PCR using the two oligonucleotide sets, P3042f*Bam*HI plus P4510b*Hin*dIII and P4510f*Hin*dIII plus P5940b*Xba*I (Table 2), and Pfx DNA polymerase (Gibco BRL). The two obtained PCR products were cloned into *Sma*I-linerarized plasmid pBluescript SK^- (Stratagene); the 1,469- and 1,430-bp PCR products were excised by restriction with *Bam*HI plus *Hind*III or with *Hind*III plus *Xba*I, respectively. Both fragments and either an about 1,000-bp *Hind*III–*Hind*III kanamycin resistance cassette (*aph*) or an about 1,000-bp *Hind*III–*Hind*III gentamycin resistance cassette (*aacC1*) were ligated to *Bam*HI–*Xba*I-linearized plasmid pCG76, yielding plasmids pCGORF6::*aph* and pCGORF6::*aacC1*, which were transferred to *E. coli* strain XL1-Blue. Kanamycin- or gentamycin-resistant transformants harboring pCGORF6::*aph* or pCGORF6::*aacC1* were obtained, respectively. These plasmids were then isolated and transferred to *G. westfalica* strain Kb1 by electroporation.

For construction of the suicide vector pBBRORF34::*aph*, the 2,192-bp sequence of the *cadA* coding region of plasmid pKB1 (position 29,826–32,018) exhibited a unique restriction site for *Eco*72I 1,711 nucleotides downstream of the start codon. The entire 2,192-bp *cadA* region was amplified by PCR using oligonucleotides P29826f*Bam*HI and P32018b*Xba*I (Table 2). The PCR product was then cloned into *Sma*I-linearized plasmid pBluescript SK^- (Stratagene), excised by restriction with *Bam*HI and *Xba*I, ligated to *Bam*HI–*Xba*I-linearized plasmid pBBR1MCS-5 (Kovach et al. 1995) and transferred into *E. coli* strain XL1-Blue. Gentamycin-resistant colonies were obtained, and hybrid plasmid pBBRORF34 was isolated and sequenced. Because plasmid pBBR1MCS-5 contains no cleavage site for *Eco*72I, pBBRORF34 was linearized by restriction with *Eco*72I at position 1,711 of *cadA*. An about 1,000-bp *Sma*I–*Sma*I kanamycin resistance cassette (*aph*) was subsequently inserted at this position of *cadA*, yielding vector pBBRORF34::*aph*, which was transferred to *E. coli* strain XL1-Blue. Gentamycin- and kanamycin-resistant colonies were obtained. The final vector pBBRORF34::*aph* was sequenced and contained a kanamycin-resistance and a gentamycin-resistance gene as selectable markers. Because pBBRORF34::*aph* lacked an origin of replication for *Gordonia* and *Mycobacterium*, it represented a suicide

Table 2 Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)	Function
P4226f*Bam*HI	AAAGGATCCCGAATCTGCGCGGTTATTC	With P4863b*Xba*I, 637-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Bam*HI restriction sites used for cloning are underlined)
P4863b*Xba*I	AAATCTAGACGGAGGGATGAGTCTCAGGCAGC	With P4226f*Bam*HI, 637-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Xba*I restriction sites used for cloning are underlined)
P39530f*Bam*HI	AAAGGATCCATGCAATTCCTCTCGACCG	With P40671b*Xba*I, 1,141-bp PCR product of pKB1 (part of ORF42) from *G. westfalica* strain Kb1 (*Hin*dIII restriction sites used for cloning are underlined)
P40671b*Xba*I	GGGTCTAGAGTGCTGCACCAAGAAGGTG	With P39530f*Bam*HI, 1,141-bp PCR product of pKB1 (part of ORF42) from *G. westfalica* strain Kb1 (*Xba*I restriction sites used for cloning are underlined)
P3042f*Bam*HI	AAAGGATCCTGATCAGCAGAGCGATGCAG	With P4510b*Hin*dIII, 1,469-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Bam*HI restriction sites used for cloning are underlined)
P4510b*Hin*dIII	CACAAAGCTTATCGCAGCGATGTACCAGC	With P3042f*Bam*H, 1,469-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Hin*dIII restriction sites used for cloning are underlined)
P4510f*Hin*dIII	ATATAAGCTTCGCGCTTCCTGCGGTTCCC	With P5940b*Xba*I, 1,430-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Hind*III restriction sites used for cloning are underlined)
P5940b*Xba*I	GCATCTAGAGATGGGTGTGAGGTCGTCG	With P4510f*Hin*dIII, 1,430-bp PCR product of pKB1 (part of ORF6) from *G. westfalica* strain Kb1 (*Xba*I restriction sites used for cloning are underlined)
P29826f*Bam*HI	AAAGGATCCATGGCTGACGCATGCTGCGG	With P32018b*Xba*I, 2,192-bp PCR product of pKB1 (part of ORF34) from *G. westfalica* strain Kb1 (*Bam*HI restriction sites used for cloning are underlined)
P32018b*Xba*I	AAATCTGAGTCAGTGGCGTTCGCGATGGG	With P32018b*Xba*I, 2,192-bp PCR product of pKB1 (part of ORF34) from *G. westfalica* strain Kb1 (*Xba*I restriction sites used for cloning are underlined)

plasmid unable to replicate in these bacteria. Construction of this plasmid is summarized in Fig. 1.

Nucleotide sequence analysis

The *cadA* containing hybrid plasmids pBBRORF34 and pBBRORF34::*aph* were sequenced, employing IRD800-labeled universal and reverse primers, the SequiTherm Excel II Long-Read L-C kit, and a LI-COR 4200 sequencer (LI-COR Biosiences, Lincoln, Nebr.).

Results

Failure to transfer plasmids stably into *G. westfalica* strain Kb1

Transformation of *G. westfalica* strain Kb1 and, therefore, engineering of pKB1 failed because of the absence of efficient gene transfer systems for this strain. It was, for example, not possible to transfer plasmids like pNC9503 or pBBRKmNC903 (Arenskötter et al. 2003) and pDBMCS-2 or pDBMCS-5 (Bröker et al. 2004), suitable for other members of the genus *Gordonia*, to *G. westfalica* strain Kb1 by electroporation or by conjugation via *E. coli* strain S17-1.

Because the thermosensitive plasmid pCG79, a derivative of plasmid pCG76 containing Tn*611* for transposition, was able to replicate autonomously in *G. polyisoprenivorans* strain VH2 (Banh et al. 2005), the *E. coli*–mycobacteria shuttle vector pCG76 was transferred to *G. westfalica* strain Kb1 by electroporation. Approximately 80% of the obtained transformants, which were selected on St-I agar plates containing streptomycin (20 μg/ml), harbored plasmid pCG76, indicating autonomous replication of this plasmid in *G. westfalica* strain Kb1 at 30°C.

The thermosensitive plasmid pCG76 maintains in cells incubated at 30°C but is rapidly lost when the culture is incubated at 39°C (Guilhot et al. 1994). As *G. westfalica* strain Kb1 is able to grow until temperatures of 42°C, plasmid pCG76 can be applied as suicide plasmid in this strain and used for plasmid integration via homologous recombination between a pKB1-specific sequence on plasmid pCG76 and the respective sequence in pKB1. As

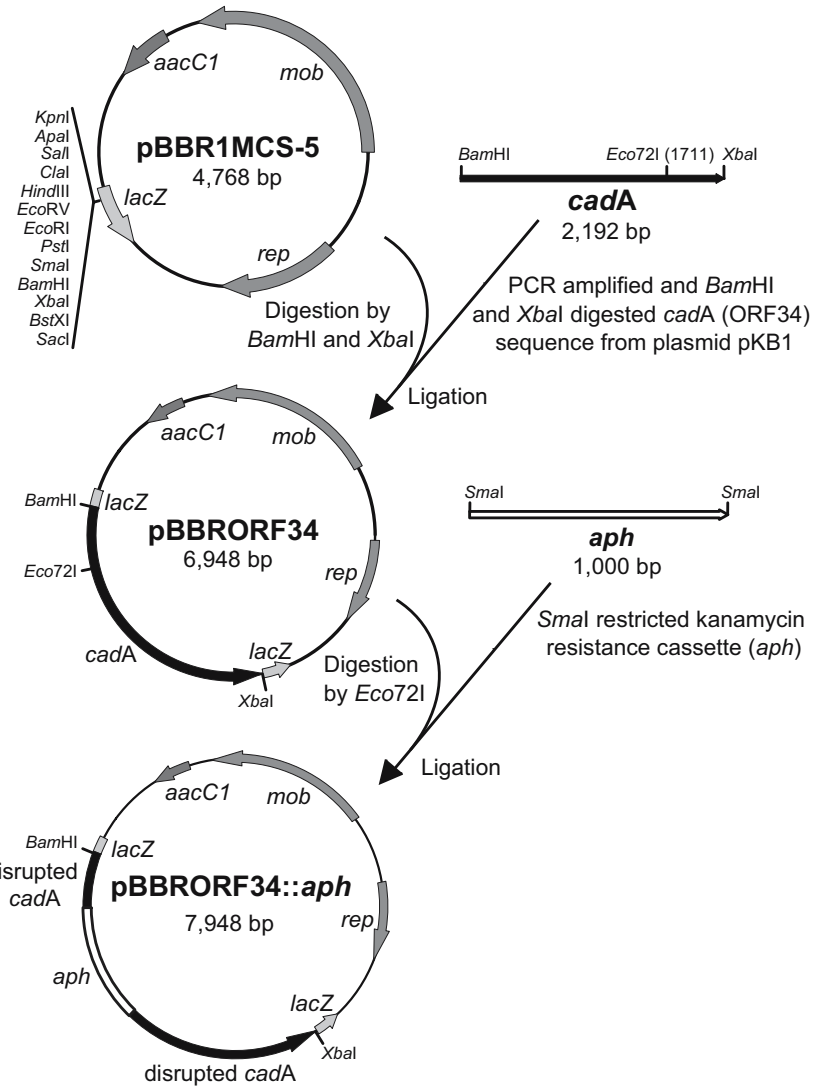

Fig. 1 Scheme for the construction of plasmid pBBRORF34:: *aph* derived from pBBR1MCS-5. Relevant cleavage sites and structural genes are indicated (*aacC1*, gentamycin-3-acetyltransferase gene or gentamycin resistance gene; *aph*, aminoglycoside phosphotransferase gene or kanamycin resistance gene; *lacZ*, β-galactosidase gene of *E. coli*; *rep*, *E. coli* origin of replication; *mob*, gene for plasmid mobilization when RK2 transfer functions are provided in *trans*; *cad*A, cadmium resistance gene of plasmid pKB1)

it was previously discussed that the putative gene products of the pKB1-encoded ORF6 (putative epoxide hydrolase) and ORF42 (putative cytochrome *c* oxidase) might have a metabolic function (Bröker et al. 2004), internal regions of ORF6 and ORF42 were used as pKB1-specific sequences to test the possibility to disrupt these ORFs by the aspired plasmid integration. Therefore, 637- and 1,141-bp sequences of ORF6 or ORF42, respectively, of plasmid pKB1 were amplified by PCR and ligated to plasmid pCG76, yielding plasmids pCGredORF6 and pCGredORF42, which were then transferred to *G. westfalica* strain Kb1 by electroporation. Selection of mutants with an integration of plasmid pCGredORF6 or pCGredORF42 and the required temperature shift to 40°C was performed as previously described (Guilhot et al. 1994; Banh et al. 2005). Plasmid DNA was isolated from 50 obtained streptomycin-resistant mutants. Only two mutants still harbored beside pKB1 episomal pCG76 derivative DNA, indicating loss or integration of pCGredORF6 or pCGredORF42, respectively, in the other mutants. Plasmid DNA isolated from the wild-type *G. westfalica* strain Kb1 and from ten mutants with putative integration of pCGredORF6 (designated ORF6M1 to ORF6M10) or pCGredORF42 (designated ORF42M1 to ORF42M10), respectively, were restricted with appropriate restriction endonucleases and separated in a 0.7% (*w*/*v*) agarose gel, which was stained with ethidium bromide. Comparison of the visualized restriction fragment pattern of plasmid DNA derived from the mutants to that of the wild type revealed the occurrence of an additional fragment with an increased size of 9.9 or 10.4 kbp, representing the size of vector pCGredORF6 or pCGredORF42, respectively, for the mutants. These results demonstrated integration of pCGredORF6 or pCGredORF42 into plasmid pKB1.

Mutants with an integration of pCG76 into plasmid pKB1 were further analyzed with respect to their capabilities to utilize rubber. Therefore, the two mutants ORF6M9 and ORF42M6 and also the wild-type *G. westfalica* strain Kb1 were exemplarily cultivated with poly(*cis*-1,4-isoprene) as the only carbon source at 40°C to prevent excision of the integrated thermosensitive vector pCG76. However, *G. westfalica* strain Kb1 wild type showed only very slow growth at 40°C with poly(*cis*-1,4-isoprene) as sole carbon source, which was also observed for *G. polyisoprenivorans* strain VH2 (Banh et al. 2005). Another problem was the acquired resistance to streptomycin of *G. westfalica* strain Kb1. In spite of selection with streptomycin (20 μg/ml), mutants ORF6M9 and ORF42M6 reverted to the wild-type genotype after two generations with a complete loss of the integrated plasmids pCGredORF6 or pCGredORF42, respectively (data not shown).

To overcome these problems, the integration of a gentamycin (*aacC1*) or kanamycin (*aph*) resistance gene into plasmid pKB1 via homologous recombination was targeted. Therefore, two pKB1-specific PCR products and a kanamycin or a gentamycin resistance cassette were ligated to pCG76, yielding plasmids pCGORF6::*aph* and pCGORF6::*aacC1*, respectively. After transfer of these hybrid plasmids to *G. westfalica* strain Kb1 by electroporation, neither kanamycin- nor gentamycin-resistant transformants were obtained, indicating that the plasmid-encoded resistance genes were not functionally expressed in *G. westfalica* strain Kb1. Therefore, direct genetic manipulation of plasmid pKB1 in its natural host *G. westfalica* strain Kb1 failed. Instead, megaplasmid pKB1 had to be transferred to genetically approachable strains to engineer pKB1 in the resulting transgenic strains.

Transfer of megaplasmid pKB1 by electroporation and transformation efficiency

From a previous study, it was known that *G. westfalica* strain Kb1 harboring megaplasmid pKB1 is able to grow in the presence of up to 800 μM $CdCl_2$ on St-I agar plates, whereas a mutant cured of this plasmid exhibited decreased cadmium resistance (Bröker et al. 2004). Tolerance for cadmium was investigated and evaluated after incubation for 3 days at 30°C on St-I agar plates containing $CdCl_2$ at concentrations between 100 and 800 μM for some other related bacteria. It was found that *G. polyisoprenivorans* strains VH2 (growth up to 150 μM cadmium) and Y2K (growth up to 150 μM cadmium) or *M. smegmatis* strain mc^2155 (growth up to 100 μM cadmium) and *R. opacus* strain PD630Km100 (growth up to 100 μM cadmium) exhibited a significantly lower cadmium resistance (Table 3). When electrocompetent cells of these three cadmium-sensitive strains were transformed with isolated megaplasmid pKB1 by electroporation, $CdCl_2$-resistant colonies were obtained on plates containing 500 μM $CdCl_2$, whereas no colonies occurred on the same plates with cells subjected to electroporation without plasmid. Transformation frequencies of plasmid pKB1 were 5.5×10^3, 1.9×10^3, and 8.3×10^2 transformants per μg plasmid DNA for *G. polyisoprenivorans* strain VH2, *G. polyisoprenivorans* strain Y2K, and *M. smegmatis* strain mc^2155, respectively, with each value representing the average from three independent experiments. Presence of

Table 3 Determination of cadmium tolerance for the different strains of *Gordonia*, *Rhodococcus* and *Mycobacterium* and for the recombinant strains harboring pKB1 or pKB1pBBRORF34::*aph*

Strains	Concentration of $CdCl_2$ (μM)									
	0	100	150	200	300	400	500	600	700	800
G. polyisoprenivorans strain VH2	++	++	+	–	–	–	–	–	–	–
G. polyisoprenivorans strain VH2 + pKB1	++	++	++	++	++	++	++	++	+	+
G. polyisoprenivorans strain VH2+pKB1pBBRORF34::*aph*	++	++	++	++	++	++	++	++	+	+
G. polyisoprenivorans strain Y2K	++	++	+	–	–	–	–	–	–	–
G. polyisoprenivorans strain Y2K+pKB1	++	++	++	++	++	++	++	++	+	+
G. polyisoprenivorans strain Y2K+pKB1pBBRORF34::*aph*	++	++	++	++	++	++	++	++	+	+
M. smegmatis strain mc^2155	++	+/–	–	–	–	–	–	–	–	–
M. smegmatis strain mc^2155+pKB1	++	++	++	++	++	++	++	+/–	+/––	+/––
M. smegmatis strain mc^2155 + pKB1pBBRORF34::*aph*	++	++	++	++	++	++	++	+/–	+/––	+/––
R. opacus strain PD630Km100	++	++	–	–	–	–	–	–	–	–
R. opacus strain PD630Km100+pKB1	++	++	++	++	++	++	++	++	+	+/–

The strains were cultivated on St-I medium agar plates containing the indicated concentration of cadmium. After an incubation period of 3 days at 30°C, growth was evaluated

++ Good growth, + growth, +/− limited growth, +/–– very limited growth, − no growth

megaplasmid pKB1 in the $CdCl_2$-resistant transformants was confirmed by isolation of plasmid DNA from cells; in addition, the restriction fragment patterns of these plasmids were identical with that of the plasmid isolated from the wild type (Fig. 2). Therefore, the pKB1-encoded cadmium resistance gene *cadA* could be used as selection marker.

Transfer of pKB1 by conjugation and megaplasmid mobilization frequency

To determine, whether megaplasmid pKB1 is transmissible by conjugation, a spontaneous kanamycin-resistant mutant of *R. opacus* strain PD630, which was referred to as PD630Km100, was generated and used as recipient for conjugative transfer of megaplasmid pKB1 by spot agar mating with *G. westfalica* strain Kb1 as donor. For selection of transconjugants from recipient and donor cells, the pKB1-encoded cadmium resistance gene *cadA* was used as selection marker. Colonies of transconjugants of *R. opacus* strain PD630Km100 appeared after 4 days of incubation on St-I agar plates containing 100 μg/ml kanamycin and 500 μM $CdCl_2$. Mobilization frequencies of megaplasmid pKB1 to the recipient *R. opacus* strain PD630Km100 were approximately 6.2×10^{-8} events per recipient cell as revealed in three independent experiments. Presence of plasmid pKB1 in the transconjugants was also confirmed physically by electrophoresis in crude lysates (Fig. 2).

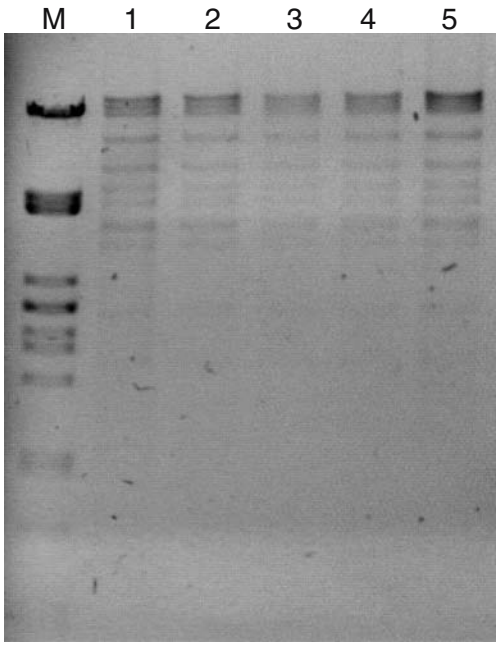

Fig. 2 Comparison of the restriction pattern of plasmid pKB1 from the native host versus the heterologous hosts. The isolated plasmid pKB1 DNA of the native host *G. westfalica* strain Kb1 and of the heterologous hosts *R. opacus* strain PD630Km100, *M. smegmatis* strain mc^2155, and *G. polyisoprenivorans* strains Y2K and VH2 was digested with *Bam*HI, separated by agarose gel electrophoresis and stained with ethidium bromide. *M* λ-DNA digested with *Pst*I, *1* pKB1 of *G. westfalica* strain Kb1, *2* pKB1 of the recombinant *R. opacus* strain PD630Km100, *3* pKB1 of the recombinant *M. smegmatis* strain mc^2155, *4* pKB1 of the recombinant *G. polyisoprenivorans* strain Y2K, and *5* pKB1 of the recombinant *G. polyisoprenivorans* strain VH2

Characterization of the recombinant plasmid pKB1-containing strains

Plasmid pKB1 conferred cadmium resistance to *G. polyisoprenivorans* strains VH2 and Y2K, as well as to *M. smegmatis* strain mc^2155 and *R. opacus* strain PD630Km100. In contrast to the parent strains, all recombinant strains of these bacteria harboring pKB1 were able to grow on St-I agar plates containing the maximum concentration of 800 μM $CdCl_2$ (Table 3).

To investigate whether plasmid pKB1 confers also poly (*cis*-1,4-isoprene) utilization to the non-rubber-degrading bacteria *M. smegmatis* strain mc^2155 and *R. opacus* strain PD630Km100, the recombinant pKB1-harboring strains were cultivated in MSM containing 0.25% (*w*/*v*) poly(*cis*-1,4-isoprene) as sole carbon and energy source. However, even after 30 days incubation the recombinant pKB1-containing strains did not exhibit any growth on or deterioration of poly(*cis*-1,4-isoprene). GPC analysis of the residual poly(*cis*-1,4-isoprene) with an average molecular mass of 800-kDa after 8 weeks of incubation did not indicate any changes in molecular weights or concentrations of the polymer (data not shown).

Modification of plasmid pKB1

The recombinant pKB1-harboring *G. polyisoprenivorans* strain VH2 and *M. smegmatis* strain mc^2155 were employed to modify pKB1. After amplification of *cadA* from *G. westfalica* strain Kb1 by PCR using pKB1 as template and primers P29826f*Bam*HI and P32018b*Xba*I, the PCR product was restricted with *Bam*HI plus *Xba*I and then ligated to *Bam*HI–*Xba*I-linearized plasmid pBBR1MCS-5 DNA, yielding plasmid pBBRORF34 as described in "Materials and methods." After restriction of pBBRORF34 with *Eco*72I, the linearized plasmid pBBRORF34 was ligated with a *Sma*I-restricted kanamycin resistance cassette (*aph*) to generate the vector pBBRORF34::*aph* (Fig. 1).

To examine the ability of the suicide plasmid pBBRORF34::*aph* to integrate into plasmid pKB1, the purified and desalted plasmid pBBRORF34::*aph* was transferred to the pKB1-harboring *G. polyisoprenivorans* strain VH2 and *M. smegmatis* strain mc^2155 by electroporation. Selection of kanamycin- (50 μg/ml) and gentamycin (10 μg/ml)-resistant transformants yielded recombinant strains in which pBBRORF34::*aph* had integrated into the target locus on plasmid pKB1 by a single-crossover event after homologous recombination. In total, 34

recombinant strains of *M. smegmatis* strain mc^{2}155, designated M1–M34, and 11 recombinant strains of *G. polyisoprenivorans* strain VH2, designated G1–G11, were obtained, which harbored the modified plasmid pKB1 with inserted vector pBBRORF34::*aph* and designated as pKB1pBBRORF34::*aph*. All 45 transformants harboring the modified plasmid pKB1pBBRORF34::*aph* were verified by isolation of plasmid DNA from kanamycin- and gentamycin-resistant cells and by PCR using primers P29826f*Bam*HI and P32018b*Xba*I. This gave two products: one 2,192-bp fragment representing the intact *cadA* gene and one 3,192-bp representing *cadA* with the inserted 1,000-bp kanamycin resistance cassette (data not shown). Therefore, plasmid pKB1pBBRORF34::*aph* resulted from a single-crossover event of pBBRORF34::*aph* into pKB1 with one wild-type copy of *cadA* plus the vector copy containing the inserted kanamycin resistance cassette, as it was expected for heterogenotes occurring after the performed selection of kanamycin and gentamycin. Thus, plasmid pKB1 was provided with these two additional antibiotic selection markers. If plasmid pKB1pBBRORF34::*aph* from transformants M1 and G1 was isolated and transferred to *G. polyisoprenivorans* strains VH2 and Y2K, and to *M. smegmatis* strain mc^{2}155 by electroporation, kanamycin- and gentamycin-resistant transformants were obtained from either strain, thus, indicating a stable propagation of plasmid pKB1pBBRORF34::*aph* in these strains. The presence of this plasmid was confirmed as described above.

Unfortunately, transfer of pKB1pBBRORF34::*aph* to *G. alkanivorans* strain 44369 by electroporation failed; no kanamycin- (50 μg/ml) and gentamycin (10 μg/ml)-resistant transformants were obtained. Plasmid pKB1pBBRORF34::*aph*, which should contain an origin of replication for *E. coli* because of the integration of vector pBBRORF34::*aph*, was also transferred to *E. coli* strain DH5α by electroporation according to a protocol described by Sheng et al. (1995). Although strain DH5α was described to be more efficient for transformation of large DNA molecules (Sheng et al. 1995), neither gentamycin- nor kanamycin-resistant transformants were obtained after transformation.

Discussion

Previous investigations provided evidence that genes essential for rubber degradation in *G. westfalica* strain Kb1 are encoded on megaplasmid pKB1 (Bröker et al. 2004). As genetic engineering of pKB1 in its host failed because of the absence of an effective gene transfer system for this strain, pKB1 had to be transferred to genetically approachable strains for engineering. The suitability of the cadmium resistance gene *cadA* (ORF34) as pKB1-specific selection marker to monitor transfer of pKB1 by electroporation and conjugation to two cadmium sensitive strains of *G. polyisoprenivorans* and two taxonomically related bacteria was unequivocally demonstrated with pKB1 mediating cadmium resistance to a concentration of 800 μM (Table 3).

Transfer of megaplasmid pKB1 by electroporation occurred at transformation frequencies of 5.5×10^3 and 1.9×10^3 transformants per microgram plasmid DNA for *G. polyisoprenivorans* strains VH2 and Y2K, and 8.3×10^2 for *M. smegmatis* strain mc^{2}155, respectively. It is described that efficiencies of plasmid DNA transfer by electroporation decrease with increasing plasmid size (Mozo and Hooykaas 1991), which is consistent with transformation frequencies for the 101,016-bp plasmid pKB1.

Conjugative transfer of megaplasmid pKB1, using the spontaneous kanamycin-resistant mutant *R. opacus* strain PD630Km100 as recipient and *G. westfalica* strain Kb1 as donor occurred at mobilization frequencies of approximately 6.2×10^{-8} events per recipient cell, demonstrating the functionality of pKB1-encoded conjugative transfer genes. Therefore, plasmid pKB1 represents the first conjugative plasmid within the genus *Gordonia*. Strains of the genus *Rhodococcus* are the closest relatives containing described conjugative megaplasmids, as for example, megaplasmids pREA400 (400 kbp) and pREA250 (250 kbp) from *R. erythropolis* strain AN12, which were shown to be conjugative for the recipient *R. erythropolis* strain SQ1 at frequencies of approximately 7×10^{-4} events per recipient cell (Yang et al. 2007). Conjugative transfer of plasmid pD188 (138 kbp) from *R. fascians* strain D188 between different *R. fascians* strains occurred at transfer frequencies of 1.5×10^{-2} to 3×10^{-7} (Desomer et al. 1988).

Plasmid pKB1 did not confer the utilization of poly(*cis*-1,4-isoprene) to the non-rubber-degrading bacteria *M. smegmatis* strain mc^{2}155 and *R. opacus* strain PD630Km100 as revealed by the lacking growth and by the GPC analysis of the residual poly(*cis*-1,4-isoprene) after 8 weeks of incubation. It is, therefore, suggested that plasmid pKB1-encoded genes are not essential for the initial poly(*cis*-1,4-isoprene) cleavage but code for enzymes required for subsequent steps of the catabolic pathway. Because the rubber-degrading strain *G. alkanivorans* strain 44187 also harbors a megaplasmid-designated pKB2, which consists of plasmid pKB1-identical sequence regions (Bröker et al., unpublished data), and because the difference between this and the non-rubber-utilizing strain 44369 of *G. alkanivorans* seems to be only the absence of plasmid pKB2 (Kummer et al. 1999), plasmid pKB1 should be transferred to strain 44369 to restore the ability for rubber degradation. Because of the cadmium resistance of *G. alkanivorans* strain 44369, plasmid pKB1 had to be provided with other resistance markers.

Thus, two additional antibiotic selection markers (kanamycin and gentamycin resistance) were accommodated in pKB1 to facilitate transfer of the plasmid into cadmium-

sensitive strains of *Gordonia* and related taxa for analysis of plasmid encoded features. As transfer of foreign DNA to *G. westfalica* strain Kb1 failed, the genetically approachable recombinant pKB1-containing *G. polyisoprenivorans* strain VH2 and *M. smegmatis* strain mc^{2}155 were used for engineering of pKB1. Methods for transformation and gene replacement were intensively studied for *M. smegmatis* strain mc^{2}155 (Husson et al. 1990; McFadden 1996; Hinds et al. 1999; Galamba et al. 2001) and for *G. polyisoprenivorans* strain VH2 (Arenskötter et al. 2003; Banh et al. 2005; Arenskötter et al., unpublished data). Heterogenotes, containing a modified plasmid pKB1, in which the suicide vector pBBRORF34::*aph* (Fig. 1) integrated into pKB1 by homologous recombination, were obtained for both recombinant strains. The modified plasmid pKB1, designated as pKB1pBBRORF34::*aph*, contained two additional useful antibiotic selection markers (kanamycin and gentamycin resistance) and mediated resistance to both antibiotics to recombinant pKB1pBBRORF34::*aph* containing strains of *G. polyisoprenivorans* strains VH2 and Y2K and *M. smegmatis* strain mc^{2}155 but unfortunately not to *G. alkanivorans* strain 44369 representing one of the closest relative of *G. westfalica* strain Kb1 (Arenskötter et al. 2005). It was suggested that plasmid-encoded kanamycin or gentamycin resistance genes were not functionally expressed neither in *G. alkanivorans* strain 44369 using plasmid pKB1pBBRORF34::*aph* nor in *G. westfalica* strain Kb1 using plasmids pCGORF6::*aph* and pCGORF6::*aacC1*. Also transfer and propagation of foreign DNA seemed to be prevented by these members of the genus *Gordonia*. Possibly, these strains possess a highly efficient protection system against foreign DNA, such as restriction–modification (R–M) systems (Wilson and Murray 1991), a histone-like nucleoid structure-like (H-NS) protein repression system (Navarre et al. 2007) or a putative RNA-interference-based immune system (Makarova et al. 2006), as it has been described or discussed for many other bacteria.

As there is obviously a lack of suitable genetic tools for *Gordonia* species, the procedures applied in this study open the possibility for transfer of large DNA molecules by conjugation and electroporation to genetically approachable strains and for their engineering in resulting transgenic strains.

Acknowledgment Financial support by a grant of the Deutsche Forschungsgemeinschaft (STE 386/10–1) is gratefully acknowledged.

References

Arenskötter M, Baumeister D, Berekaa MM, Pötter G, Kroppenstedt RM, Linos A, Steinbüchel A (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

Arenskötter M, Baumeister D, Kalscheuer R, Steinbüchel A (2003) Identification and application of plasmids suitable for transfer of foreign DNA to members of the genus *Gordonia*. Appl Environ Microbiol 69:4971–4974

Arenskötter M, Bröker D, Steinbüchel A (2004) Biology of the metabolically diverse genus *Gordonia*. Appl Environ Microbiol 70:3195–3204

Arenskötter M, Linos A, Schumann P, Kroppenstedt RM, Steinbüchel A (2005) *Gordonia nitida* Yoon et al. 2001 is a later synonym of *Gordonia alkanivorans* Kummer et al. 1999. Int J Syst Evol Microbiol 55:695–697

Banh Q, Arenskötter M, Steinbüchel A (2005) Establishment of Tn*5096*-based transposon mutagenesis in *Gordonia polyisoprenivorans*. Appl Environ Microbiol 71:5077–5084

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7:1513–1523

Bröker D, Arenskötter M, Legatzki A, Nies DH, Steinbüchel A (2004) Characterization of the 101.016-kbp megaplasmid pKB1 isolated from the rubber degrading bacterium *Gordonia westfalica* Kb1. J Bacteriol 186:212–225

Bullock WO, Fernandez JM, Stuart JM (1987) XL1-Blue: a high efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection. Bio Techniques 5:376–379

Byrd DR, Matson SW (1997) Nicking by transesterification: the reaction catalysed by a relaxase. Mol Microbiol 25:1011–1022

Desomer J, Dhaese P, van Montagu M (1988) Conjugative transfer of cadmium resistance plasmids in *Rhodococcus fascians* strains. J Bacteriol 170:2401–2405

Dogan I, Pagilla KR, Webster DA, Stark BC (2006) Expression of *Vitreoscilla* hemoglobin in *Gordonia amarae* enhances biosurfactant production. J Ind Microbiol Biotechnol 33:693–700

Friedrich B, Hogrefe C, Schlegel HG (1981) Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J Bacteriol 147:198–205

Galamba A, Soetaert K, Wang X-M, De Bruyn J, Jacobs P, Content J (2001) Disruption of *adhC* reveals a large duplication in the *Mycobacterium smegmatis* mc^{2}155 genome. Microbiology 147:3281–3294

Gilbert SC, Morton J, Buchanan S, Oldfield C, McRoberts A (1998) Isolation of a unique benzothiophene-desulphurizing bacterium, *Gordona* sp. strain 213E (NCIMB 40816), and characterization of desulphurization pathway. Microbiology (SGM) 144:2545–2553

Grohmann E, Muth G, Espinosa M (2003) Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev 67:277–301

Guilhot C, Otal I, van Rompaey I, Martìn C, Gicquel B (1994) Efficient transposition in mycobacteria: construction of *Mycobacterium smegmatis* insertional mutant libraries. J Bacteriol 176:535–539

Hernandez-Perez G, Fayolle F, Vandecasteele J-P (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

Hinds J, Mahenthiralingam E, Kempsell KE, Duncan K, Stokes RW, Parish T, Stoker NG (1999) Enhanced gene replacement in mycobacteria. Microbiology 145:519–527

Husson RN, James BE, Young RA (1990) Gene replacement and expression of foreign DNA in mycobacteria. J Bacteriol 172:519–524

Ibrahim EMA, Arenskötter M, Luftmann H, Steinbüchel A (2006) Identification of poly(*cis*-1,4-isoprene) degradation intermediates during growth of moderately thermophilic Actinomycetes on

rubber and cloning of a functional *lcp* homologue from *Nocardia farcinica* strain E1. Appl Environ Microbiol 72:3375–3382

Kalscheuer R, Arenskötter M, Steinbüchel A (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 Biotech 52:508–515

Kim SB, Brown R, Oldfield C, Gilbert SC, Goodfellow M (1999) *Gordonia desulfuricans* sp. nov., a benzothiophene-desulfurizing actinomycete. Int J Syst Bacteriol 49:1845–1851

Kim SB, Brown R, Oldfield C, Gilbert SC, Iliarionov S, Goodfellow M (2000) *Gordonia amicalis* sp. nov., a dibenzothiophene–desulfurizing actinomycete. Int J Syst Bacteriol 50:2031–2036

Kotani T, Yamamoto T, Yurimoto H, Sakai Y, Kato N (2003) Propane monooxygenase and NAD^+-dependent secondary alcohol dehydrogenase in propane metabolism by *Gordonia* sp. strain TY-5. J Bacteriol 185:7120–7128

Kotani T, Yurimoto H, Kato N, Sakai Y (2007) Novel acetone metabolism in a propane-utilizing bacterium, *Gordonia* sp. strain TY-5. J Bacteriol 189:886–893

Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM (1995) Four new derivates of the broad host range cloning vector pBBR1MCS, carrying different antibiotic resistance cassettes. Gene 166:175–176

Kummer C, Schumann P, Stackebrandt E (1999) *Gordonia alkanivorans* sp. nov., isolated from tar-contaminated soil. Int J Syst Bacteriol 49:1513–1522

Linos A, Steinbüchel A (1998) Microbial degradation of natural and synthetic rubbers by novel bacteria belonging to the genus *Gordona*. Kautsch Gummi Kunstst 51:496–499

Linos A, Steinbüchel A, Spröer C, Kroppenstedt RM (1999) *Gordonia polyisoprenivorans* sp. nov., a rubber degrading actinomycete isolated from automobile tire. Int J Syst Bacteriol 49:1785–1791

Linos A, Berekaa MM, Steinbüchel A, Kim KK, Spröer C, Kroppenstedt RM (2002) *Gordonia westfalica* sp. nov., a novel rubber-degrading actinomycete. Int J Syst Evol Microbiol 52:1133–1139

Makarova KS, Grishin NV, Shabalina SA, Wolf YI, Koonin EV (2006) A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. Biol Direct 1:7–32

Mozo T, Hooykaas PJJ (1991) Electroporation of megaplasmids into *Agrobacterium*. Plant Mol Biol 16:917–918

McFadden J (1996) Recombination in mycobacteria. Mol Microbiol 21:2056–211

Navarre WW, McClelland M, Libby SJ, Fang FC (2007) Silencing of xenogeneic DNA by H-NS—facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. Genes Dev 21:1456–1471

Pansegrau W, Lanka E (1996) Enzymology of DNA transfer by conjugative mechanisms. Prog Nucleic Acid Res Mol Biol 54:197–251

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schlegel HG, Kaltwasser H, Gottschalk G (1961) Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch Mikrobiol 38:209–222

Sheng Y, Mancino V, Birren B (1995) Transformation of *Escherichia coli* with large DNA molecules by electroporation. Nucleic Acids Res 23:1990–1996

Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR Jr (1990) Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol Microbiol 4:1911–1919

Wilson GG, Murray NE (1991) Restriction and modification systems. Annu Rev Genet 25:585–627

Yang JC, Lessard PA, Sengupta N, Windsor SD, O'Brien XM, Bramucci M, Tomb J-F, Nagarajan V, Sinskey AJ (2007) TraA is required for megaplasmid conjugation in *Rhodococcus erythropolis* AN12. Plasmid 57:55–70

Veiga-Crespo P, Feijoo-Siota L, de Miguel T, Poza M, Villa TG (2006) Proposal of a method for the genetic transformation of *Gordonia jacobae*. J Appl Microbiol 100:608–614

Yoon J-H, Lee JJ, Kang SS, Takeuchi M, Shin YK, Lee ST, Kang KH, Park YH (2000) *Gordonia nitida* sp. nov., a bacterium that degrades 3-ethylpyridine and 3-methylpyridine. Int J Syst Evol Microbiol 50:1203–1210