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The Genomes of the Non-Clearing-Zone-Forming and Natural-Rubber-Degrading Species *Gordonia polyisoprenivorans* and *Gordonia westfalica* Harbor Genes Expressing Lcp Activity in *Streptomyces* Strains[▽]†

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The latex-clearing protein (Lcp_{K30}) from the rubber-degrading bacterium *Streptomyces* sp. strain K30 is involved in the cleavage of poly(*cis*-1,4-isoprene), yielding isoprenoid aldehydes and ketones. Lcp homologues have so far been detected in all investigated clearing-zone-forming rubber-degrading bacteria. Internal degenerated oligonucleotides derived from *lcp* genes of *Streptomyces* sp. strain K30 (lcp_{K30}), *Streptomyces coelicolor* strain A3(2), and *Nocardia farcinica* strains IFM10152 and E1 were applied in PCR to investigate whether *lcp* homologues occur also in the non-clearing-zone-forming rubber-utilizing bacteria *Gordonia polyisoprenivorans* strains VH2 and Y2K, *Gordonia alkanivorans* strain 44187, and *Gordonia westfalica* strain Kb1, which grow adhesively on rubber. The 1,230- and 1,224-bp *lcp*-homologous genes from *G. polyisoprenivorans* strain VH2 (lcp_{VH2}) and *G. westfalica* strain Kb1 (lcp_{Kb1}) were obtained after screening genomic libraries by degenerated PCR amplification, and their translational products exhibited 50 and 52% amino acid identity, respectively, to Lcp_{K30}. Recombinant lcp_{VH2} and lcp_{Kb1} harboring cells of the non-rubber-degrading *Streptomyces lividans* strain TK23 were able to form clearing zones and aldehydes on latex overlay-agar plates, thus indicating that lcp_{VH2} and lcp_{Kb1} encode functionally active proteins. Analysis by gel permeation chromatography demonstrated lower polymer concentrations and molecular weights of the remaining polyisoprenoid molecules after incubation with these recombinant *S. lividans* strains. Reverse transcription-PCR analysis demonstrated that lcp_{VH2} was transcribed in cells of *G. polyisoprenivorans* strain VH2 cultivated in the presence of poly(*cis*-1,4-isoprene) but not in the presence of sodium acetate. Anti-Lcp_{K30} immunoglobulin Gs, which were raised in this study, were rather specific for Lcp_{K30} and did not cross-react with Lcp_{VH2} and Lcp_{Kb1}. A lcp_{VH2} disruption mutant was still able to grow with poly(*cis*-1,4-isoprene) as sole carbon source; therefore, lcp_{VH2} seems not to be essential for rubber degradation in *G. polyisoprenivorans*.

Investigations of bacterial degradation of natural rubber (NR) revealed two groups of NR-degrading bacteria according to their strategy for substrate utilization. (i) Members of the first group form clearing zones on latex overlay-agar plates, indicating an extracellular enzyme activity. Representatives belong to the genera *Actinomadura*, *Actinoplanes*, *Dactylosporangium*, *Micromonospora*, *Microtetraspora*, *Nocardia*, and *Streptomyces* (22, 23). (ii) Members of the second group exhibit adhesive growth with direct contact of the cells with the NR material and extensive disintegration of the substrate. Representatives belong to the genera *Gordonia*, *Mycobacterium*, and *Nocardia* (2, 28, 29, 30, 41).

Lcp (latex-clearing protein) has been considered a key enzyme in NR degradation by clearing-zone-forming gram-positive bacteria (33), and RoxA (rubber oxygenase) has been considered a key enzyme in NR degradation by the gram-negative bacterium *Xanthomonas* sp. strain 35Y (24). RoxA is an extracellular diheme protein secreted by this strain during growth on NR (24). Purified RoxA degraded poly(*cis*-1,4-isoprene) by oxidative cleavage at the double bonds, yielding 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al as the main cleavage product; other minor cleavage products differed only in the number of repetitive isoprene units (9). In vitro experiments also revealed occurrence of two ^{18}O atoms in the reduced degradation product 12-hydroxy-4,8-dimethyltrideca-4,8-diene-1-ol, thereby disclosing a dioxygenase mechanism (10).

The *lcp* gene was identified in the gram-positive *Streptomyces* sp. strain K30, which belongs to the first group of NR-degrading bacteria, by Rose et al. (33). UV mutagenesis produced mutants with a clearing-zone-negative phenotype on latex overlay-agar plates and the inability to mineralize NR. A genomic DNA fragment from *Streptomyces* sp. strain K30, which restored the latex-positive phenotype in the mutants, comprised three open reading frames possibly involved in NR degradation. The translational product of one gene exhibited similarities to a putatively secreted protein of *Streptomyces coelicolor* strain A3(2) and was designated Lcp (latex-clearing protein). The translational products of the two other open reading frames exhibited strong similarities to putative heterodimeric molybdenum hydroxylases (OxiAB) representing some isochinoline oxidoreductases and aldehyde dehydrogenases. Heterologous expression of *lcp* in *Streptomyces lividans* strain TK23, which is not able to utilize rubber, enabled this strain to form clearing zones on latex overlay-agar plates and to degrade NR as demonstrated by gel permeation chromatography (GPC) analysis and by staining with Schiff's reagent indicating the pres-

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ence of compounds with aldehyde groups among the degradation products of NR. According to a hypothetical degradation pathway, the rubber molecules are cleaved by Lcp to aldehydes and ketones with low molecular weights which are then possibly further oxidized by OxiAB to the corresponding acids and activated and metabolized via the β-oxidation pathway in *Streptomyces* sp. strain K30 (34).

Very little is known about the biochemical mechanism of rubber degradation in adhesively growing bacteria. Members of the genus *Gordonia* serve as model organisms to investigate this aspect (2, 5, 12, 28, 29, 30). Due to the importance of Lcp for biodegradation of NR in *Streptomyces* sp. strain K30, the occurrence and diversity of Lcp homologues in members of the genus *Gordonia* were investigated in this study. This study should unravel whether Lcp homologues occur only in clearing-zone-forming bacteria or in any rubber-degrading bacteria. One Lcp homologue was recently identified in a thermophilic adhesively growing strain of *Nocardia farcinica* (20). To identify Lcp homologues in *Gordonia* sp., degenerate PCR primers specific for genes coding for Lcp were designed based on DNA sequences from *Streptomyces* sp. strain K30, *S. coelicolor* strain A3(2), and the *N. farcinica* strains IFM10152 and E1. Two identified *lcp*-homologous genes were then further characterized.

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*, *Nocardia*, and *Streptomyces* were grown at 30°C on either standard I complex nutrient broth (St-I; Merck, Darmstadt, Germany) or mineral salts medium (MSM) (38). Carbon sources were added to liquid MSM as indicated in the text. Liquid cultures in Erlenmeyer flasks were incubated on a horizontal rotary shaker. Solid media were prepared by addition of 1.5% (wt/vol) agar-agar. For preparation of latex overlay-agar plates, MSM agar plates, containing 1% (wt/vol) glucose, were covered with an overlay of MSM agar containing 0.2% (vol/vol) latex concentrate (Neotex Latz; Weber & Schaer, Hamburg, Germany) plus 1% (wt/vol) glucose. *Escherichia coli* was cultivated at 37°C in Luria-Bertani broth (LB) (36). Antibiotics were applied according to the method of Sambrook et al. (36) and as indicated in the text. Protoplasts of *S. lividans* strain TK23 were prepared from cells grown in modified YEME (3% [wt/vol] yeast extract, 5% [wt/vol] Bacto peptone, 3% [wt/vol] malt extract, 34% [wt/vol] sucrose) medium (19). R5 agar plates were used for protoplast regeneration (25).

Isolation, analysis, and manipulation of DNA. Plasmid DNA was prepared from crude lysates by the alkaline extraction method (8). Total DNA of *Streptomyces*, *Gordonia*, and *Nocardia* was prepared as described by Ausubel et al. (4) with modifications as recently described (12). Recombinant DNA techniques for *S. lividans* strain TK23 were performed as described previously (25). DNA was transferred to *Gordonia polyisoprenivorans* strain VH2 by electroporation (3). DNA was restricted with restriction endonucleases (Gibco/BRL, Gaithersburg, MD) under conditions recommended by the manufacturer. All other genetic procedures and manipulations were conducted as described by Sambrook et al. (36).

GPC. Cleavage of poly(*cis*-1,4-isoprene) by recombinant strains of *S. lividans* strain TK23 was verified by GPC. After a cultivation period of 8 weeks, samples were prepared in chloroform and analyzed as previously described (20) employing a Waters-GPC system (Waters, Milford, CT) consisting of a 515 high-pressure liquid chromatography 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, and 10^6 Å, respectively). Molecular weights of poly(*cis*-1,4-isoprene) and of cleavage products were calculated from retention times of defined poly(*cis*-1,4-isoprene) standards (PSS Polymer Standards Service GmbH, Mainz, Germany).

Cosmid cloning and sequencing. A cosmid library of *Gordonia westfalica* strain Kb1 was prepared from partially restricted (EcoRI) genomic DNA yielding preferentially fragments in the 30- to 40-kbp size range. The latter were ligated to pHC79 (18) and packaged into λ particles (17), and ampicillin-plus-tetracycline-resistant transductants of *E. coli* strain DH5α were selected. Clones containing hybrid cosmids of this cosmid library of *G. westfalica* strain Kb1 and of another cosmid library of *G. polyisoprenivorans* strain VH2 (Quyen Banh, unpublished data) were selected on LB-ampicillin-tetracycline agar plates. All the colonies from several plates (around 50 on each plate) were pooled, plasmid DNA was prepared from each pool, and putative fragments containing the *lcp* genes were amplified by PCR at an annealing temperature of 61°C using the ImmoMix ready-to-use PCR mixture (BioLine, Randolph, MA) and the degenerated primers P265f and P701r (Table 1). The number of colonies from the master plate of the identified pool was decreased and used for further screening by the described PCR amplification. Using this method, 35 and 25 pools comprising 700 and 500 clones of *G. westfalica* strain Kb1 or *G. polyisoprenivorans* strain VH2, respectively, were screened for the presence of *lcp*-containing hybrid plasmids. Hybrid cosmids pHC79::535, pHC79::549, and pHC79::632 from *G. westfalica* strain Kb1 and pHC79::290 from *G. polyisoprenivorans* strain VH2 contained *lcp*-homologous sequences. These hybrid cosmids were used as template DNA for sequencing reactions by primer walking. DNA was sequenced in a 48-capillary 3730 DNA Analyzer electrophoresis system (Applied Biosystems, Foster City, CA). Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). For sequencing of inserts in the pGEM-T Easy vector (Promega, Madison, WI), the universal M13 forward primer and M13 reverse primer were used.

Nested PCR. The 3′ region of *lcp* from *G. westfalica* strain Kb1 (lcp_{Kb1}) was amplified by nested PCR employing one biotinylated oligonucleotide, which binds closely to the 3′ end of the known lcp_{Kb1} sequence, plus four degenerated walker primers, which were expected to anneal in the 3′-flanking region of known *lcp* sequences according to the work of Mishra et al. (32). A set of four PCRs was carried out using genomic DNA of *G. westfalica* strain Kb1 as template and each single biotinylated primer in combination with each of the four walking primers under conditions described by Mishra et al. to amplify the flanking region (32). Streptavidin-coupled magnetic beads (Roche, Switzerland) were then applied according to the manufacturer's protocol to isolate biotinylated PCR products which were subsequently used as template for a second set of PCRs. For this a specific nested primer was designed, binding even more closely to the 3′ region of the known sequence than the corresponding biotinylated oligonucleotide. The specific nested primer was used together with the general nested primer, also taken from the work of Mishra et al. (32), for PCR, and the obtained amplification products were then cloned into the vector pGEM-T Easy (Promega, Madison, WI) and sequenced (see "Cosmid cloning and sequencing"). The designed biotinylated and nested oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

Sequence analyses. Database searches of the predicted protein sequences were performed employing the BLAST program provided by EMBL/Heidelberg (1). Multiple sequence alignments and the generation of sequence contigs were carried out using the program ClustalX (39). Phylogenetic trees were constructed using the program TREE 1.6.5. The SignalP 3.0 server was used for prediction of the presence and location of signal peptide cleavage sites in amino acid sequences (15).

Construction of plasmids. The coding regions of lcp_{VH2} from *G. polyisoprenivorans* strain VH2 and lcp_{Kb1} from *G. westfalica* strain Kb1, including regions approximately 400 bp upstream of the start codons and approximately 100 bp downstream of the stop codons, were amplified by PCR using oligonucleotides PVH2_117fBglII plus PVH2_1752rBglII for lcp_{VH2} and PKb1_57fBglII plus PKb1_1789rBglII for lcp_{Kb1} (Table 1) and Pfx DNA polymerase (Gibco BRL) according to the manufacturer's instructions. For complementation experiments in *S. lividans* strain TK23, the *E. coli-Streptomyces* shuttle vector pIJSK::lcp_{K30} was used as a positive control and also for cloning of lcp_{VH2} and lcp_{Kb1}. The vector pIJSK::lcp_{K30} conferred ampicillin resistance for selection in *E. coli* and thiostrepton resistance for selection in *Streptomyces* (Henrike Wernsmann, unpublished data). To obtain pIJSK from pIJSK::lcp_{K30}, lcp_{K30} was excised by restriction with BglII. It was then also ligated to lcp_{VH2} or lcp_{Kb1}, yielding pIJSK::lcp_{VH2} or pIJSK::lcp_{Kb1}, respectively (see Fig. S1 in the supplemental material). All plasmids were transferred to *S. lividans* strain TK23 by protoplast transformation (25).

The coding region of lcp_{K30} from *Streptomyces* sp. strain K30 was amplified by PCR using oligonucleotides Lcp_NtermNdeI and Lcp_CtermBamHI (Table 1) and Pfx DNA polymerase (Gibco BRL) according to the manufacturer's instructions. The PCR product was then cloned into the NdeI-BamHI-linearized plasmid pET-23a, yielding plasmid pET-23a::lcp_{K30}his, which was subsequently transferred to *E. coli* BL21(DE3).

To study the loss of functionality of the disrupted lcp_{VH2} gene, it was amplified from the disruption mutant A17 by PCR using primers PVH2_117fBglII and PVH2_1752bBglII (Table 1). The resulting 2,635-bp lcp_{VH2}::*aph* fragment was cloned into the BglII-linearized pIJSK vector. The hybrid plasmid

TABLE 1. Bacterial strains and plasmids and oligonucleotides used in this study

Strain, plasmid, or oligonucleotide	Relevant marker(s) or sequence (5′→3′)	Reference, source, or function
Strains		
Gordonia species		
G. polyisoprenivorans strain VH2	Poly(*cis*-1,4-isoprene)-degrading wild type	2
G. polyisoprenivorans strain Y2K	Poly(*cis*-1,4-isoprene)-degrading wild type	2
G. polyisoprenivorans mutant A12	lcp_{VH2} disruption mutant of *G. polyisoprenivorans* strain VH2; Km^r	This study
G. polyisoprenivorans mutant A17	lcp_{VH2} disruption mutant of *G. polyisoprenivorans* strain VH2; Km^r	This study
G. polyisoprenivorans mutant A29	lcp_{VH2} disruption mutant of *G. polyisoprenivorans* strain VH2; Km^r	This study
G. polyisoprenivorans mutant A34	lcp_{VH2} disruption mutant of *G. polyisoprenivorans* strain VH2; Km^r	This study
G. alkanivorans strain 44187	Poly(*cis*-1,4-isoprene)-degrading wild type	26
G. westfalica strain Kb1	Poly(*cis*-1,4-isoprene)-degrading wild type	30
Nocardia farcinica strain IFM10152	Poly(*cis*-1,4-isoprene)-degrading wild type	21
Streptomyces species		
Streptomyces sp. strain K30	Poly(*cis*-1,4-isoprene)-degrading and clearing-zone-forming wild type	33
S. lividans strain TK23	Non-poly(*cis*-1,4-isoprene)-degrading and non-clearing-zone-forming wild type	19
E. coli strains		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_k^- m_k^+) *supE44 relA1* λ⁻ *lac* [F′ *proAB lacI*^{q} *lacZ*Δ*M15* Tn*10*(Tc^r)]	13
DH5α	F⁻ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_k^- m_k^+) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Roche Applied Science, Penzberg, Germany
BL21(DE3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm lacY1*(DE3)	Novagen, Madison, WI
Plasmids		
pBluescript SK⁻	Ap^r *lacPOZ′*	Stratagene, San Diego, CA
pHC79	Cosmid (Ap^r Tc^r)	18
pGEM-T Easy	*E. coli* cloning vector (Ap^r T-tailing)	Promega, Madison, WI
pGEM-T Easy::lcp_{VH2}::*aph*	*E. coli* cloning vector (Ap^r), containing the cloned gene lcp_{VH2} from *G. polyisoprenivorans* strain VH2 with an inserted *aph* (Km^r)	This study
pET-23a	*E. coli* expression vector (Ap^r T7 promoter)	Novagen, Madison, WI
pET-23a::lcp_{K30}his	*E. coli* expression vector (Ap^r T7 promoter), containing the cloned gene lcp_{K30} from *Streptomyces* sp. strain K30	This study
pIJSK::lcp_{K30}	*E. coli-Streptomyces* shuttle vector, containing the cloned gene lcp_{K30} from *Streptomyces* sp. strain K30 (Ap^r Tsr^r *melC1 melC2*)	Henrike Wernsmann, unpublished data
pIJSK	*E. coli-Streptomyces* shuttle vector (Ap^r Tsr^r *melC1 melC2*)	This study
pIJSK::lcp_{VH2}	*E. coli-Streptomyces* shuttle vector, containing the cloned gene lcp_{VH2} from *G. polyisoprenivorans* strain VH2 (Ap^r Tsr^r *melC1 melC2*)	This study
pIJSK::lcp_{VH2}::*aph*	*E. coli-Streptomyces* shuttle vector, containing the cloned gene lcp_{VH2}::*aph from G. polyisoprenivorans* mutant A17 (Ap^r Tsr^r Km^r *melC1 melC2*)	This study
pIJSK::lcp_{Kb1}	*E. coli-Streptomyces* shuttle vector, containing the cloned gene lcp_{Kb1} from *G. westfalica* strain Kb1 (Ap^r Tsr^r *melC1 melC2*)	This study
Oligonucleotides		
P265f	CGCTGCCCG(AG)CGGA(CT)T(GC)CC(GC)	Degenerated PCR primer based on *lcp*-homologous sequences, with P701r amplification of a 436-bp PCR product
P701r	CTGTG(GC)(AC)AGGTGACCA(AGT)GCATGTCG	Degenerated PCR primer based on *lcp*-homologous sequences, with P265f amplification of a 436-bp PCR product

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TABLE 1—*Continued*

Strain, plasmid, or oligonucleotide	Relevant marker(s) or sequence (5′→3′)	Reference, source, or function
PVH2_117fBglII	ATATAGATCTGCGAGGTGCGTTGAATACCG	With PVH2_1752rBglII, 1,635-bp lcp_{VH2} PCR product of *G. polyisoprenivorans* strain VH2 (BglII restriction sites used for cloning are underlined)
PVH2_1752rBglII	ATATAGATCTAGGACCTCGGTTCCGGTGAAC	With PVH2_117fBglII, 1,635-bp lcp_{VH2} PCR product of *G. polyisoprenivorans* strain VH2 (BglII restriction sites used for cloning are underlined)
PVH2_360f(RT)	TTCAATCAGCGACGGGGCAC	With PVH2_911r(RT), 551-bp PCR product of *G. polyisoprenivorans* strain VH2 used for RT-PCR
PVH2_911r(RT)	AGCATGTCGGCGAGTTTCTGG	With PVH2_360f(RT), 551-bp PCR product of *G. polyisoprenivorans* strain VH2 used for RT-PCR
PVH2_449f	TGACGAAGGCCAGCAGCAGG	With PVH2_2392r, 1,944-bp lcp_{VH2} PCR product of *G. polyisoprenivorans* strain VH2
PVH2_2392r	AGGAAGTGCAGTTGCGCGGTC	With PVH2_449f, 1,944-bp lcp_{VH2} PCR product of *G. polyisoprenivorans* strain VH2
PKb1_57fBglII	ATATAGATCTCTGGCGTTGATTGGATTCGGG	With PKb1_1789rBglII, 1,732-bp lcp_{Kb1} PCR product of *G. westfalica* strain Kb1 (BglII restriction sites used for cloning are underlined)
PKb1_1789rBglII	ATATAGATCTGCGTCTCCCGTTCAGCAAATGG	With PKb1_57fBglII, 1,732-bp lcp_{Kb1} PCR product of *G. westfalica* strain Kb1 (BglII restriction sites used for cloning are underlined)
PLcp_NtermNdeI	GGCATATGGACGGTTCAGCAG	With PLcp_CtermBamHI, 1,235-bp lcp_{K30} PCR product of *Streptomyces* sp. strain K30 (NdeI restriction sites used for cloning are underlined)
PLcp_CtermBamHI	AAAGGATCCGGACGGGCGGTTGACGTCCGG	With PLcp_NtermNdeI, 1,235-bp lcp_{K30} PCR product of *Streptomyces* sp. strain K30 (BamHI restriction sites used for cloning are underlined)

pIJSK::lcp_{VH2}::*aph* was subsequently transferred to *S. lividans* strain TK23 by protoplast transformation (25).

Expression of six-His-tagged Lcp_{K30} in *E. coli* strain BL21(DE3), isolation of IBs, and generation of anti-Lcp_{K30} antibodies. *E. coli* strain BL21(DE3) harboring plasmid pET-23a::lcp_{K30}his was cultivated in LB medium at 37°C to an optical density at 600 nm of 0.5, and then expression was induced by addition of IPTG (isopropyl-β-D-thiogalactopyranoside) to a final concentration of 1 mM for 3 h yielding cells with inclusion bodies (IBs). For isolation of IBs, the cells of a 100-ml culture were harvested, resuspended in 4 ml 20 mM Tris-HCl (pH 8.0) buffer, and disrupted by a twofold French press passage at 1,000 MPa. The disrupted cells were centrifuged at 25,000 × *g* for 15 min at 4°C. The obtained pellet was resuspended in 3 ml cold IB wash buffer (2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100, pH 8.0) by sonication (1 min/ml with an amplitude of 40 μm) with a Bandelin Sonopuls GM200 ultrasonic disintegrator. After 15 min of centrifugation at 4°C and 25,000 × *g*, treatment with IB wash buffer, resuspension by sonication, and centrifugation were repeated three times. The purified IBs were dissolved in sodium dodecyl sulfate (SDS) denaturation buffer (27). A sample, consisting of the dissolved IBs containing the extracted Lcp protein, was separated by SDS-polyacrylamide gel electrophoresis and excised from the gel, and its identity was confirmed by matrix-assisted laser desorption ionization–time of flight analysis, before it was used for generation of custom polyclonal antibodies in rabbits by Eurogentec (Seraing, Belgium). Purified polyclonal rabbit anti-Lcp_{K30} immunoglobulin G (IgG) antibodies were obtained from the serum by chromatography on protein A-Sepharose (16).

Preparation of crude cell extracts and extracellular protein fractions. For preparative purposes, cells of *G. polyisoprenivorans* strain VH2, *G. westfalica* strain Kb1, *Streptomyces* sp. strain K30, and recombinant strains of *S. lividans* TK23 were grown in MSM (38) containing the carbon sources and for the periods described in the text. Cells were sedimented from the aqueous phase by 30 min of ultracentrifugation at 100,000 × *g* at 4°C to remove cells and as many particles as possible from the supernatant. Cells were used for preparation of crude cell extracts by a threefold passage through a French pressure cell (1,000 MPa). The clear supernatant was filtrated using a 0.22-μm membrane filter (Roth, Karlsruhe, Germany); the proteins were subsequently concentrated by precipitation with 0.015% (vol/vol) sodium deoxycholate plus 10% (wt/vol) trichloric acid as previously described (31) and then resuspended in an appropriate volume of distilled water and 2% (vol/vol) 1 M Tris-HCl (pH 9.0). Small amounts of extracellular proteins were also concentrated from these supernatants by applying Vivaspin 500 centrifugal filter units with a 10-kDa-cutoff polyethersulfone membrane (Sartorius, Göttingen, Germany).

SDS-polyacrylamide gel electrophoresis, Western blot analysis, and other immunological analyses. Protein contents of crude cell extracts and extracellular protein fractions were estimated by the dye binding principle method using bovine serum albumin as standard (11). Samples of crude cell extracts and

extracellular protein fractions representing a quantity of 50 μg protein were separated in 11.5% (wt/vol) SDS-polyacrylamide gels (27). Proteins were visualized with Coomassie brilliant blue R250 (42). For immunological detection of Lcp, the proteins were transferred from gels onto polyvinylidene difluoride (PVDF) membranes, according to the work of Towbin et al. (40) and the manufacturer (GE Healthcare, Buckinghamshire, United Kingdom). Proteins on the membrane were stained with Ponceau S and analyzed immunologically employing 400 μl of the polyclonal rabbit anti-Lcp_{K30} IgG solution. IgG antibodies were visualized on immunoblots using anti-rabbit IgG-alkaline phosphatase conjugates (Sigma-Aldrich), converting 5-bromo-4-chloro-3-indolyl-phosphate dipotassium/nitrotetrazolium blue chloride (Sigma-Aldrich) into an insoluble dark product. Dot blotting experiments were done as described by the manufacturer of the PVDF membrane (GE Healthcare, Buckinghamshire, United Kingdom).

RT-PCR analysis of total RNA from *G. polyisoprenivorans* strain VH2. DNA-free total RNA of *G. polyisoprenivorans* strain VH2 was prepared by DNase I treatment of an RNA sample kindly provided by Quyen Banh in our laboratory. For identification of lcp_{VH2}-derived mRNA, reverse transcription-PCR (RT-PCR) was applied using oligonucleotides PVH2_360f(RT) and PVH2_911r(RT) (Table 1). RT-PCR was carried out using a commercial kit (One Step RT-PCR kit; Qiagen, Hilden, Germany) according to the manufacturer's protocol and 0.5 ng RNA as template. To exclude any DNA contamination that could serve as template for PCR, template RNA was added in a control experiment, after inactivation of reverse transcriptase for 15 min at 95°C in the presence of *Taq* polymerase: the absence of PCR products indicated that the RT-PCR products were not derived from contaminating DNA.

Nucleotide sequence accession numbers. The nucleotide sequences of lcp_{VH2} and lcp_{Kb1} investigated in this study have been deposited in the GenBank database under accession numbers EU013941 and EU013942, respectively.

RESULTS

Identification of *lcp* homologues in rubber-degrading species of the genus *Gordonia*. The occurrence of *lcp*-homologous genes in the genomes of rubber-utilizing species belonging to the genus *Gordonia* was investigated. Two degenerated PCR primers, P265f and P701r (Table 1), were designed based on conserved sequences within the known *lcp* genes from *Streptomyces* sp. strain K30, *S. coelicolor* strain A3(2), and *N. farcinica* strains IFM10152 and E1. Total DNA of the rubber-utilizing bacteria *G. polyisoprenivorans* strains VH2 and Y2K, *Gordonia alkanivorans* strain 44187, and *G. westfalica* strain Kb1 was isolated and used as template DNA in a gradient PCR (annealing temperature of 49 to 61°C) applying the ImmoMix ready-to-use PCR mixture (BioLine, Randolph, MA) and primers P265f and P701r. Specific PCR products were obtained at an annealing temperature of 61°C from all four templates as well as from *N. farcinica* strain IFM10152 DNA, which was used as a positive control. The PCR products were then cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced. Comparison of the sequences obtained to the NCBI protein database showed significant similarities to the already-known *lcp* genes. The translational products of the 433-bp sequences from *G. polyisoprenivorans* strains VH2 and Y2K exhibited 60% amino acid identity and those of the 425- and 431-bp sequences from *G. alkanivorans* strain 44187 and *G. westfalica* strain Kb1 showed 63 and 65% amino acid identity, respectively, to the corresponding region of Lcp from *Streptomyces* sp. strain K30. This provided for the first time strong evidence that Lcp homologues occur also in rubber-degrading bacteria belonging to the genus *Gordonia* that grow adhesively on the polymer (see Fig. S2 in the supplemental material).

Identification of the complete nucleotide sequences of lcp_{VH2} from *G. polyisoprenivorans* strain VH2 and lcp_{Kb1} from *G. westfalica* strain Kb1. To obtain the complete nucleotide sequences of *lcp* homologues from two *Gordonia* species, which have been studied in most detail in the past (2, 5, 12, 29, 30), cosmid libraries of *G. westfalica* strain Kb1 or *G. polyisoprenivorans* strain VH2 were screened for occurrence of recombinant *E. coli* clones harboring *lcp* homologues by clones harboring a degenerated PCR amplification. Hybrid cosmids containing *lcp*-homologous sequences were then used as template DNA for sequencing reactions by primer walking. Using cosmid pHC79::290, a 2,987-bp sequence was determined containing the complete lcp_{VH2} from *G. polyisoprenivorans* strain VH2. Cosmids pHC79::535, pHC79::549, and pHC79::632, which were all identified in the library of *G. westfalica* strain Kb1, did not yield the complete lcp_{Kb1} sequence due to an internal EcoRI restriction site at position 1130 of lcp_{Kb1}. Therefore, the missing nucleotides encoding the C-terminal region of Lcp_{Kb1} were amplified by nested PCR as described in Materials and Methods (32). The obtained PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced. Two steps of nested PCRs were performed to obtain a 2,505-bp sequence containing the complete lcp_{Kb1}.

Phylogenetic analysis of the amino acid sequence derived from the *lcp* homologues of *G. polyisoprenivorans* strain VH2 (lcp_{VH2}), *G. westfalica* strain Kb1 (lcp_{Kb1}), the *N. farcinica* strains E1 (lcp_{E1}) and IFM10152 ($lcp_{IFM10152}$), *Streptomyces* sp. strain K30 (lcp_{K30}), and *S. coelicolor* strain A3(2) [$lcp_{A3(2)}$] revealed the closest relationship between Lcp_{VH2} and Lcp_{Kb1} (see Fig. S3 in the supplemental material); this is not surprising since their hosts belong to the same genus. Both are also more closely related to the *Nocardia* Lcps than to the Lcp of *Streptomyces*. Lcp_{VH2} and Lcp_{Kb1} exhibited 50 and 52% amino acid identity, respectively, to Lcp_{K30}.

Cloning of lcp_{VH2} and lcp_{Kb1} and complementation experiments in *S. lividans* strain TK23. To test the functionality of the two *Gordonia lcp* genes, fragments containing the 1,635-bp lcp_{VH2} and the 1,732-bp lcp_{Kb1} sequences were amplified by PCR. Both fragments comprised beside the entire *lcp* genes about 400 bp of the region upstream of the respective start codons to include their own promoter regions and about 100 bp of the region downstream of the putative stop codon. Both PCR products were cloned via the pGEM-T Easy vector into the newly constructed *E. coli-Streptomyces* shuttle vector pIJSK, yielding plasmids pIJSK::lcp_{VH2} and pIJSK::lcp_{Kb1}. Plasmid pIJSK::lcp_{K30}, containing the *lcp* gene of *Streptomyces* sp. strain K30 including 300 bp upstream and 100 bp downstream of the start and stop codon, was used as positive control. All plasmids were transferred to *S. lividans* strain TK23, and the resulting recombinant strains were incubated on latex overlay-agar plates containing 1% (wt/vol) glucose for 10 days and stained with Schiff's reagent, to detect aldehydes. Aldehydes occurred around colonies of the positive control (*S. lividans* strain TK23 containing pIJSK::lcp_{K30} [Fig. 1A]), and in addition clearing zones became visible after an incubation of 30 days (Fig. 1E) as observed previously in our laboratory (Henrike Wernsmann, unpublished data), whereas the negative control, *S. lividans* strain TK23 harboring only the vector pIJSK, did not form aldehydes (Fig. 1D) or clearing zones (Fig. 1H) in latex overlay-agar plates. Since the recombinant strains of *S. lividans* strain TK23 harboring pIJSK::lcp_{VH2} (Fig. 1B and 1F) or pIJSK::lcp_{Kb1} (Fig. 1C and 1G) formed aldehydes after 10 days of incubation and clearing zones after 30 days, the homologues

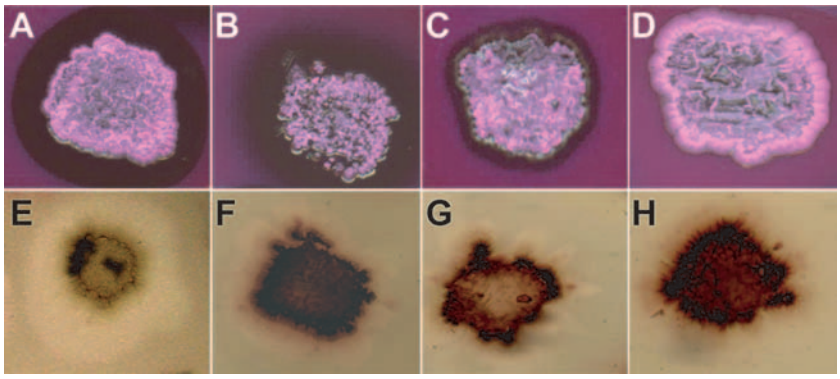

FIG. 1. Formation of aldehydes and clearing zones on latex overlay-agar plates. Recombinant strains of *S. lividans* strain TK23 harboring plasmid pIJSK::lcp_{K30} (A), pIJSK::lcp_{VH2} (B), pIJSK::lcp_{Kb1} (C), or pIJSK (D) were incubated for 10 days at 30°C on latex overlay-agar plates containing 0.5% (wt/vol) glucose and stained with Schiff's reagent to detect aldehydes; results are shown in the upper part of the figure. Clearing-zone formation was documented for the same recombinant strains of *S. lividans* strain TK23 in the corresponding panels at the bottom of the figure after 30 days of incubation at 30°C on latex overlay-agar plates containing 0.5% (wt/vol) glucose. Thiostrepton (25 μg/ml) was added to the medium for plasmid maintenance.

lcp_{VH2} and lcp_{Kb1} from both *Gordonia* species code for active enzymes.

The region with stained aldehydes appeared diffuse without a clearly visible border on latex overlay-agar plates incubated with *S. lividans* strain TK23 harboring pIJSK::lcp_{VH2} (Fig. 1B), as with latex overlay-agar plates incubated with *S. lividans* strain TK23 pIJSK::lcp_{K30} (Fig. 1A). Furthermore, the stained regions were bigger with recombinant strains of *S. lividans* strain TK23 harboring pIJSK::lcp_{K30} or pIJSK::lcp_{VH2} than with *S. lividans* strain TK23 harboring pIJSK::lcp_{Kb1}, where the stained region was much more closely restricted to the cells (Fig. 1C).

It was also tested if the wild-type *G. polyisoprenivorans* strain VH2 and *G. westfalica* strain Kb1 were able to form aldehydes on latex overlay-agar plates. For this, both strains were incubated on latex overlay-agar plates containing 1% (wt/vol) glucose for 10 days and stained with Schiff's reagent, to detect the formation of aldehydes. Little aldehyde formation very close to the cells could be detected on latex overlay-agar plates incubated with *G. polyisoprenivorans* strain VH2. With *G. westfalica* strain Kb1 only very weak aldehyde formation occurred. Both strains did not form clearing zones even after 30 days of incubation (data not shown).

GPC analysis of poly(*cis*-1,4-isoprene) after incubation with recombinant strains of *S. lividans* strain TK23. Cleavage of poly(*cis*-1,4-isoprene) by recombinant strains of *S. lividans* strain TK23 harboring pIJSK::lcp_{VH2} or pIJSK::lcp_{Kb1} was verified by GPC. *S. lividans* strain TK23 containing plasmid pIJSK::lcp_{K30} or pIJSK was used as a positive or negative control, respectively. Cells of these recombinant strains were cultivated in 50 ml MSM in the presence of 0.25% (wt/vol) poly(*cis*-1,4-isoprene) with an average molecular mass of 800 kDa (catalog no. 182141; Sigma-Aldrich, Steinheim, Germany) and 0.5% (wt/vol) glucose. GPC analysis of the residual poly(*cis*-1,4-isoprene) after 8 weeks of incubation was performed as described in Materials and Methods. Poly(*cis*-1,4-isoprene) incubated with *S. lividans* strain TK23 harboring pIJSK, which was used as a negative control, showed no change in peak height and area and thus in molecular mass of the 800-kDa polymer (Fig. 2). In contrast, a decrease of 43.3% in height and 27.1% in the peak area (total weight loss of 33.88 mg) occurred after incubation of poly(*cis*-1,4-isoprene) with strain TK23 harboring pIJSK::lcp_{Kb1}, and a decrease of 43.6% in height and 53.8% in the peak area (total weight loss of 67.25 mg) occurred with strain TK23 containing pIJSK::lcp_{VH2}. With the positive control, i.e., strain TK23 harboring pIJSK::lcp_{K30}, the poly(*cis*-1,4-isoprene) peak showed a decrease in height of 54.6% and in area of 56.7% (total weight loss of 70.88 mg) in comparison to the reference. This clearly indicated that these strains were able to cleave the polyisoprene polymer (Fig. 2). These results agree with observations made for poly(*cis*-1,4-isoprene) incubated with recombinant strains of *S. lividans* TK23 strains harboring lcp_{K30} (33) or lcp_{E1} (20).

Transcription analysis of lcp_{VH2} in *G. polyisoprenivorans* strain VH2. The occurrence of an *lcp*-homologous gene in *G.*

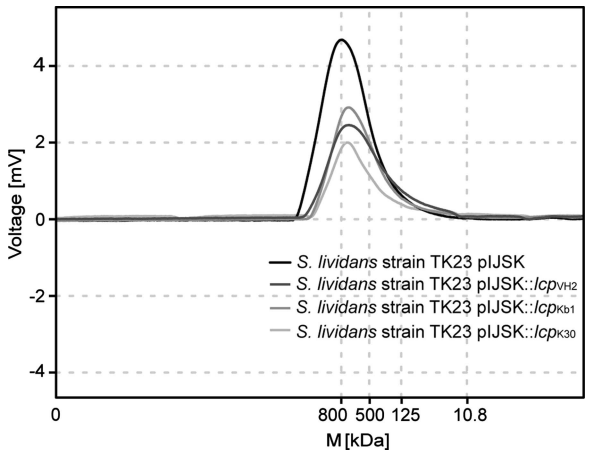

FIG. 2. Changes of molecular masses of poly(*cis*-1,4-isoprene) after incubation with recombinant strains of *S. lividans* strain TK23. The diagram represents GPC elution profiles for residual chloroform-soluble polymers after incubation of synthetic poly(*cis*-1,4-isoprene) with an average molecular mass (M) of 800 kDa (catalog no. 182141; Sigma-Aldrich, Steinheim, Germany) with recombinant strains of *S. lividans* strain TK23 harboring plasmid pIJSK::lcp_{K30}, pIJSK::lcp_{VH2}, pIJSK::lcp_{Kb1}, or pIJSK for 8 weeks.

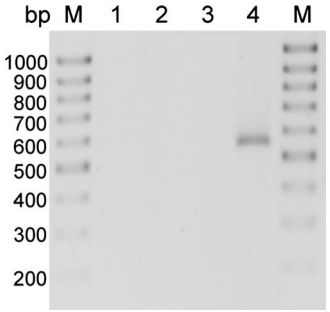

FIG. 3. Transcription analysis of lcp_{VH2} in *G. polyisoprenivorans* strain VH2. Expression of lcp_{VH2} was analyzed by RT-PCR of samples containing total RNA isolated from cells of *G. polyisoprenivorans* strain VH2 in the logarithmic growth phase. The resulting PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide, and a negative image is presented. Cells were grown in MSM with either 0.2% (wt/vol) sodium acetate (lanes 1 and 2) or 0.25% (wt/vol) poly(*cis*-1,4-isoprene) (lanes 3 and 4) as sole carbon source. Lanes 1 and 3 represent the controls to detect DNA contamination, whereas lanes 2 and 4 represent the RT-PCR assay. A 100-bp DNA ladder (lanes M; MBI Fermentas, Germany) was used for size comparison.

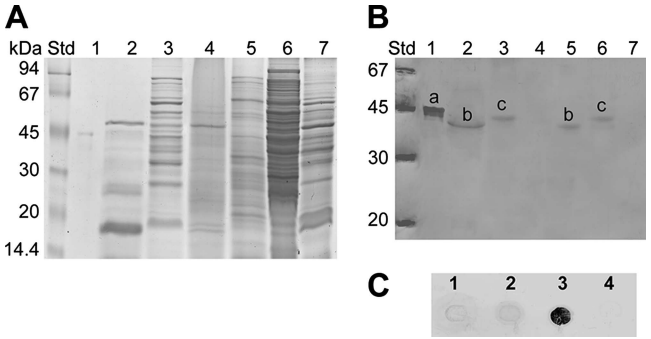

FIG. 4. Immunological detection of Lcp from *Gordonia* species. (A) Electropherogram of an SDS-polyacrylamide gel after separation of proteins from crude cell extracts and extracellular protein fractions. Proteins in the gel were stained with Coomassie brilliant blue R250. (B) Western blot employing anti-Lcp_{K30} IgG antibodies prepared from an SDS-polyacrylamide gel. Std, molecular mass standard; lanes 1, six-His-tagged Lcp_{K30} protein; lanes 2, extracellular protein fraction of *S. lividans* strain TK23 harboring pIJSK::lcp_{K30}; lanes 3, crude cell extract of *S. lividans* strain TK23 harboring pIJSK::lcp_{K30}; lanes 4, crude cell extract of *S. lividans* strain TK23 harboring pIJSK; lanes 5, extracellular protein fraction of *Streptomyces* sp. strain K30 wild type grown on latex; lanes 6, crude cell extract of *Streptomyces* sp. strain K30 wild type grown on latex; lanes 7, crude cell extract of *Streptomyces* sp. strain K30 wild type grown on glucose. *Streptomyces* sp. strain K30 was grown in MSM either with 0.2% (wt/vol) latex or with 0.5% glucose. The recombinant strain of *S. lividans* strain TK23 containing plasmid pIJSK::lcp_{K30} was cultivated in MSM with 0.5% (wt/vol) glucose plus 0.2% (wt/vol) latex as carbon sources. In the Western blot the anti-Lcp_{K30} IgG antibodies recognized the approximately 46-kDa six-His-tagged Lcp_{K30} protein (a), the approximately 45-kDa Lcp_{K30} with signal peptide (b), and the approximately 42-kDa Lcp_{K30} after signal peptide cleavage (c). Furthermore, Lcp present in crude cell extracts was detected in dot blot assays. (C) Crude cell extracts of the recombinant strains of *S. lividans* strain TK23 containing plasmids pIJSK::lcp_{VH2} (lane 1), pIJSK::lcp_{Kb1} (lane 2), pIJSK::lcp_{K30} (lane 3), or pIJSK (lane 4), cultivated for 1 week at 30°C in MSM containing 0.5% (wt/vol) glucose plus 0.2% (wt/vol) latex as carbon sources, were applied to a PVDF membrane, and the immunological analysis was done as described in the manual from the manufacturer (GE Healthcare, Buckinghamshire, United Kingdom).

polyisoprenivorans strain VH2 and other species of *Gordonia* and the finding that this lcp_{VH2} gene was functional in *S. lividans* strain TK23 harboring pIJSK::lcp_{VH2} raised the question whether lcp_{VH2} is also expressed in its natural host. Therefore, RT-PCR was employed to investigate transcription of lcp_{VH2} in *G. polyisoprenivorans* strain VH2 qualitatively. RT-PCR was done with DNA-free total RNA obtained after DNase I treatment and with primers PVH2_360f(RT) and PVH2_911r(RT) (Table 1). This yielded a PCR product of the expected size of 551 bp representing the central region of lcp_{VH2} which was not obtained if RNA isolated from acetate-grown cells of strain VH2 was analyzed. This clearly demonstrated that lcp_{VH2} was transcribed in cells of *G. polyisoprenivorans* strain VH2 cultivated in the presence of poly(*cis*-1,4-isoprene) and not in the presence of acetate. The absence of PCR products in the control indicated that the RT-PCR product was not derived from contaminating DNA (Fig. 3).

Application of antibodies raised against Lcp of *Streptomyces* sp. strain K30 for detection of Lcp proteins. Because the genomes of *G. polyisoprenivorans* strain VH2 and *G. westfalica* strain Kb1 encode definitely a functional *lcp* gene that is transcribed, it should be investigated if detectable Lcp protein occurs in the intra- and extracellular protein fractions of these bacteria. Cells of both strains were grown in MSM, either for 8 weeks in medium containing 0.25% (wt/vol) poly(*cis*-1,4-isoprene) or for 3 days in medium containing 0.2% (wt/vol) sodium acetate as sole carbon source. To test the functionality of the available antibodies raised against the *Streptomyces* sp. strain K30 Lcp_{K30}, cells of this bacterium were grown in MSM, either for 8 weeks in medium containing 0.2% (wt/vol) latex or for 4 days in medium containing 0.5% (wt/vol) glucose as sole carbon source. To determine heterologous expression of *lcp* in the recombinants of *S. lividans* strain TK23 harboring pIJSK::lcp_{VH2}, pIJSK::lcp_{Kb1}, or pIJSK::lcp_{K30}, these strains and the negative control, *S. lividans* strain TK23 harboring pIJSK, were cultivated in MSM with 0.5% (wt/vol) glucose plus 0.2% (wt/vol) latex for 4 days. Crude cell extracts and extracellular protein fractions were prepared and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4A) and Western blotting (Fig. 4B). The Western blot prepared with antibodies raised against Lcp_{K30} gave an intensive immunoreaction signal with a protein exhibiting a size of approximately 46 kDa with the purified six-His-tagged Lcp_{K30} protein. This molecular mass corresponded well to that calculated for the six-His-tagged Lcp_{K30}. Applying anti-Lcp_{K30} IgG antibodies to the extracellular protein fraction of *S. lividans* strain TK23 harboring pIJSK::lcp_{K30}, a signal from an approximately 42-kDa protein occurred as was expected for the extracellular Lcp_{K30}. Considering cleavage of the signal peptide of Lcp_{K30} between amino acid positions 30 and 31, the latter represented the mature protein as calculated with the SignalP program (15). Western blots of the crude cell extract prepared from this strain revealed a signal representing a protein of approximately 45 kDa. The anti-Lcp_{K30} IgG antibodies also recognized the approximately 42- and 45-kDa proteins in Western blots obtained from the extracellular fraction or from the crude cell extracts of the wild-type *Streptomyces* sp. strain K30 grown on latex. Crude cell extracts of the negative controls, i.e., *S. lividans* strain TK23 harboring pIJSK and *Streptomyces* sp. strain K30

grown on 0.5% (wt/vol) glucose, revealed no immunoreaction signal (Fig. 4B), thus demonstrating that the anti-Lcp_{K30} IgG antibodies were useful and specific against Lcp_{K30}.

No immunoreactions occurred with crude cell extracts or extracellular protein fractions of *G. polyisoprenivorans* strain VH2 and *G. westfalica* strain Kb1 or of the recombinant *S. lividans* TK23 strain harboring pIJSK::lcp_{VH2} or pIJSK::lcp_{Kb1} (data not shown). Thus, dot blot experiments should demonstrate if the generated antibodies raised against the *Streptomyces* sp. strain K30 Lcp_{K30} were suitable for detection of the Lcp homologues of *G. polyisoprenivorans* strain VH2 and *G. westfalica* strain Kb1. Due to possible proteolysis in extracellular fractions, only crude cell extracts were analyzed in order to obtain clear results. Crude cell extracts were obtained from the recombinant *S. lividans* TK23 strains harboring pIJSK::lcp_{VH2}, pIJSK::lcp_{Kb1}, pIJSK::lcp_{K30}, or pIJSK after cultivation in MSM with 0.5% (wt/vol) glucose plus 0.2% (wt/vol) latex for 1 week and were analyzed in a dot blot assay. We applied 5 μl of each sample to a PVDF membrane and used anti-Lcp_{K30} IgG antibodies as described in the manual from the manufacturer (GE Healthcare, Buckinghamshire, United Kingdom). No immunoreactions occurred with crude cell extracts obtained from the recombinant *S. lividans* TK23 strains harboring pIJSK::lcp_{VH2}, pIJSK::lcp_{Kb1}, or pIJSK, whereas the crude cell extract of *S. lividans* strain TK23 harboring pIJSK::lcp_{K30} gave an intensive signal (Fig. 4C), thus demonstrating that the anti-Lcp_{K30} IgG antibodies were specific against Lcp_{K30} and did not cross-react with the Lcp homologues.

Construction of a lcp_{VH2} disruption mutant. To investigate the relevance of lcp_{VH2} for degradation of poly(*cis*-1,4-isoprene) by *G. polyisoprenivorans* strain VH2, this gene was disrupted. A 1,635-bp sequence, comprising the complete coding region of lcp_{VH2} (positions 313 to 1590) and adjacent regions, was amplified by PCR using oligonucleotides PVH2_117fBglII and PVH2_1752rBglII (Table 1). As lcp_{VH2} exhibited a unique restriction site for XhoI 719 nucleotides downstream of the putative start codon, whereas the pGEM-T Easy vector (Promega, Madison, WI) did not contain a cleavage site for XhoI, the 1,635-bp lcp_{VH2} PCR fragment was cloned into pGEM-T Easy after addition of 3′-A overhangs (A-tailing). The resulting hybrid plasmid containing lcp_{VH2} was then linearized with XhoI, the 3′-protruding ends were blunted with T4 DNA polymerase, and an approximately 1,000-bp SmaI-SmaI kanamycin resistance cassette (*aph*) was subsequently inserted at position 719 of lcp_{VH2}. Primers PVH2_117fBglII and PVH2_1752rBglII were then used to amplify the resulting 2,635-bp lcp_{VH2}::*aph* DNA fragment by PCR, and the fragment was then transferred to *G. polyisoprenivorans* strain VH2 by electroporation. Recombinant clones with integration of the 2,635-bp lcp_{VH2}::*aph* fragment into the chromosome were selected on St-I medium agar plates containing kanamycin (50 μg/ml). About 200 kanamycin-resistant colonies were obtained and suspended in 50 μl TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0), boiled for 15 min, and then used as template for colony PCR. Use of primers PVH2_449f and PVH2_2392r (Table 1), which bind 180 bp upstream of PVH2_117fBglII and 130 bp downstream of PVH2_1752rBglII, yielded four clones (A12, A17, A29, and A34) possessing the 2,944-bp lcp_{VH2}::*aph* knockout PCR product as expected for the desired lcp_{VH2} disruption mutants. PCRs employing total DNA of the wild-type strain VH2 and of the four disruption mutants as templates confirmed these results (see Fig. S4A in the supplemental material). Total KpnI-digested DNA isolated from the wild type and the four mutants was separated in an agarose gel, stained with ethidium bromide, and transferred to a nylon membrane and then hybridized with digoxigenin-labeled lcp_{VH2}. DNA also confirmed disruption of lcp_{VH2} in the mutants since the hybridizing KpnI fragments of these mutants were approximately 1 kbp larger than that of the wild type due to the inserted kanamycin resistance cassette (see Fig. S4B and C in the supplemental material).

Complementation experiments in *S. lividans* strain TK23 using the 2,635-bp lcp_{VH2}::*aph* knockout DNA fragment. The hybrid plasmid pIJSK::lcp_{VH2}::*aph* was transferred to *S. lividans* strain TK23. The obtained recombinant strains were incubated on latex overlay-agar plates containing 1% (wt/vol) glucose for 10 days and were stained with Schiff's reagent; no aldehyde formation was detected. Also no clearing-zone formation was observed after 30 days of incubation (data not shown). Therefore, the disrupted lcp_{VH2}::*aph* gene could, in contrast to lcp_{VH2}, not confer aldehyde or clearing-zone formation on *S. lividans* strain TK23, thereby clearly demonstrating the inactivation of lcp_{VH2} by the disruption performed with *aph* in mutant A17.

Phenotypic analysis of the mutants. To investigate the effect of *lcp* disruption on the utilization of poly(*cis*-1,4-isoprene), mutants A12, A17, A29, and A34 and the wild-type strain VH2 were cultivated in MSM containing 0.25% (wt/vol) poly(*cis*-1,4-isoprene) as sole carbon and energy source. However, after 30 days of incubation no effect of the inactivation of lcp_{VH2} on growth with poly(*cis*-1,4-isoprene) was observed. Both the mutants and the wild type showed similar adhesive growth on the rubber substrate (data not shown). Mutants A12, A17, A29, and A34 were also able to utilize acetonylacetone and methyl-branched isoprenoid compounds such as geranylacetone, farnesol, and squalene like the wild-type strain VH2. These volatile carbon sources were added to MSM agar plates which were sealed with Parafilm.

DISCUSSION

Recently, the *lcp* gene from the clearing-zone-forming bacterium *Streptomyces* sp. strain K30 was identified, and its involvement in degradation of poly(*cis*-1,4-isoprene) was unequivocally demonstrated (33). Lcp was considered a key protein occurring solely for clearing-zone-forming, rubber-degrading gram-positive bacteria, whereas polyisoprenoid degradation by gram-positive bacteria growing adhesively on rubber was considered to rely on a different type of protein. The present study was carried out to investigate whether Lcp homologues occur also in rubber-degrading bacteria belonging to the genus *Gordonia*, which serve as model organisms to study rubber degradation in adhesively growing bacteria, and whether Lcp is essential for rubber degradation in these bacteria. *G. polyisoprenivorans* strain VH2 is for example a much better rubber degrader, growing about six times faster in MSM containing poly(*cis*-1,4-isoprene) as sole carbon source than the clearing-zone-forming *Streptomyces* sp. strain K30. Recently, the occurrence of *lcp*-homologous genes was also described for thermophilic adhesively growing strains of *N. farcinica* (20). Rubber degradation in both clearing-zone-form-

ing and adhesively growing bacteria is still only slightly understood (34).

As *G. polyisprenivorans* strains VH2 and Y2K, *G. alkanivorans* strain 44187, and *G. westfalica* strain Kb1 contain *lcp*-homologous genes, it must be concluded that Lcp is widespread in gram-positive, rubber-utilizing bacteria exhibiting an adhesive growth on rubber and that Lcp is not restricted to clearing-zone-forming actinobacteria. It is noticeable that so far no rubber-degrading gram-positive bacterium without Lcp has been found (20, 33; this study) and that Lcp comprises obviously a highly conserved type of a novel protein.

The functionality of the *lcp* homologues from *Gordonia* sp. was unequivocally demonstrated by clearing-zone and aldehyde formation. The specific characteristics of aldehyde formation regarding size and shape of the stained aldehyde regions in the recombinant strains of *S. lividans* strain TK23 harboring the *lcp* homologues from *Gordonia* sp. in comparison to strain TK23 containing lcp_{K30} from *Streptomyces* sp. strain K30 indicate a lower rate of protein secretion in the recombinant lcp_{VH2}- and lcp_{Kb1}-containing strains than in the recombinant lcp_{K30}-harboring strain. Due to the present signal sequence there are differences in native protein secretion and especially for secretion of heterologous proteins in *S. lividans*, as previously described (6, 14, 35, 37). These differences in heterologous protein secretion might also explain the different GPC profiles of poly(*cis*-1,4-isoprene) molecules after incubation with the recombinant lcp_{VH2}-, lcp_{Kb1}-, or lcp_{K30}-harboring strains of *S. lividans* TK23 (Fig. 2). However, this might be also due to different enzyme activities of the various Lcp homologues.

Furthermore, induction of lcp_{VH2} transcription in cells of *G. polyisoprenivorans* strain VH2 during growth on poly(*cis*-1,4-isoprene) but not on acetate (Fig. 3) confirmed again that Lcp is related to rubber degradation.

Although the Lcp homologues from *Gordonia* sp. exhibited significant amino acid identity to Lcp_{K30}, anti-Lcp_{K30} IgG antibodies raised against Lcp from *Streptomyces* sp. strain K30 did not cross-react with these Lcp homologues. However, this was the first time that antibodies to an Lcp protein became available, and these antibodies were obviously rather specific for Lcp_{K30} (Fig. 4B).

Disruption of lcp_{VH2} demonstrated that Lcp_{VH2} has no essential role for rubber or isoprenoid degradation in *G. polyisoprenivorans* strain VH2. It cannot be excluded that through gene duplication at least one further *lcp*-homologous gene is present in this bacterium. However, completely sequenced genomes of *N. farcinica* strain IFM10152 (21) and *S. coelicolor* strain A3(2) (7) contain only a single *lcp* gene. Another explanation is the involvement of a different protein type in parallel to Lcp during rubber degradation in *Gordonia*. The latter could upon inactivation of the lcp_{VH2} compensate for its rubber-degrading capacity to allow full growth on natural rubber. This assumption is supported by a previously described transposon mutagenesis of *G. polyisoprenivorans* strain VH2 (5). Interestingly, none of 25,000 characterized mutants were defective in genes whose translational products were homologous to the two known enzymes, Lcp_{K30} and RoxA, catalyzing the primary poly(*cis*-1,4-isoprene)-cleaving reaction identified in *Streptomyces* sp. strain K30 (33) and in *Xanthomonas* sp. strain 35Y (9), respectively. Therefore, it was suggested that the well-characterized rubber-cleaving enzyme RoxA (10) exclusively occurs in gram-negative bacteria (5). Further studies will be necessary to unravel rubber degradation in the gram-positive *Gordonia* sp. strains.

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