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Characterization of the 101-Kilobase-Pair Megaplasmid pKB1, Isolated from the Rubber-Degrading Bacterium *Gordonia westfalica* Kb1

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The complete sequence of the circular 101,016-bp megaplasmid pKB1 from the *cis*-1,4-polyisoprene-degrading bacterium *Gordonia westfalica* Kb1, which represents the first described extrachromosomal DNA of a member of this genus, was determined. Plasmid pKB1 harbors 105 open reading frames. The predicted products of 46 of these are significantly related to proteins of known function. Plasmid pKB1 is organized into three functional regions that are flanked by insertion sequence (IS) elements: (i) a replication and putative partitioning region, (ii) a putative metabolic region, and (iii) a large putative conjugative transfer region, which is interrupted by an additional IS element. Southern hybridization experiments revealed the presence of another copy of this conjugational transfer region on the bacterial chromosome. The origin of replication (*oriV*) of pKB1 was identified and used for construction of *Escherichia coli-Gordonia* shuttle vectors, which was also suitable for several other *Gordonia* species and related genera. The metabolic region included the heavy-metal resistance gene *cadA*, encoding a P-type ATPase. Expression of *cadA* in *E. coli* mediated resistance to cadmium, but not to zinc, and decreased the cellular content of cadmium in this host. When *G. westfalica* strain Kb1 was cured of plasmid pKB1, the resulting derivative strains exhibited slightly decreased cadmium resistance. Furthermore, they had lost the ability to use isoprene rubber as a sole source of carbon and energy, suggesting that genes essential for rubber degradation are encoded by pKB1.

The genus *Gordonia* was proposed by Tsukamura for coryneform bacteria isolated from sputa of patients with pulmonary disease or from soil (65–67). *Gordonia* belongs to the so-called CMN group (*Corynebacterium*, *Mycobacterium*, and *Nocardia*) of actinomycetes, which synthesize mycolic acids (13, 59). *Gordonia* strains also play an important role in bioremediation and biodegradation of pollutants and have attracted much interest in recent years due to their unusual and diverse metabolic capabilities (23, 29, 30, 70). Three strains of *Gordonia polyisoprenivorans* (2, 37) and the novel species *G. westfalica* Kb1 (39) were described as bacteria able to degrade natural rubber and synthetic *cis*-1,4-polyisoprene, which allows species of this genus to serve as model organisms for the investigation of the hitherto unknown biochemical and molecular mechanisms of rubber biodegradation (36). So far, no native plasmids of the genus *Gordonia* have been detected. Since factors encoded by linear or circular plasmids are often involved in degradation of complex xenobiotics (62), rubber-degrading bacterial strains were screened for the occurrence of extrachromosomal DNA. This publication gives the first example and the complete DNA sequence of a native *Gordonia* plasmid, which also provides the basis for the development of shuttle vectors that may serve as a novel genetic tool for the study of *Gordonia* and related genera.

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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 genera *Gordonia* and *Rhodococcus* were grown at 30°C in standard I complex nutrient broth (St-I; E. Merck AG, Darmstadt, Germany), mycobacterial strains were grown at 30°C in Luria-Bertani broth (LB) (52) containing Tween 80 (50 ml/liter of broth), and strains of *Streptomyces* were cultivated at 30°C in yeast extract-malt extract medium (24). For growth experiments with *cis*-1,4-polyisoprene as the sole carbon source, *G. westfalica* Kb1, *G. westfalica* Kb1-K38, and *G. westfalica* Kb1-K43 were cultivated at 30°C on mineral salts medium (54). Cells of *Escherichia coli* were cultivated at 37°C in LB broth. Antibiotics were applied according to the method of Sambrook et al. (52) and as indicated in the text. Carbon sources were added as indicated in the text. Liquid cultures were made in Erlenmeyer flasks and incubated on a horizontal rotary shaker. Solid media were prepared by the addition of agar (15 g/liter).

Determination of metal tolerance. For the gram-positive bacteria of the genera *Gordonia*, *Rhodococcus*, and *Mycobacterium*, St-I agar plates containing 100 to 800 μM $CdCl_2$ were prepared in a 0.1 M stock solution of $CdCl_2$ H_2O (E. Merck AG) which was sterilized by autoclaving. The plates were inoculated with the bacterial strains and tolerance for cadmium was evaluated after incubation for 3 days at 30°C. Resistance of *E. coli* strains was determined by use of dose-response curves generated by growth in Lennox medium (Becton-Dickinson, Sparks, Md.). The medium contained 50 μg of anhydrotetracycline per liter to induce expression of the plasmid-encoded genes in these strains. Overnight cultures of *E. coli* strains were used to inoculate parallel cultures with increasing metal concentrations. Cells were cultivated for 16 h with shaking at 37°C, and the optical density was determined at 600 nm.

Cadmium uptake experiments. Cadmium uptake experiments were performed with *E. coli* cells in Tris buffer (10 mM; pH 7.0) by filtration, as published previously (44). The cells were cultivated in Tris-buffered mineral salts medium in the presence of 2 g of glucose per liter and 1 g of yeast extract per liter, up to 100 Klett units, when 200 μg of anhydrotetracycline per liter was added, and incubation was continued with shaking for 3 h at 30°C. Cells were harvested by centrifugation, washed, and suspended in 10 mM Tris-HCl buffer (pH 7.0). Radioactive $^{109}Cd^{2+}$ (87.4 GBq/g) was added at a concentration of 10 μM, incubation was continued with shaking at 30°C, and the metal content in washed cells (dry weight) was determined at various time points by use of an equilibration curve. Background binding was not subtracted.

TABLE 1. Bacterial strains and plasmids used for this study

Strain or plasmid	Relevant characteristic(s)	Reference or source (strain no.)
Strains		
Gordonia species		
G. alkanivorans HKI 0136	Hexadekane-degrading wild type	33 (DSM 44369)
G. amicalis IEGM^T	Dibenzothiophene-desulfurizing strain	30 (DSM 44461^T)
G. bronchialis		(DSM 43247^T)
G. desulfuricans 213E	Benzothiophene-desulfurizing wild type	29 (NCIMB 40816)
G. nitida LE31^T	3-Ethylpyridine- and 3-methylpyridine-degrading strain	70 (DSM 44499)
G. polyisoprenivorans Kd2	*cis*-1,4-Polyisoprene-degrading wild type	37 (DSM 44302)
G. polyisoprenivorans VH2	*cis*-1,4-Polyisoprene-degrading wild type	2 (DSM 44266)
G. polyisoprenivorans Y2K	*cis*-1,4-Polyisoprene-degrading wild type	2
G. sputi		(DSM 43896^T)
G. terrae	Ethyl *t*-butyl ether-degrading strain	23 (DSM 43249^T)
G. westfalica Kb1/Kb2	*cis*-1,4-Polyisoprene-degrading wild type	38 (DSM 44215)
G. westfalica Kb1-K12	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K34	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K35	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K36	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K37	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K38	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K39	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K40	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K41	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K42	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K43	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K44	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K45	pKB1-deficient mutant of *G. westfalica* Kb1	This work
G. westfalica Kb1-K46	pKB1-deficient mutant of *G. westfalica* Kb1	This work
Mycobacterium species		
M. smegmatis mc^{2}155	Transformation-efficient mutant	58
Rhodococcus species		
R. opacus PD630	Wild type; TAG$^+$	(DSMZ 44193)
R. rhodochrous RNMS1	Wild type	61 (ATCC 13808^T)
R. ruber	Wild type; PHA$^+$ TAG$^+$	22
Streptomyces species		
S. coelicolor A3(2)	Wild type	56 (DSMZ 40783)
S. lividans TK23	Wild type	John Innes Institut, Norwick, England
E. coli strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi1 hsdR17*(r_K^- m_K^+) *supE44 relA1* λ^- *lac* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10* (Tc^r)]	12
E. coli S17-1	*thi1 proA hsdR17*(r_K^- m_K^+) *recA1 tra* gene of plasmid RP4 integrated into the chromomsome	57
E. coli W3110	Wild type	20
E. coli RW3110	*zntA*	50
Plasmids		
pASK3	*E. coli* vector	IBA-GmbH, Göttingen, Germany
pBBR1MCS-1	Cm^r *lacPOZ′*, Mob	32
pBBR1MCS-2	Km^r *lacPOZ′*, Mob	31
pBBR1MCS-3	Tc^r *lacPOZ′*, Mob	31
pBBR1MCS-4	Ap^r, *lacPOZ′*, Mob	31
pBBR1MCS-5	Gm^r, *lacPOZ′*, Mob	31
pBBRKB1:*Xho*I7	Km^r, *lacPOZ′*, Mob, *oriV* pKB1	This work
pDBMCS-2	Km^r *lacPOZ′*, Mob, *oriV* pKB1	This work
pDBMCS-5	Gm^r *lacPOZ′*, Mob, *oriV* pKB1	This work

Isolation, analysis, and manipulation of DNA. Plasmid DNA was prepared from crude lysates by the alkaline extraction method (8). Before lysis, cells of *Gordonia*, *Rhodococcus*, and *Mycobacterium* were incubated in the presence of lysozyme (2 g/liter) for 2 h at 37°C. Total DNA of *Gordonia* was prepared as described by Ausubel et al. (4), with modifications as follows: cells of 50-ml cultures were harvested by centrifugation and suspended in 8.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and 1 ml of lysozyme solution (10 g of TE per liter). After incubation at 37°C for 2 h, 500 μl of a sodium dodecyl sulfate solution (100 g/liter) and 50 μl of a proteinase K solution (20 g of TE per liter) were added and mixed gently. After additional incubation at 37°C for 1 h, 5 ml of 5 M NaCl and 1.5 ml of a CTAB solution (100 g of cetyltrimethylammonium bromide per liter of 0.7 M NaCl) were added and the solution was incubated at 65°C for 20 min. DNA was digested with various restriction endonucleases (Gibco/BRL, Gaithersburg, Md.) under the conditions recommended by the manufacturer. All other genetic procedures and manipulations were conducted as described by Sambrook et al. (52).

TABLE 2. PCR and sequencing primers used in this study

Oligonucleotide	Sequence (5′ → 3′)	Position in pKB1[a]	Function
P1	AAA GAA TTC GCT GAC GCA TGC TGC GGA	29829–29846	2,003-bp PCR product from *cadA* gene from *G. westfalica* (*Eco*RI and *Bam*HI restriction sites used for cloning are underlined)
P2	AAA GGA TCC GTG GCG TTC GCG ATG GGG	31998–32015c	
P3	TCG GGC AGC GTA CTC GGC CGG	801–821	720-bp PCR product from IS1 (comprising ORF18)
P4	TCG TCA ACT GCC GCA AGC GCA	1501–1521c	
P5	CGC TCA AGC GCG GAC GAG CAG	50301–50321	720-bp PCR product from IS3 (comprising ORF50)
P6	CGG CAA GCC GCT GTG CGC GGC	51001–51021c	
P7	TCG TGA TGG GAG CAG GCT GGC	2801–2821c	With P3, 2,020-bp PCR product from IS1, with contiguous region (comprising ORF18)
P8	GTC GAT GCA ATA CGA CCG CTC	49001–49021	With P6, 2,020-bp PCR product from IS3, with contiguous region (comprising ORF50)
P9	GCC CTA TAC CTT GTC TGC CTC CCC G	2520–2544[b]	5,134-bp PCR product of the vector pBBR1MCS-2 and 4,758-bp PCR product of the vector pBBR1MCS-5
P10	GCT ACA GCC GAT AGT CTG GAA CAG C	2510–2484c[b]	
P11	AAG ACC ACG ATC CAG TCG GC	5101–5120	2,332-bp PCR product of pKB1 comprising *oriV* of pKB1
P12	TTA ACT ATC GGG CGG AGT CG	7414–7433c	

[a] c, complementary strand.
[b] Numbering is based on the vectors pBBR1MCS-2 and pBBR1MCS-5 (31).

Extrachromosomal DNA was detected by pulsed-field gel electrophoresis (PFGE). Preparation of total DNA embedded in low-melting-temperature agarose and linearization of circular plasmid DNA were done according to the methods of Barton et al. (5). Electrophoresis was performed in the CHEF-DR III system (Bio-Rad GmbH, Munich, Germany).

Plasmid curing. For generation of plasmid-free mutants of *G. westfalica* Kb1, heat curing was performed (43). Fifty milliliters of St-I medium in 300-ml Erlenmeyer flasks was inoculated with cells of *G. westfalica* Kb1 and incubated at 42°C. Every 2 days, Erlenmeyer flasks with fresh St-I medium were inoculated with 1 ml of the grown culture and subsequently cultivated at 42°C. After 20 passages, the cells were diluted and spread on St-I agar plates. The colonies obtained were screened for the absence of plasmid pKB1 as described previously (27).

Cloning procedures. The *cadA* gene from *G. westfalica* Kb1 was amplified by PCR from plasmid pKB1 with the primers P1 and P2 (listed in Table 2) and was cloned downstream of the *tet* promoter in plasmid pASK3 (IBA GmbH, Göttingen, Germany), leading to plasmid pECA34. The two insertion sequence (IS) elements, IS1 and IS3 (comprising ORF1/ORF2 and ORF53), were amplified from total DNA of wild-type *G. westfalica* Kb1 and the pKB1-free mutants with the primer sets P3 plus P4 and P5 plus P6 (listed in Table 2). The two IS elements, IS1 and IS3, with their contiguous regions, were then amplified with the two primer sets P3 plus P7 and P8 plus P6 (Table 2).

Gene transfer. Hybrid plasmids containing *oriV* of pKB1 were transferred to species of the genera *Gordonia*, *Mycobacterium*, and *Rhodococcus* by electroporation in a Model 2510 electroporator (Eppendorf-Netheler-Hinz, Hamburg, Germany). Preparation of the electrocompetent cells and the execution of electroporation were done as described recently (3, 28). For transformation of *Streptomyces coelicolor* strain A3(2) and *Streptomyces lividans* strain TK23, protoplasts of these strains were prepared as described by Okanishi et al. (47), Bibb et al. (7), and Thompson et al. (63). Transformations were done according to the rapid small procedure, as described by Bibb et al. (6), Thompson et al. (63), and Okanishi et al. (46). Conjugational plasmid transfer was carried out, applying a previously described protocol (18) employing *E. coli* S17-1 as donor and *G. polyisoprenivorans* as recipient.

DNA sequence analysis. To obtain the complete sequence of megaplasmid pKB1, we constructed a shotgun library. Plasmid DNA was fragmented by hydroshearing, cloned into pGEM-T vector DNA (Promega, Madison, Wis.), and sequenced by MWG Biotech (Ebersberg, Germany), resulting in fivefold sequence coverage. A few regions for which only uncertain sequences were obtained were sequenced by employing individual primers. Hybrid plasmids containing *oriV*-comprising DNA fragments were sequenced with IRD800-labeled universal and reverse primers, using the SequiTherm EXCEL II Long-Read L-C kit and a LI-COR 4200 sequencer (LI-COR Biosciences, Lincoln, Nebr.).

Open reading frames (ORFs) were identified by use of the program GeneMark (http://opal.biology.gatech.edu/GeneMark/) to indicate start codons, stop codons, and codon usage statistics for each reading frame (41). Database searches of the predicted protein sequences were performed with the BLAST program provided by EMBL/Heidelberg (1). Multiple sequence alignments were carried out with the program BioEdit (21). Protein sequences were also analyzed for functionally important motifs by use of SMART software (http://smart.embl-heidelberg.de/) (35, 55).

Nucleotide sequence accession number. The DNA sequence of pKB1 has been deposited in the EMBL database under accession number AJ576039.

RESULTS AND DISCUSSION

Detection and characterization of the megaplasmid pKB1. Since metabolic pathways involved in degradation of unusual compounds may be encoded by bacterial plasmids, the rubber-degrading bacteria *G. polyisoprenivorans* strains Kd2^T, VH2, and Y2K and *G. westfalica* strain Kb1 were screened for the occurrence of plasmids. Cells were embedded in low-melting-temperature agarose and lysed, and the immobilized total DNA was treated with *Aspergillus oryzae* S1 nuclease to linearize possible circular plasmids. When the DNA samples were separated by PFGE, only those from *G. westfalica* Kb1 displayed a distinct band, which was visible only after S1 treatment and corresponded to a size of about 100 kbp (data not shown). Consequently, this strain contained a circular megaplasmid that we named pKB1. Plasmid pKB1 represents the first extrachromosomal DNA identified for the genus *Gordonia*.

To investigate the possible metabolic function of pKB1, we completely sequenced it (Fig. 1). Plasmid pKB1 is a circular DNA molecule with a size of 101,016 bp, which confirmed the PFGE results. The G+C content was 66 mol%, as is expected for DNA from a bacterium of the CMN group. The plasmid carried 105 ORFs, 47 on one DNA strand and 58 on the other. The predicted products of 67 ORFs were related to proteins in the current databases, as indicated by a BLAST analysis, of which 46 were homologous to proteins with putative functions (Table 3). According to the predicted functions of their products, the genes located on plasmid pKB1 were clustered into

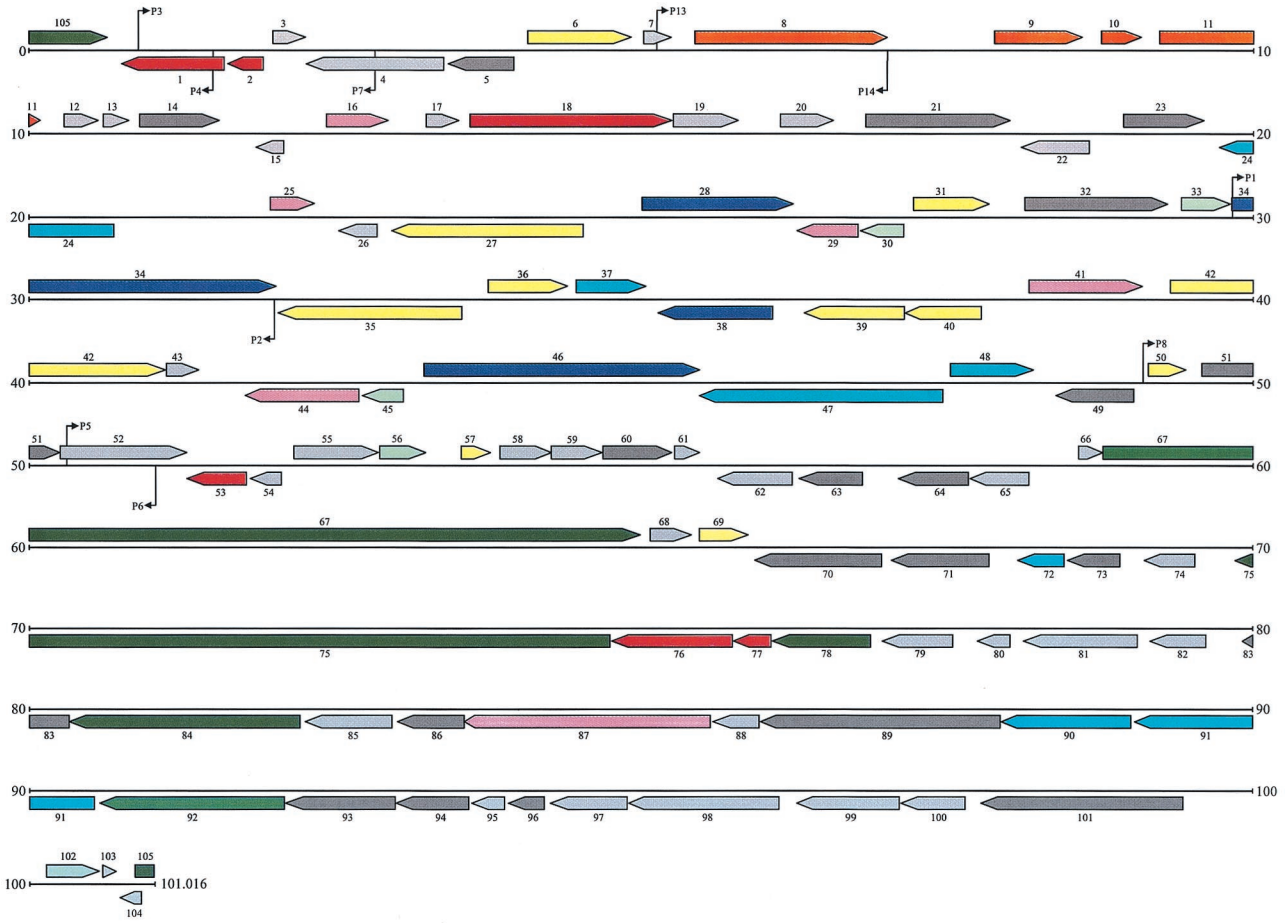

FIG. 1. Schematic representation of the ORFs of the 101,016-bp plasmid pKB1. The predicted ORFs located on plasmid pKB1 are shown as arrows. The direction of the arrow indicates the DNA strand on which the ORF is located. The colors of the arrows group the assigned functions of the putative ORF products as follows: orange, ORFs related to replication and partitioning; yellow, ORFs with putative metabolic functions; blue, ORFs related to heavy-metal resistance; dark green, ORFs related to conjugation; red, ORFs related to IS elements and transposons; green, putative regulator proteins; pink, putative peptidases; light blue, putative membrane proteins; dark gray, conserved hypothetical ORFs; light gray, ORFs with no known function. The small black arrows indicate binding sites and orientations of pKB1-specific primers (Table 2).

three major regions that were all flanked by putative IS elements: a replication and partitioning region, a metabolic region, and a conjugational transfer region (Fig. 1). Investigations were carried out with ORF8 within the replication and partitioning region, which encodes a replication gene and mediates autonomous replication, and with ORF34, which encodes *cadA*, a cadmium P-type ATPase.

Identification of *oriV* of plasmid pKB1. The replication and partitioning region of plasmid pKB1 was further characterized, with the intention of constructing *E. coli-Gordonia* shuttle vectors. For cloning and identification of the origin of replication, *oriV*, of plasmid pKB1, a plasmid library of pKB1 was generated by use of the mobilizable vector pBBR1MCS-2, which is not able to replicate in *Gordonia* strains (unpublished data). The DNA of pKB1 was partially digested with *Xho*I and ligated to *Xho*I-linearized pBBR1MCS-2 plasmid DNA. Since plasmid transfer into *G. westfalica* yielded no transformants, the DNA mixture was electroporated into *G. polyisoprenivorans* strain VH2. The cells were screened for 4 days at 30°C for kanamycin resistance, which is encoded by plasmid pBBR1MCS-2.

The resulting kanamycin-resistant colonies of *G. polyisoprenivorans* strain VH2 contained hybrid plasmids that were composed of at least three *Xho*I fragments: plasmid pBBR1MCS-2 DNA plus two additional fragments, of 2,331 and 4,927 bp (data not shown). Neither fragment alone was able to mediate replication in *G. polyisoprenivorans* VH2 (data not shown), indicating that essential elements were located on both fragments, which together correspond to the 7,258-bp region from nucleotides 3,743 to 11,000 on the sequence map of plasmid pKB1. This region contains nine ORFs (ORF 6 to ORF 14) (Fig. 2A) and five tandem repeats, with a period size of 16 bp, downstream of ORF8 (nucleotides 7,558 to 7,637) (Fig. 2B).

To identify the genes and *cis*-acting elements essential for replication and partitioning, we deleted the 7,258-bp gene region from both ends by exonuclease III and endonuclease treatment. The autonomous replication ability of the resulting fragments was tested (Fig. 2A). The smallest of the fragments able to mediate stable plasmid replication in *G. polyisoprenivorans* strain VH2 had a size of 2,332 bp and harbored

TABLE 3. Summary of ORFs identified by significant homology (BLAST search) or GENEMARK prediction

ORF	No. of amino acids	Coding sequence position (start codon–stop codon)[a]	Gene or function of closest relative (source), identified protein domains	Data bank reference no.	No. of amino acids with identity/total (%)	E value[b]
1	278	759–1592c	Hypothetical protein (*Rhodopseudomonas palustris*), related to IS*511*, transposase OrfB (*Caulobacter crescentus* CB15), pfam00665	ZP_00008656	111/265 (41%)	7e−45
				AAK24707	103/271 (38%)	1e−37
2	97	1622–1912c	Hypothetical protein (*Magnetospirillum magnetotacticum*), related to IS*1477* transposase (*Xanthomonas campestris* pv. *campestris*), pfam01527	ZP_00053525	38/90 (42%)	3e−09
				NP_637161	29/69 (42%)	4e−04
3	89	1992–2258	GENEMARK prediction; no homology			
4	398	2241–3434c	GENEMARK prediction; no homology			
5	177	3431–3961c	Cinorf13 protein (*Streptomyces cinnamoneus*)	CAD60535	45/161 (27%)	5e−06
6	282	4068–4913	Epoxide hydrolase homolog YfhM (*Bacillus subtilis*), pfam00561	BAA24479	91/277 (32%)	1e−32
7	76	5017–5244	GENEMARK prediction; no homology			
8	680	5404–7443	GENEMARK prediction; no homology			
9	238	7882–8595	Putative chromosome-partitioning ATPase-like protein (*R. equi*), COG1192	BAB16660	144/232 (62%)	2e−70
10	109	8754–9080	Hypothetical protein (*R. equi*)	NP_858507	25/59 (42%)	5e−04
11	293	9214–10092	Putative mycobacteriophage excisionase (*R. equi*)	NP_066806	51/121 (42%)	1e−10
12	109	10285–10611	GENEMARK prediction; no homology			
13	69	10608–10814	GENEMARK prediction; no homology			
14	217	10901–11551	p24 (human immunodeficiency virus 1)	CAB87182	25/62 (40%)	0.037
15	78	11867–12100c	GENEMARK prediction; no homology			
16	169	12431–12937	gp8 (mycobacteriophage Bxb1), related to L-alanoyl-D-glutamate peptidase (bacteriophage A500)	NP_075275	44/132 (33%)	2e−10
				CAA59365	25/71 (35%)	4e−04
17	90	13247–13516	GENEMARK prediction; no homology			
18	547	13601–15241	IS*1554*, transposase (*M. tuberculosis* CDC1551)	AAK45194	358/415 (86%)	0.0
19	176	15242–15769	GENEMARK prediction; no homology			
20	145	16137–16571	GENEMARK prediction; no homology			
21	392	16831–18006	Hypothetical protein (*Nitrosomonas europaea* ATCC 19718)	CAD84656	100/340 (29%)	1e−27
22	186	18097–18654c	GENEMARK prediction; no homology			
23	220	18932–19591	Predicted protein (*Methanosarcina acetivorans* C2A)	AAM07556	36/100 (36%)	1e−05
24	323	19721–20689c	Hypothetical transmembrane protein (*Bifidobacterium longum* NCC2705), COG3021, related to iron deficiency-induced protein A (*Synechococcus* sp. PCC7942), pfam03372	AAN25527	74/244 (30%)	2e−18
				CAC40996	57/204 (27%)	0.13
25	121	21894–22256	Conserved hypothetical protein (*M. bovis* subsp. *bovis* AF2122/97), related to membrane proteins related to metalloendopeptidases (*Corynebacterium glutamicum* ATCC 13032), pfam01551	CAD93836	66/117 (56%)	5e−35
				BAB98251	66/115 (57%)	5e−32
26	105	22532–22846c	GENEMARK prediction; no homology			
27	520	22970–24529c	Conserved hypothetical protein (*M. tuberculosis* CDC1551), related to ResB protein required for cytochrome *c* biosynthesis (*C. glutamicum* ATCC 13032), COG1333, pfam05140	AAK44773	254/488 (52%)	e−134
				NP_599688	237/491 (48%)	e−117
28	415	25001–26245	Divalent cation-transport integral membrane protein MNTH (*M. bovis* subsp. *bovis* AF2122/97), pfam01566, COG1914	CAD93809	233/391 (59%)	e−111
29	166	26272–26769c	Lipoprotein signal peptidase (*Brucella melitensis* 16M), pfam01252	AAL52980	56/127 (44%)	1e−10
30	118	26766–27119c	Hypothetical protein (*Thermobifida fusca*), related to transcriptional regulator (*Nostoc* sp. PCC 7120), smart00418, pfam01022	ZP_00056748	67/97 (69%)	3e−27
				BAB74465	27/67 (40%)	1e−07
31	207	27221–27841	Hypothetical methyltransferase (*Shewanella oneidensis* MR-1), COG2226, pfam01209, related to menaquinone biosynthesis methyltransferase (2-heptaprenyl-1,4-naphthoquinone methyltransferase) (*Methanosarcina acetivorans* C2A)	NP_717595	70/189 (37%)	1e−27
				NP_619210	35/116 (30%)	3e−05
32	391	28126–29298	Probable conserved lipoprotein LppS (*M. bovis* subsp. *bovis* AF2122/97), pfam03734, COG1376	CAD97408	174/386 (45%)	6e−88
33	140	29414–29833	*cadC* (*Listeria monocytogenes*), pfam01022	AAA25276	36/98 (36%)	4e−11
34	731	29826–32018	Probable cation transport ATPase (*M. tuberculosis*), COG2217, pfam00122	F70757	378/647 (58%)	e−151
35	501	32034–33536c	Putative polyprenol-phosphate-mannose synthase 2 (Ppm2) (*M. smegmatis*), COG0815, pfam00745	CAC15462	205/483 (42%)	5e−86
36	215	33754–34398	Putative membrane protein (*Corynebacterium efficiens*), COG1651, related to thiol-disulfide oxidoreductase BdbD (*B. cereus* ATCC 14579), pfam01323	BAC19796	95/209 (45%)	8e−40
				NP_830362	49/217 (22%)	6e−06
37	191	34464–35036	Hypothetical integral membrane protein (*M. smegmatis*), COG4243	AAG30410	93/190 (48%)	1e−41
38	314	35135–36076c	Hypothetical protein (*Bifidobacterium longum* DJO10A), related to cobalt-zinc-cadmium resistance protein (*Xanthomonas axonopodis* pv. *citri* 306), COG1230, pfam01545	ZP_00120702	125/297 (42%)	1e−49
				NP_641652	106/298 (35%)	5e−38

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TABLE 3—*Continued*

ORF	No. of amino acids	Coding sequence position (start codon–stop codon)[a]	Gene or function of closest relative (source), identified protein domains	Data bank reference no.	No. of amino acids with identity/total (%)	E value[b]
39	271	36335–37147c	Possible cytochrome *c*-type biogenesis protein CCDA (*M. bovis* subsp. *bovis*), related to a cytochrome *c* biogenesis protein (*C. glutamicum* ATCC 13032), pfam02683, COG0785	CAD93402	120/233 (51%)	4e−49
				NP_599687	116/254 (45%)	1e−45
40	209	37144–37770c	Hypothetical protein (*M. leprae*), COG0526, COG1225, related to thiol-disulfide isomerase and thioredoxins (*C. glutamicum* ATCC 13032)	S72901	94/179 (52%)	4e−41
				BAB97832	73/174 (41%)	1e−27
41	309	38162–39088	Hypothetical protein (*M. leprae*), COG0739, related to membrane proteins related to metalloendopeptidases (*C. glutamicum* ATCC 13032), pfam01551	CAB36664	64/120 (53%)	1e−29
				BAB98251	65/120 (54%)	5e−27
42	597	39321–41111	Cytochrome *c* oxidase, subunit 1 (*M. tuberculosis* CDC1551), COG0843, pfam00115	NP_337644	442/568 (77%)	0.0
43	91	41108–41380	GENEMARK prediction; no homology			
44	311	41762–42694c	Hypothetical protein (*T. fusca*), COG0501, related to peptidase M48 (*C. glutamicum*)	ZP_00058818	47/117 (40%)	3e−08
				AAL31539	37/111 (33%)	3e−04
45	113	42699–43037c	Hypothetical protein (*T. fusca*), COG3680, pfam03965, related to methicillin resistance regulatory protein (*S. aureus* subsp. *aureus* N315)	ZP_00056896	45/109 (41%)	8e−13
				NP_373280	20/87 (22%)	0.007
46	730	43290–45479	Cation-transporting ATPase (*M. leprae*), COG2216, COG2217, pfam00122	NP_302350	337/724 (46%)	3−148
47	663	45417–47405c	Possible membrane protein (*M. leprae*), related to CtaG (*B. subtilis*), COG3336	NP_302349	212/598 (35%)	8e−86
				NP_389376	50/234 (21%)	0.004
48	226	47520–48197	Probable conserved integral membrane protein (*M. bovis* subsp. *bovis* AF2122/97)	CAD93856	50/183 (27%)	3e−04
49	155	48266–48730c	Hypothetical protein (*Cytophaga hutchinsonii*)	ZP_00117563	29/79 (36%)	2e−08
50	104	49143–49454	Putative modification methylase (*Streptomyces lividans*)	AAO61179	22/64 (34%)	0.19
51	223	49580–50248	Hypothetical protein Rv1044 (*M. tuberculosis* H37Rv)	NP_215560	62/201 (30%)	3e−05
52	344	50248–51279	GENEMARK prediction; no homology			
53	164	51284–51775c	GENEMARK prediction; no homology			
54	85	51807–52061c	GENEMARK prediction; no homology			
55	231	52162–52854	GENEMARK prediction; no homology			
56	125	52851–53225	Helix-turn-helix protein (*Pyrobaculum aerophilum* IM2), related to SgraIC control protein (*Streptomyces griseus*), pfam01381	AAL64755	29/61 (47%)	1e−04
				AAG31560	24/60 (40%)	0.002
57	79	53531–53767	Glutaredoxin electron transport component of NrdEF (*M. leprae*), COG0695, pfam00462	NP_302197	60/77 (77%)	4e−25
58	138	53846–54259	GENEMARK prediction; no homology			
59	141	54256–54678	GENEMARK prediction; no homology			
60	189	54682–55248	gp82 (mycobacteriophage CJW1), GerE (*Corynebacterium striatum*, plasmid pTP10), COG0305, pfam00772	AAN01696	41/147 (27%)	0.008
				AAG03386	5/73 (34%)	0.014
61	69	55270–55476	GENEMARK prediction; no homology			
62	180	55631–56170c	GENEMARK prediction; no homology			
63	171	56299–56811c	Hypothetical protein (*Nostoc punctiforme*)	ZP_00106356	34/95 (35%)	5e−05
64	192	57097–57672c	Putative bacteriophage-related protein (*Ralstonia solanacearum*), related to gene 2.8 (enterobacteria phage T7)	NP_521357	33/95 (34%)	5e−04
				NP_041971	30/97 (30%)	0.010
65	113	57690–58028c	GENEMARK prediction; no homology			
66	65	58573–58767	GENEMARK prediction; no homology			
67	2,073	58773–64991	TraA-like protein (*R. equi*), related to TraA (*C. glutamicum*), COG0507	NP_066783	329/987 (33%)	e−105
				NP_776232	253/878 (28%)	3e−56
68	113	65072–65410	GENEMARK prediction; no homology			
69	133	65473–65871	Putative glycosyl transferase (*Streptomyces nogalater*)	AAF01809	28/82 (34%)	0.032
70	345	65928–66962c	Hypothetical protein (*Microbulbifer degradans* 2-40)	ZP_00068299	71/150 (47%)	2e−27
71	275	67047–67871c	Conserved hypothetical protein (*C. efficiens* YS-314)	NP_736653	48/181 (26%)	1e−09
72	128	68071–68454c	Putative membrane protein (*Streptomyces avermitilis* MA-4680)	NP_825985	29/82 (35%)	0.002
73	144	68480–68911c	WD40-repeat-containing protein (*Methanosarcina acetivorans* C2A)	NP_617428	22/70 (31%)	0.30
74	139	69109–69525c	GENEMARK prediction; no homology			
75	1,631	69852–74744c	Putative methylase (or helicase) (*R. equi*), related to helicase SNF2 family (*Agrobacterium tumefaciens* C58), COG4646	BAB16635	489/1302 (37%)	0.0
				AAL46337	399/1325 (30%)	e−125
76	331	74754–75746c	Putative transposase (*C. efficiens* YS-314), related to IS*1601*-D (*Mycobacterium avium*), pfam00665, COG2801	NP_739229	186/284 (65%)	7e−96
				AAD44200		7e−55
77	104	75743–76054c	Putative transposase (*C. efficiens* YS-314), related to transposase IS*911* helix-turn-helix and LZ region (*N. europaea* ATCC 19718), pfam01527	NP_739228	64/103 (62%)	1e−23

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TABLE 3—*Continued*

ORF	No. of amino acids	Coding sequence position (start codon–stop codon)[a]	Gene or function of closest relative (source), identified protein domains	Data bank reference no.	No. of amino acids with identity/total (%)	E value[b]
78	266	76073–76870c	Putative methylase (or helicase) (*R. equi*), related to DNA methylase (*Listeria innocua*), COG2263	BAB16635	110/264 (41%)	6e−41
				NP_569161	65/182 (35%)	5e−27
79	193	76999–77577c	GENEMARK prediction; no homology			
80	90	77862–78131c	GENEMARK prediction; no homology			
81	312	78251–79186c	GENEMARK prediction; no homology			
82	154	79275–79736c	GENEMARK prediction; no homology			
83	136	79916–80323c	Hypothetical protein (*Arthrobacter nicotinovorans*)	CAD47981	30/68 (44%)	2e−08
84	630	80323–82212c	Conjugative transfer gene complex protein-like protein (*R. equi*), related to transfer complex protein TrsK (*S. aureus*), pfam02534, COG3505	NP_066787	187/531 (35%)	9e−77
				C56976	28/78 (35%)	8e−07
85	238	82251–82964c	GENEMARK prediction; no homology			
86	183	83008–83556c	Hypothetical protein (*R. equi*)	NP_066788	40/161 (24%)	0.037
87	675	83553–85577c	Putative peptidase (*R. equi*), related to peptidase M23/M37 family (*Bacillus anthracis* Ames), pfam01551, COG0739	NP_066789	150/318 (47%)	4e−59
				NP_844314	38/104 (36%)	1e−11
88	133	85570–85968c	GENEMARK prediction; no homology			
89	653	85965–87923c	Hypothetical protein (*R. equi*), related to ATP binding protein-like protein (*R. equi*)	NP_858493	192/560 (34%)	1e−70
				NP_066791	192/560 (34%)	1e−70
90	353	87943–89001c	Integral membrane protein-like protein (*R. equi*)	NP_066792	71/266 (26%)	3e−06
91	499	89029–90525c	Integral membrane protein-like protein (*R. equi*)	NP_066792	174/473 (36%)	2e−68
92	505	90570–92084c	Putative transfer gene complex protein-like protein (*A. nicotinovorans*)	CAD47985	27/72 (37%)	2e−05
93	301	92078–92980c	Hypothetical protein (*R. equi*)	NP_066795	76/258 (29%)	2e−10
94	198	92985–93578c	Hypothetical protein (*R. equi*)	NP_066796	65/191 (34%)	3e−18
95	90	93617–93886c	GENEMARK prediction; no homology			
96	97	93912–94202c	Hypothetical protein (*Haemophilus somnus* 129PT), related to preprotein translocase SecY subunit (*Haemophilus influenzae* Rd)	ZP_00123677	29/80 (36%)	0.16
				NP_438957	27/75 (36%)	0.31
97	211	94249–94881c	GENEMARK prediction; no homology			
98	413	94887–96125c	GENEMARK prediction; no homology			
99	279	96272–97108c	GENEMARK prediction; no homology			
100	109	97105–97431c	GENEMARK prediction; no homology			
101	551	97778–99430c	Hypothetical protein (*R. equi*)	NP_066802	151/332 (45%)	3e−58
102	144	100135–100566	Putative repressor protein (*R. equi*), related to transcriptional repressor (*B. cereus* ATCC 14579), pfam1381, COG1476	NP_066803	49/141 (34%)	6e−16
				NP_830818	20/61 (32%)	5e−05
103	37	100591–100701	GENEMARK prediction; no homology			
104	59	100804–100980c	GENEMARK prediction; no homology			
105	227	100858–522	gp48 (mycobacteriophage Che8), related to methyltransferase-helicase polyprotein (grapevine rootstock stem lesion-associated virus)	NP_817386	36/91 (39%)	4e−08
				AAN63466	27/92 (29%)	0.052

[a] c, complementary strand.
[b] An E value of >0.4 indicates no homology.

only the predicted ORF8 plus a sequence of 39 bp (nucleotides 5,327 to 5,365) (Fig. 2B). Further reduction of the 2,332-bp fragment by use of restriction enzymes (*Bcl*I, *Bgl*II, and *Kpn*I) (Fig. 2B) or by amplification of ORF8 by PCR did not generate fragments that were able to maintain stable plasmid replication. The ORF8 product had a predicted size of 680 amino acid (aa) residues. Neither the sequence of this fragment nor the predicted amino acid sequence of ORF8 showed similarities to DNA elements or proteins known to be involved in plasmid replication. The only element associated with replication function was the 39-bp sequence. Thus, the 39-bp sequence was also required for replication. This sequence was 92% identical at the nucleotide level to a region of plasmid pSOX from *Rhodococcus* sp. which is located between the replication genes of that plasmid (14). The tandem repeats downstream of ORF8 were not essential for autonomous replication.

ORF9 may encode an ATPase (238 aa) involved in chromosome partitioning. The predicted protein, containing a *parA* domain, was 62% identical to a hypothetical protein of a virulence plasmid from *Rhodococcus equi* (60). ORF10 encodes a predicted protein (109 aa) comprising a helix-turn-helix-like motif of the CopG family, which is involved in dimerization of RepA proteins but not in DNA binding. Homologies were obtained to hypothetical proteins of the virulence plasmids p33701 and p103 of *R. equi* strains ATCC 33701 and 103, respectively. These are plasmids with an origin of replication (*oriV*) that may belong to a novel type (60). The predicted ORF11 product (293 aa) was 42% identical to a putative pro-

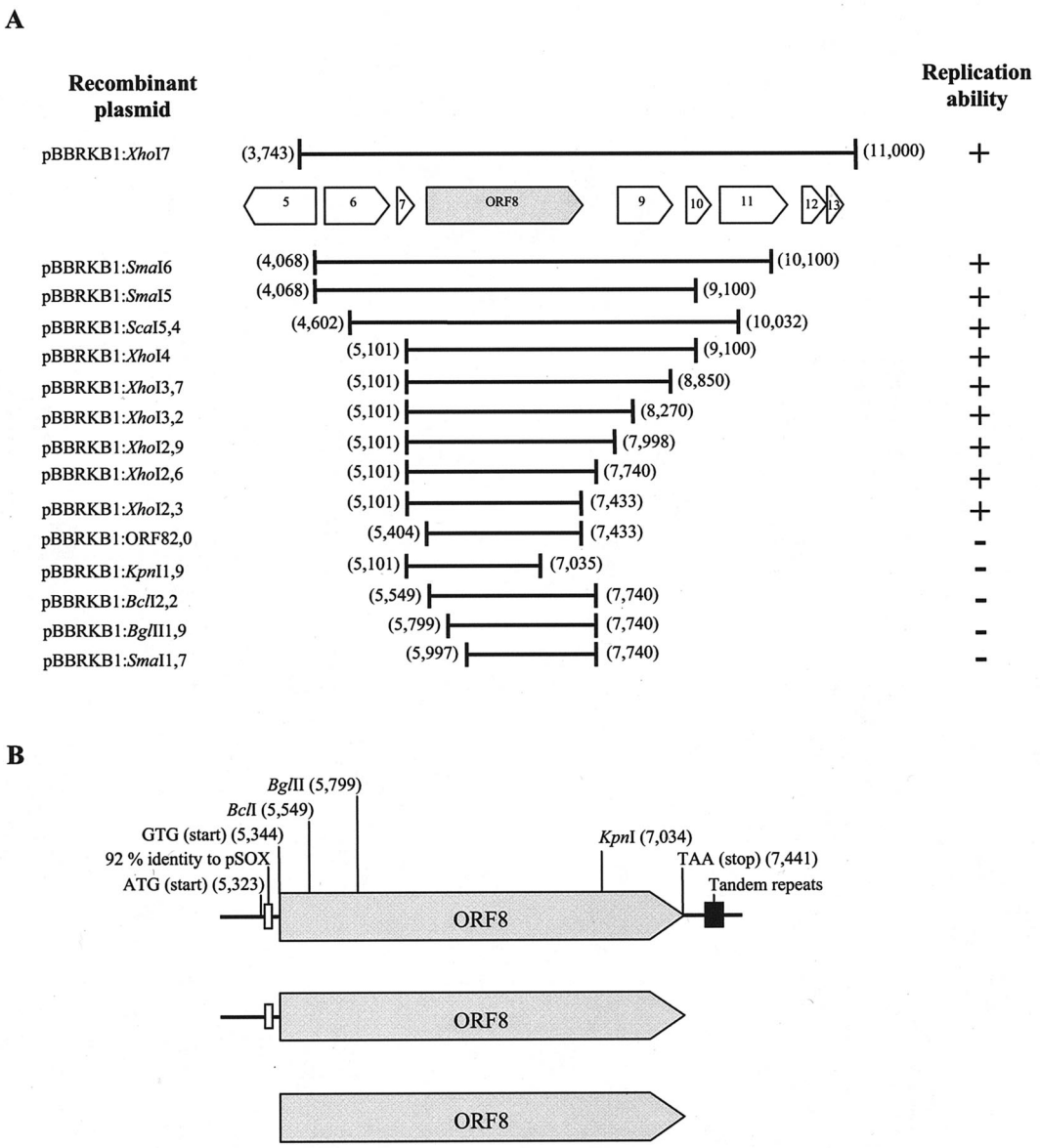

FIG. 2. Mapping of the *oriV* region of plasmid pKB1. (A) The ability of DNA fragments of plasmid pKB1 to confer autonomous replication in *Gordonia* to a suicide plasmid was tested in *G. polyisoprenivorans*. Successfully replicating hybrid plasmids conferred kanamycin resistance to the host, as indicated on the right. Initially, a 7-kbp dual *Xho*I fragment of plasmid pKB1 was identified in a gene bank of this plasmid. A hybrid plasmid containing this 7-kbp *Xho*I region (pBBRKB1:*Xho*I7) and the ORFs carried by it is diagrammed at the top. Below, the sizes and locations of derivatives of the 7-kbp DNA fragment are given. The numbers at the right and left margins of the fragments indicate the exact positions of these fragments in the map of plasmid pKB1. (B) To identify the smallest DNA region of plasmid pKB1 that is essential to confer stable replication, three fragments were amplified by PCR: pBBRKB1:*Xho*I2,6 (top), pBBRKB1:*Xho*I2,3 (middle), and pBBRKB1:ORF82,0 (bottom). The gray arrows indicate the size and position of ORF8, which encodes a protein with unknown function. The ORF8 product may be translated by two possible start codons, ATG (at position 5,323 on the pKB1 map) and GTG (at position 5,344). The stop codon, TAA, of ORF8 is at position 7,441. Moreover, the positions of two possible *cis*-acting sites are given, one a 39-bp sequence upstream of ORF8 with 92% identity to a region of plasmid pSOX from *Rhodococcus* sp. strain X309 (nucleotides 5,327 to 5,365) and the other downstream tandem repeats (nucleotides 7,558 to 7,637). Single restriction sites for *Bcl*I (5,549), *Bgl*II (5,799), and *Kpn*I (7,034) are also shown.

phage excisionase, which again is harbored by the virulence plasmids p33701 and p103 as part of a cluster of replication genes (60). The functions of the other ORFs in this region remained unknown or could be not related to replication.

Host range of pKB1 replicon. The broad-host-range cloning vectors pBBR1MCS-2 (Km^r) and pBBR1MCS-5 (Gm^r), mediating resistance to kanamycin and gentamicin, respectively, were chosen for construction of *E. coli-Gordonia* shuttle vectors. Both plasmids and the 2,332-bp *oriV*-containing fragment of plasmid pKB1 were amplified by PCR (primer pairs P9-P10 and P11-P12, respectively) (Table 2), ligated, and tested for replication in *E. coli* and *Gordonia*, leading to the shuttle vectors pDBMCS-2 and pDBMCS-5 (Fig. 3). Both new vector plasmids possess an extended multiple cloning site (MCS),

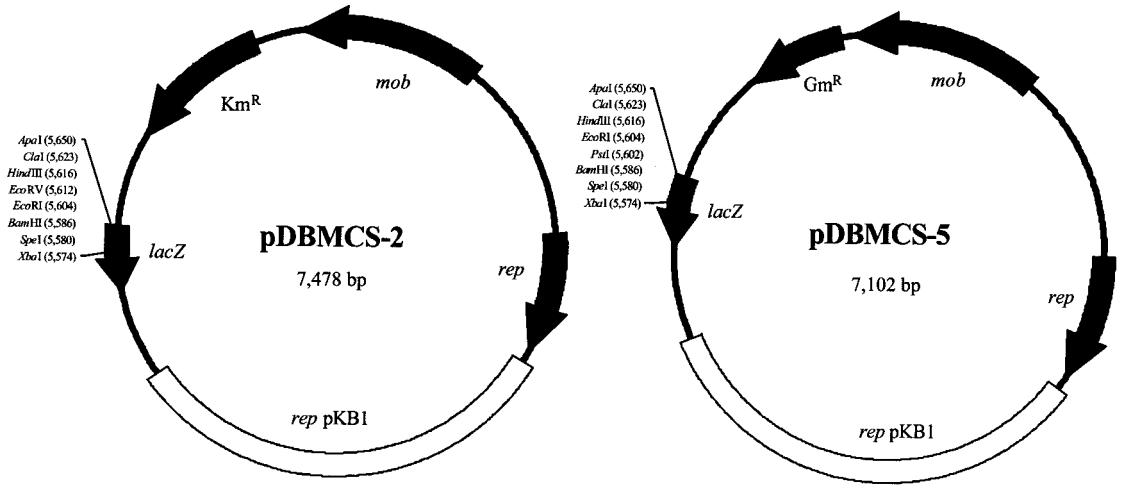

FIG. 3. Physical maps of two *E. coli-Gordonia* shuttle vectors. The 2,332-bp fragment of plasmid pKB1 that is the smallest essential part necessary to confer stable replication in *Gordonia* was amplified by PCR (open white bar) and cloned into suicide plasmids pBBR1MCS-2 and pBBR1MCS-5 (31), leading to the new vector plasmids pDBMCS-2 and pDBMCS-5, respectively. The genes located on the suicide plasmids are indicated by black arrows. Unique restriction sites present in the MCS of each plasmid are shown.

allow for blue-white selection in *E. coli* via alpha complementation, are compatible with IncP, IncQ, IncW, ColE1, and P15a group plasmids, and can be mobilized if the RK2 transfer functions are provided in *trans.* We also constructed derivatives of plasmids pBBR1MCS (Cm^r), pBBR1MCS-3 (Tc^r), and pBBR1MCS-4 (Ap^r), containing the 2,332-bp fragment comprising *oriV* cloned into the MCS, which did not mediate resistance to the respective antibiotics in *G. polyisoprenivorans*.

To test the host range of *oriV* from plasmid pKB1, the *oriV*-carrying hybrid plasmid pBBRKB1:*Xho*I7 was transferred to different species of the genera *Gordonia*, *Rhodococcus*, and *Mycobacterium* by electroporation as well as to two species of the genus *Streptomyces* by transformation of protoplasts (Table 4). Kanamycin-resistant derivatives of *Mycobacterium smegmatis* mc^2155, *Rhodococcus opacus* PD630, *G. polyisoprenivorans* VH2, and *G. polyisoprenivorans* Y2K were obtained, indicating functional expression of the pDBMCS-2-encoded kanamycin resistance gene and stable propagation of the vector plasmid. For all other strains listed in Table 4, no kanamycin-resistant colonies could be obtained. This indicates a rather narrow host range of the *oriV* from plasmid pKB1 that is limited to *Gordonia* strains and closely related bacteria. In addition to the *E. coli-Rhodococcus* shuttle vector systems based on pNC903, which were previously described as functional replicons for *G. polyisoprenivorans* Y2K and VH2 (3), the two *E. coli-Gordonia* shuttle vectors constructed in this study represent a second functional replication system.

TABLE 4. Transfer of pKb1 *ori*[a]

Strain	Method of DNA transfer	Autonomous replication[b]
G. alkanivorans HKI 0136	Electroporation	−
G. desulfuricans 213E	Electroporation	−
G. polyisoprenivorans Kd2	Electroporation	−
G. polyisoprenivorans VH2	Electroporation	+
G. polyisoprenivorans Y2K	Electroporation	+
G. westfalica Kb1	Electroporation	−
G. westfalica Kb2	Electroporation	−
M. smegmatis mc^2155	Electroporation	+
R. opacus PD630	Electroporation	+
R. rhodochrous RNMS1	Electroporation	−
R. ruber	Electroporation	−
S. coelicolor A3(2)	Protoplast transformation	−
S. lividans TK23	Protoplast transformation	−

[a] The *oriV*-containing hybrid plasmid pBBRKB1:*Xho*I7 (Fig. 2A) was transferred to different members of the genera *Gordonia*, *Rhodococcus*, and *Mycobacterium* by electroporation and of *Streptomyces* by protoplast transformation. Resistant colonies were screened for the presence of pBBRKB1:*Xho*I7 by plating on St-I agar plates with 50 mg of kanamycin per liter.

[b] +, positive DNA transfer; −, negative DNA transfer.

Generation of a plasmid-free mutant strain of *G. westfalica* Kb1. Thirty-four ORFs were localized within the metabolic region of pKB1. Three ORFs represented putative regulators, four represented putative membrane proteins, four represented putative peptidases, eight represented putative metabolic functions, and four were putatively involved in heavy-metal resistance. No function could be assigned to 11 ORFs. For identification of the functions encoded by plasmid pKB1, a plasmid-cured derivative strain of *G. westfalica* Kb1 was generated by heat curing (43). This method was used because the bacterium is very sensitive to mitomycin C. The curing procedure was successful, and 14 of 50 tested mutant strains did not contain plasmid pKB1 DNA (data not shown). The plasmid-free *G. westfalica* strains Kb1-K38 and Kb1-K43 were further characterized.

Since plasmid pKB1 contains many putative IS elements, total DNA isolated from the plasmid-free strain Kb1-K43 was analyzed for possible chromosomal insertions of parts of pKB1. Southern hybridization experiments were performed with the complete plasmid pKB1 as probe and total DNA from the plasmid-containing wild-type strain *G. westfalica* Kb1 serving as a positive control, and all 11 *Eco*RI fragments of plasmid pKB1 were visible (Fig. 4). An additional fragment (8,000 bp) for the positive control was observed which could not be derived from plasmid pKB1. When total DNA from the plasmid-free derivative strain Kb1-K43 was used instead of wild-type

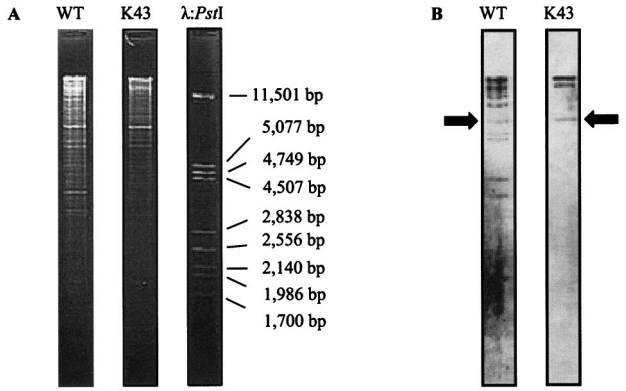

FIG. 4. Southern hybridization analysis for detection of pKB1 in total DNA of pKB1-deficient mutant Kb1-K43 and *G. westfalica* Kb1. The isolated total DNA of the *G. westfalica* Kb1 wild type and of the pKB1-deficient mutant Kb1-K43 was digested with *Eco*RI, separated by agarose gel electrophoresis, and stained with ethidium bromide (A), and the fragments were transferred to a nylon membrane for Southern blotting (B). The DNA of complete plasmid pKB1 was used as a digoxigenin-labeled probe. λ:*Pst*I, λ DNA digested with *Pst*I; WT, total DNA of *G. westfalica* Kb1 wild type; K43, total DNA of *G. westfalica* K43.

DNA, signals corresponding to the two largest *Eco*RI fragments (16,247 and 24,349 bp) of plasmid pKB1 and the signal corresponding to the 8,000-bp fragment were still obtained. This indicated the presence of DNA that was highly homologous to parts of plasmid pKB1 in the chromosome of the cured derivative strain Kb1-K43.

The two IS elements that are putatively responsible for integration, comprising ORF1/ORF2 and ORF53 (only weak homologies to *tnpA* from *Arthrobacter nicotinivorans*, GenBank CAA65743), respectively, coding for putative transposases, were identified by PCR (Fig. 1). The PCR products generated with the primer sets P3-P4 and P5-P6 (Table 2; Fig. 1), containing only the sequences of the putative IS elements (ORF1/ORF2 and ORF53), could be amplified from both the wild type and the mutant Kb1-K43, whereas the PCR products generated with the primer sets P3-P7 and P8-P6 (Table 2; Fig. 1), containing the complete sequences of the two putative IS elements and parts of the adjacent regions, which were expected to be absent from the mutant Kb1-K43 (Fig. 4), could indeed only be amplified from the genome of the wild type and not from that of the mutant Kb1-K43. Therefore, an insertion into the chromosome of the two IS elements harboring ORF1/ORF2 and ORF53 and of the 51,527-bp region between these two IS elements must have already occurred in the wild-type strain Kb1 of *G. westfalica*. The insertion was then maintained in the chromosomes of the pKB1-free mutant; however, this insertion contained the complete conjugation region only. This demonstrates that the metabolic and heavy-metal resistance genes were eliminated during plasmid curing and that insertion of the conjugation region may have occurred early in the history of *G. westfalica*.

In conclusion, the metabolic region may constitute a catabolic transposon, which is a widespread occurrence among eubacteria and is found in gram-negative as well as gram-positive bacteria. Composite transposons are flanked by related, but not necessarily identical, IS elements and may be very large, exceeding 50 kbp (62).

The plasmid-free mutant strain of *G. westfalica* was not able to grow on *cis*-1,4-polyisoprene as sole carbon source. Since several examples for plasmid-encoded degradation pathways are known (62), the possible involvement of plasmid pKB1 in rubber degradation was tested. The *G. westfalica* strain Kb1 wild type and plasmid-free mutant derivatives were cultivated for 40 days in the presence of *cis*-1,4-polyisoprene as the sole carbon source. While the turbidity at 600 nm of the wild-type culture increased from 0.15 to 0.75 during incubation, that of the mutant strains did not. This indicated that the mutant strains were not able to use *cis*-1,4-polyisoprene as a sole carbon source and that, therefore, at least some genes of plasmid pKB1 of *G. westfalica* must be involved in rubber degradation.

Genes essential for rubber biodegradation are probably encoded by the 49,489-bp region that is definitively missing from the cured mutants (Fig. 4). Since little is known about the biodegradation of rubber (25, 36) and proteins involved in this biochemical process have not been identified, it is not surprising that many ORFs with a hitherto unknown function are located in this metabolic region. These are strong candidates for rubber degradation genes. Electron transport proteins are often involved in catabolic and anabolic reactions and may also be involved in the initiation of biodegradation of rubber. Three ORFs (ORF27, ORF39, and ORF42) encode putative proteins that may be involved in cytochrome c biosynthesis. ORF27 encodes a protein of 520 aa, exhibiting 52% identity to a conserved hypothetical protein from *Mycobacterium tuberculosis*, which is putatively involved in cytochrome c biosynthesis. ORF39 encodes a protein of 271 aa, whose predicted protein comprises a DsbD (cytochrome c biogenesis) domain, and exhibits 51% identity to CCDA from *Mycobacterium bovis*. ORF42 encodes a protein of 597 aa which contains a COX1 domain and exhibits 77% identity to the cytochrome c oxidase subunit I isolated from *M. tuberculosis*. Cytochromes are involved in various electron transport systems and do not function only in aerobic or anaerobic respiration (17), e.g., cytochrome c catalyses peroxidase-like reactions in the presence of an electron acceptor like hydrogen peroxide (68). During rubber biodegradation, cytochrome c may catalyze epoxidation of the *cis*-1,4-polyisoprene molecule.

A putative epoxide hydrolase encoded by ORF6, which is localized about 10 kbp downstream of the metabolic region, may subsequently catalyze hydrolysis of the epoxide to the corresponding diol as has been shown for other epoxide hydrolases (69). A similar initiation of isoprene degradation was described by Johan et al. (26) for *Rhodococcus* sp. strain AD45. Such a sequence of reactions would in principal be consistent with the occurrence of cleavage products identified during biodegradation of natural rubber or related compounds (9, 10, 16, 53, 64). In addition, ORF28 and ORF40 encode proteins which are putatively necessary for the transport of electrons. The predicted ORF40 product (209 aa) contains a thioredoxin domain and exhibits 52% identity to a hypothetical protein isolated from *Mycobacterium leprae*.

Since biodegradation of rubber must occur outside of the cell or at the cell surface, membrane and transport proteins most probably perform a crucial function in rubber biodegradation. Furthermore, special proteins or other biopolymers

TABLE 5. Determination of cadmium tolerance for different strains of *Gordonia*, *Rhodococcus*, and *Mycobacterium*[a]

Strain	Growth in the presence of $CdCl_2$ (μM)[b]									
	0	100	150	200	300	400	500	600	700	800
G. alkanivorans	++	++	++	++	+	+	+/−	+/−−	+/−−	+/−−
G. amicalis	++	++	++	++	++	++	+	+/−	+/−	+/−
G. bronchialis	++	++	++	++	++	++	++	++	++	++
G. desulfuricans	++	++	++	+	+/−−	−	−	−	−	−
G. nitida	++	++	++	++	++	++	+	+	+	+
G. polyisoprenivorans VH2	++	++	+	−	−	−	−	−	−	−
G. polyisoprenivorans Y2K	++	++	+	−	−	−	−	−	−	−
G. sputi	++	+/−	+/−−	−	−	−	−	−	−	−
G. terrae	++	−	−	−	−	−	−	−	−	−
G. westfalica K43	++	++	++	++	++	++	++	++	++	++
G. westfalica Kb1	++	++	++	++	++	++	++	++	++	+
M. smegmatis mc^2155	++	+/−	−	−	−	−	−	−	−	−
R. opacus PD630	++	+	−	−	−	−	−	−	−	−
R. rhodochrous	++	+/−	−	−	−	−	−	−	−	−

[a] The strains were cultivated on St-I agar plates containing the indicated concentration of cadmium. After an incubation period of 3 days at 30 °C, growth was evaluated.
[b] ++, good growth; +, growth; +/−, limited growth; +/−−, very limited growth; −, no growth.

may be required to establish a tight contact of the cells with the rather hydrophobic rubber molecules, in particular for those bacteria exhibiting adhesive growth as a biofilm on natural rubber, such as all species of the genus *Gordonia* (36). The protein encoded by ORF4, which is located 10 kbp downstream of the metabolic region, exhibited weak homology to TmtpC from *M. smegmatis*, which is involved in the transport of glycopeptidolipids through the cytoplasm membrane. Glycopeptidolipids are especially necessary for the formation of biofilms on polyvinylchloride (48). Finally, the probably complex biochemical process of rubber biodegradation and of other functions encoded by pKB1 will most probably be strongly regulated, thus explaining the occurrence of several genes for putative regulator proteins on pKB1. Thus, plasmid pKB1 encodes numerous candidate proteins that may be involved in the initial attack on natural rubber and/or in facilitating physical contact between the degrading bacterium and its substrate.

Furthermore, the metabolic region harbors a putative methyltransferase (ORF31, isolated from *Shewanella oneidensis*; 37% identity), a putative polyprenol-phosphate-mannose synthase 2 (ORF35, isolated from *M. smegmatis*; 42% identity), and a putative oxidoreductase (ORF36, isolated from *Bacillus cereus*; 22% identity).

Cadmium sensitivity of the wild type and the plasmid-free mutant. In addition to genes encoding putative degradation pathways, plasmid pKB1 contains several genes that might be involved in heavy-metal homeostasis (ORFs 27, 29, 32, 33, 37, and 45) (Table 3). Moreover, *G. westfalica* was able to grow on St-I agar plates in the presence of 800 μM Cd(II) (Table 5). The bacterial species *G. amicalis*, *G. bronchialis* 43341, and *G. nitida* exhibited similar cadmium resistance, while the tolerance of cadmium under the same conditions of other related bacteria, *G. alkanivorans* 44369 (growth up to 600 μM cadmium), *G. desulfuricans* (growth up to 300 μM cadmium), *G. polyisoprenivorans*, *G. sputi*, *M. smegmatis* mc155, *R. opacus* PD630, *Rhodococcus rhodochrous* (growth up to 200 μM cadmium), *G. terrae* (growth up to 100 μM cadmium), was significantly lower. Thus, *G. westfalica* was more resistant to cadmium than 8 of 12 bacteria tested.

To investigate whether plasmid pKB1 mediates cadmium resistance in this bacterium, we compared growth of *G. westfalica* and the plasmid-free mutant strain Kb1-K43 in liquid medium in the presence of increasing cadmium concentrations (Fig. 5). A concentration of about 300 μM cadmium was required to decrease growth of the *G. westfalica* wild type to 50%, while about 40 μM cadmium was sufficient to reduce growth of the plasmid-free strain to half. Thus, the absence of plasmid pKB1 diminished cadmium resistance of *G. westfalica* by a factor of about 7, indicating that plasmid pKB1 is involved in cadmium resistance.

The ORF34 gene product CadA may contribute to pKB1-mediated cadmium resistance. ORF34 from *G. westfalica* Kb1 plasmid pKB1 encodes a putative Cd^{2+}/Zn^{2+}-transporting P-type ATPase (Table 3). The predicted protein exhibits the closest similarity (58% identity) to a cation-transporting P-type

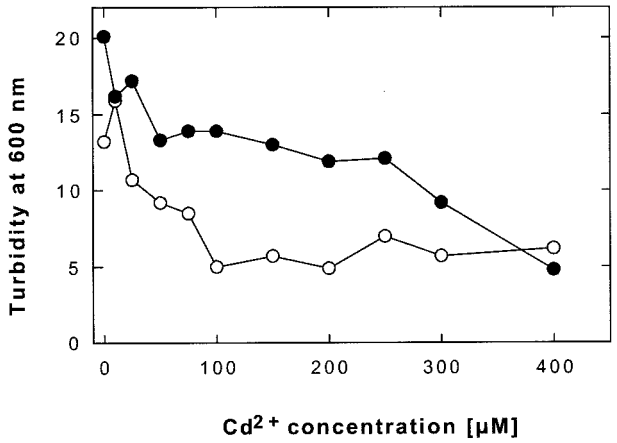

FIG. 5. Effect of Cd^{2+} on growth of *G. westfalica*. Dose-response curves for cadmium were done with the *G. westfalica* Kb1(pKB1) wild type (●) and the plasmid-cured derivative strain K43 (○).

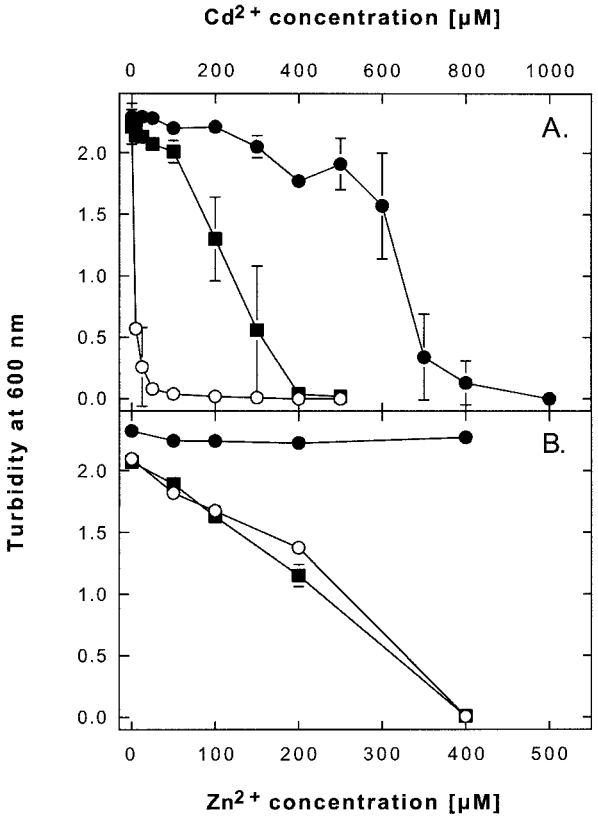

FIG. 6. Cadmium and zinc resistance of *E. coli* strains expressing a P-type ATPase from *G. westfalica* Kb1, GoCadA. Dose-response curves for cadmium (A) and zinc (B) are shown for *E. coli* strain RW3110 (Δ*zntA*) complemented in *trans* with the *cadA* gene of *G. westfalica* Kb1 cloned into plasmid pASK3 (■). The negative control strain is RW3110(pASK3) (○), and the positive control is the wild-type strain W3110(pASK3) (●). The mean results of three (cadmium) or two (zinc) independent experiments are shown, with standard deviation bars.

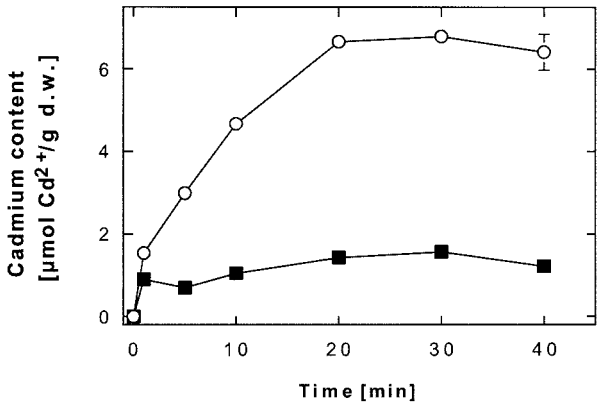

FIG. 7. Presence of P-type ATPase GoCadA from *G. westfalica* Kb1 diminishes accumulation of Cd^{2+} in *E. coli* strain RW3110. *E. coli* strain RW3110 (Δ*zntA*) was complemented in *trans* with the gene encoding GoCadA (■). The negative control (○) contained only the vector plasmid, pASK3. The mean values of two independent experiments with 10 μM $^{109}Cd^{2+}$ are shown. d.w., dry weight.

ATPase G (CtpG; NP_216508) of *M. tuberculosis*, another gram-positive bacterium with high GC content, and to other P-type ATPases, e.g., CadA from *Staphylococcus aureus*, the first described example of a heavy-metal effluxing P-type or CPx-type ATPase (45).

ORF34 was amplified by PCR from plasmid DNA of *G. westfalica* Kb1 and the gene was cloned into plasmid pASK3. The resulting plasmid, pECA34, was transferred into *E. coli* strain RW3110 (Δ*zntA*), a metal-sensitive strain with a deletion in the native Zn^{2+}/Cd^{2+}-effluxing P-type ATPase ZntA of this bacterium (50). The *G. westfalica* Kb1 gene corresponding to ORF34 conferred cadmium resistance to *E. coli* RW3110, but not zinc resistance (Fig. 6). It was therefore designated *cadA* and its product was designated GoCadA.

Half-maximal inhibition of *E. coli* strain RW3110 (Δ*zntA*) and wild-type strain W3110 occurred at about 5 and 650 μM Cd^{2+}, respectively (Fig. 6). Complementation in *trans* with *cadA* from *G. westfalica* Kb1 led to half-maximal inhibition at about 250 μM Cd^{2+}. Thus, GoCadA was able to protect *E. coli* cells against cadmium about half as efficiently as the native efflux system ZntA.

The metal cation uptake into cells of *E. coli* strain RW3110 expressing GoCadA was examined (Fig. 7). Cells of metal-sensitive *E. coli* strain RW3110 accumulated 6.6 μmol of $^{109}Cd^{2+}$ per g of cell dry weight within 20 min in an assay buffer containing 10 μM cadmium. In contrast, cells containing GoCadA accumulated only 1.4 μmol of cadmium per g of cell dry weight, only 20% of the amount bound to metal-sensitive control cells. Thus, GoCadA was functionally expressed in *E. coli* and decreased the accumulation of cadmium by *E. coli* cells, probably by cadmium efflux. These data indicate that GoCadA may contribute to plasmid pKB1-mediated cadmium resistance in this bacterium.

Plasmid pKB1 harbored putative heavy-metal resistance genes in addition to *cadA*, which is a notable situation. ORF28 encodes a protein of 415 aa containing a domain of the Nramp family and exhibiting 59% identity to a divalent cation transporter integral membrane protein isolated from *M. bovis*. ORF38 encodes a predicted protein (314 aa) comprising a domain of the cation efflux family, and 35% identity was observed to a cobalt-zinc-cadmium resistance protein from *Xanthomonas axonopodis*. ORF46 encodes a protein of 730 aa and exhibits highest identity (46%) to a cation-transporting ATPase of *M. leprae*.

Thus, plasmid pKB1 seems to harbor two genes for P-type ATPases. Bacteria that contain more than one CPx-type ATPase of the Zn/Cd/Pb group of proteins may exhibit differentiation of the functions of these proteins, while in bacteria that contain only one of these proteins, the substrate specificity of the single transporter should be broader. In *E. coli*, ZntA is responsible for detoxification of Zn^{2+}, Cd^{2+}, and Pb^{2+} (49–51). In contrast, in the gram-negative bacterium *Ralstonia metallidurans*, the three CPx-type ATPases of the Zn/Cd/Pb group mainly concentrate on Zn^{2+} (ZntA), Cd^{2+} (CadA), or Pb^{2+} (PbrA) with respect to the regulation of their expression and their substrate specificities (11, 34). CadA from *Bacillus subtilis* confers resistance to cadmium, zinc, and cobalt (19), and Bxa1 from *Oscillatoria brevis* confers resistance to zinc and cadmium (40) in *E. coli* (34).

Conjugation region. The third region of the plasmid appears to be concerned with conjugation, a complex process which involves many genes (39). This region is divided into two parts by the presence of an IS element comprising ORF76 and ORF77. This conjugation region contains 49 detected ORFs and the putative transcriptional products of 6 ORFs exhibiting homologies to proteins putatively involved in conjugational processes. ORF67 presumably encodes a TraA-like protein with 33% identity to a protein from *R. equi* that contains an ATP/GTP binding motive. The transcriptional product predicted for ORF75 represents a protein of 1,631 aa, comprising a DEAD-like helicase superfamily domain, and 37% identity was observed to a putative methylase from *R. equi*. Further similarities were observed in particular to the plasmid-encoded conjugative transfer gene complexes (*trs*) of *Lactococcus lactis* DPC3147 (15) and *S. aureus* (42). This provides evidence of possible pKB1-encoded conjugative transfer and should be further investigated.

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