Identification and Characterization of Genes from *Streptomyces* sp. Strain K30 Responsible for Clear Zone Formation on Natural Rubber Latex and Poly(*cis*-1,4-isoprene) Rubber Degradation

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Streptomyces sp. strain K30 was isolated from soil next to a city high way in Münster (Germany) according to its ability to degrade natural and synthetic poly(*cis*-1,4-isoprene) rubber and to form clear zones on natural rubber latex agar plates. The clear zone forming phenotype was used to clone the responsible gene by phenotypic complementation of a clear zone negative mutant. An open reading frame (*lcp*) of 1,191 bp was identified, which was preceded by a putative signal sequence and restored the capability to form clear zones on natural rubber latex in the mutant. The putative translation product exhibited strong homologies (50% aa identity) to a putative secreted protein from *Streptomyces coelicolor* strain A3(2), another clear zone forming strain. Heterologous expression of *lcp* of *Streptomyces* sp. strain K30 in *Streptomyces lividans* strain TK23 enabled the latter to form clear zones on latex-overlay agar plates and to accumulate a degradation product of about 12 kDa containing aldehyde groups. Two ORFs putatively encoding a heterodimeric molybdenum hydroxylase (*oxiAB*) were identified downstream of *lcp* in *Streptomyces* sp. strain K30 strain which exerted a positive effect on clear zone formation and enabled the strain to oxidize the resulting aldehydes. Heterologous expression of a fragment harboring *lcp* plus *oxiAB* in *S. lividans* TK23 resulted in accumulation of aldehydes only in the presence of 10 mM tungstate. Determination of protein content during cultivation on poly(*cis*-1,4-isoprene) revealed an increase of the cellular protein, and gel permeation chromatography analysis indicated a shift of the molecular weight distribution of the rubber to lower values in the transgenic *S. lividans* strains and in the wild type, thus confirming utilization and degradation of rubber. Therefore, for the first time, genes responsible for clear zone formation on natural rubber latex and synthetic *cis*-1,4-polyisoprene degradation in Gram-positive bacteria were identified and characterized.

Introduction

Poly(*cis*-1,4-isoprene) is the main constituent (>90% of dry weight) of natural rubber (NR), a biopolymer synthesized by many plant species and some fungi in varying degrees of quality and quantity.[1] For technical applications NR is obtained from the rubber tree *Hevea brasiliensis*.[2] Alternatively, poly(*cis*-1,4-isoprene) (IR) is synthesized chemically from isoprene.[3]

Rubber degrading microorganisms are known and studied for a long time, and two different groups can be distinguished according to their decomposition strategies.[4] Bacteria of the first group belong to the CMN group (*Corynebacterium*, *Mycobacterium*, *Nocardia*) and need direct contact to the rubber material showing adhesive growth. Rubber degrading bacteria from the genus *Gordonia*, for example, belong to this group and are currently intensively studied in our laboratory.[5−8] These strains show good rubber degradation activities in submers culture but do not grow and do not form clear zones on latex overlay agar plates containing rubber as the sole carbon source. Members of the second group show weaker growth in submers culture but grow and form clear zones on NR latex-overlay agar plates. These bacteria belong to streptomycetes and related genera.[4,9,10]

In contrast to many reports about identification and classification of new isolated rubber degrading bacteria, only little is known about the biochemical mechanism of rubber disintegration, and up to now, only one gene product of *Xanthomonas* sp. secreted during growth on NR was purified and the structural gene was cloned.[11] An oxidative cleavage of the double bond in the polymer backbone for initiation of rubber degradation has been suggested by Tsuchii and Takeda,[12] and diketone derivatives of oligo(*cis*-1,4-isoprene) were identified as metabolites of rubber degradation.[13,14] Recently, enzyme-mediator systems for in vitro oxidative degradation of polyisoprene were described based on free radical chain reactions of lipids using different known enzymes such as manganese peroxidase, laccase, horseradish peroxidase, and lipoxigenase/linoleic acid.[15,16] Gel permeation chromatography (GPC) analysis of the treated rubber showed degradation of IR after 48 h, and scanning electron micrography of latex gloves showed formation of holes at the glove

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surface after 2−7 days of treatment with these enzymes.[15,16] However, these studies did not prove the biological relevance of these enzyme mechanisms in rubber degrading microorganisms. In this report, we identified for the first time a gene which is responsible for clear zone formation on latex and which is essential for the process of rubber degradation by clear zone forming actinomycetes.

Materials and Methods

1. Strains and Plasmids. *Streptomyces* sp. strain K30 was isolated, and mutant strain 6/1 was derived from this wild type as described in this study. *S. lividans* strain TK23 was used for heterologous production of the clear zone forming enzyme. *Actinoplanes missouriensis* (DSM 43046), *Streptomyces griseus* (DSM 4069) and *Streptomyces antibioticus* (DSM 40725) were used for growth experiments and transformations, respectively. Plasmid pIJ702 was used for construction of gene libraries and for heterologous expression;[17] pBluescript SK$^-$ (Stratagene, Heidelberg, Germany) was used for cloning.

2. Media and Culture Conditions. Strains of *Streptomyces* were grown in tryptic soy broth (TSB) medium [Merck, Darmstadt, Germany]. For plates, media were solidified with 2% (w/v) agar. For preparation of protoplasts, *Streptomyces* strains were grown in modified YEME (3%, w/v, yeast extract, 5%, w/v, Bacto Peptone, 3%, w/v, malt extract, 34%, w/v, sucrose) medium.[17] For protoplast regeneration, R5 agar plates were used.[17] Thiostrepton was added to solid and liquid media at a concentration of 25 mg/L during growth of recombinant strains. *Streptomyces* strains were cultivated at 30 °C. For growth experiments with natural and synthetic polyisoprene, cells were cultivated in mineral salts medium (MSM).[18]

3. Chemicals and Polymers. Restriction enzymes and T4 DNA ligase were obtained from MBI FERMENTAS (St. Leon-Rot, Germany). Purified natural rubber latex from *H. brasiliensis* was a gift from Weber & Schaer (Hamburg, Germany). Synthetic poly(*cis*-1,4-isoprene) (IR) was obtained from Aldrich (Milwaukee, USA). Powder-free latex gloves were obtained from Kimberly-Clark (Zaventem, Belgium). Latex was used for overlay plates as described previously.[9] These plates were used to screen for strains with an altered clear zone forming phenotype as indicated by formation or size of translucent halos around the colony. IR was cut into pieces or was dissolved in $CHCl_3$, spread on the bottom of Erlenmeyer flasks, evaporated, and autoclaved together with the MSM (solution cast films). The latex gloves were cut into pieces and were treated as described previously.[19]

4. Strain Identification and Isolation of Mutants with Defects in Clear Zone Formation on NR Latex. The purified 16S rDNA was partially sequenced by the dideoxy chain-termination method using a LICOR model 4000 automatic sequencer (MWG-Biotech, Ebersberg, Germany). The sequence was compared with available 16 S rRNA gene sequences.

A suspension of *Streptomyces* spores (10^9−10^{10} spores per mL in 15%, w/v, glycerol) was irradiated with UV light in a glass Petri dish to give a survival rate of 1%. The mutagenized spores were directly plated on MSM with latex-overlay agar plates without applying any enrichment technique, and the phenotypes were analyzed.

5. General DNA Techniques. Recombinant DNA techniques in *Streptomyces* were performed as described by Kieser et al.[17] Total DNA from *Streptomyces* was isolated by the versatile quick-prep method for Gram-positive bacteria according to Pospiech and Neumann.[20]

To clone the gene(s) responsible for clear zone formation from *Streptomyces* sp. strain K30, total DNA was partially digested with *Sau*3A, and 3- to 10-kbp fragments were ligated to the multicopy plasmid pIJ702, which was cleaved at the unique *Bgl*II site within the *melC1* gene. The ligation mixture was used to transform *Streptomyces* sp. strain K30 6/1, and regenerated transformants were screened for clear zones occurring around colonies on NR latex agar plates.

6. Protein Determination. Protein contents of lyophilized samples of cells grown on different rubber material were determined according to the method of Bradford as described previously.[13]

7. Scanning Electron Microscopy. For scanning electron microscopy (SEM), the samples were directly gold sputtered with the Emitech vacuum sputter device K550x, without any preceding fixation or drying procedures. The samples were examined with a Hitachi S-3000N scanning electron microscope at 15 kV and high vacuum conditions. Micrographs were directly electronically recorded.

8. Molecular Weight Measurement. The polymers were dissolved in $CHCl_3$ and passed through a 0.45 μm pore size filter (Sartorius, Göttingen, Germany). 100 μL of the solution was separated in a row of four styragel-colums (HR3, HR4, HR5, and HR6 with 10^3−10^6 Å; (Waters, Milford, USA)) accommodated in a Waters (Milford, USA) gel permeation chromatography (GPC) system consisting of a model 515 HPLC pumps, a model 410 differential refractometer and a model 717plus autosampler.

9. Staining with Schiff's Reagent. Staining with Schiff's reagent was performed to detect aldehyde groups in polyisoprene degradation products or on latex-overlay agar plates. 30 μL of a solution containing polymers and degradation products was dissolved in $CHCl_3$ and spread on a glass slide, and the $CHCl_3$ was evaporated at room temperature. The staining was performed as described previously.[4]

10. Oligonucleotides. PSPNter (5′-ccgagatctcggcaggacgaactcccg-3′) and PSPCter (5′-ccgagatctggtgcgtcgagg-3′) were used to amplify *lcp*.

11. Data Deposition. The nucleotide sequences of *lcp*, *oxiB*, and *oxiA* investigated in this study have been deposited in the GenBank database under the GenBank accession numbers AY387589, AY388475, and AY388476. The partial 16S ribosomal RNA gene sequence has been deposited in the GenBank database under accession number AY587549.

Results

Isolation of the Rubber Degrading Strain *Streptomyces* sp. strain K30. Strain K30 was isolated from soil next to a city highway in Münster (Germany) as a rubber degrading and clear zone forming bacterium. The clear zone formation

Table 1. Determination of Protein Contents after 12 Weeks of Incubation of Cells on IR in MSM[a]

strain	protein conc. [μg/mL] after inoculation	protein conc. [μg/mL] after 12 weeks of cultivation
A. missouriensis	10	320
Streptomyces sp. K30	4	180
Streptomyces sp. K30 6/1	8	27
S. lividans TK23	11	15

[a] A 100 mL culture was harvested and lyophilized. Subsequently, 5 mL water and 2 mL of 1 M NaOH were added to the samples, and they were boiled in a water bath for 30 min. Protein concentration was measured photometrically.

on NR latex agar plates occurred after 3−5 days incubation. Cells of strain K30 stained Gram positive. The color of the spore mass is yellow-grey, and the configuration of the spore chains is rectiflexibilis. The strain grew well at 4, 17, 30 and 37 °C, but showed no growth at 45 °C. Partial 16S rRNA gene sequence analysis revealed significant homologies to *Streptomyces setonii* (99.2%), *S. griseus* (99.1%), and *S. flavogriseus* (99.0%). Isolate K30 was therefore referred to as *Streptomyces* sp. strain K30.

Isolation of Mutants Defective in Rubber Degradation. After UV mutagenesis of spores of *Streptomyces* sp. strain K30, over 7000 clones were screened for a latex negative phenotype and 69 mutants with defects in clear zone formation were identified. Interestingly, 66 of these mutants were pleiotropic and exhibited a defect in clear zone formation on latex as well as on xylan (65 mutants) and Tween 40 (one mutant). This indicated defects in protein secretion in these mutants. Only three of these mutants (1/1, 4/1, and 6/1) exhibited no defect in clear zone formation on other biopolymers like xylan, starch, casein, and Tween 40 (lipase activity) in addition to that on latex. Only after prolonged incubation for 3−4 weeks on NR latex did these mutants form small clear zones. Of these three mutants with an identical phenotype strain, 6/1 was used for further investigations and complementation studies.

To compare growth of *Streptomyces* sp. strain K30 and of the mutant strain 6/1 on IR with growth of the rubber degrading clear zone forming *A. missouriensis*[9] and the non clear zone forming *S. lividans* TK23, the total protein contents of cultures after 12 weeks of incubation were determined (Table 1). *A. missouriensis* showed strong growth on IR, whereas *S. lividans* TK23 did not grow on IR and did also not form clear zones on NR latex. A culture of the wild type of *Streptomyces* sp. K30 showed a significant increase in protein content on IR, and a weight loss of latex gloves of 13.4% (w/w) was recorded in a different experiment. In contrast, a much smaller increase of protein content and a weight loss of latex gloves of only 1.3% (w/w) was observed with cultures of mutant 6/1 derived from strain K30 under identical conditions. These results paralleled those obtained in rubber degradation studies recently performed with latex negative mutants of *S. griseus* 1D and *S. coelicolor* 1A.[13] Altogether, these data confirmed the involvement of the clear zone forming enzyme in rubber degradation.

Cloning and Characterization of Genes Responsible for Clear Zone Formation on NR Latex. DNA fragments (3−10 kbp) from *Streptomyces* sp. strain K30 were inserted into pIJ702 and transformed into mutant 6/1 of *Streptomyces* sp. K30. Transformants were selected on thiostrepton-containing latex overlay agar plates. Of about 6000 clones tested, one showed clear zone formation on NR latex. A plasmid referred to as pR was isolated from the recombinant mutant and characterized.

Plasmid pR contained an 8-kbp *Sau*3A insert of *Streptomyces* sp. K30 genomic DNA. A 4.8-kbp subfragment obtained by restriction of pR with *Bam*HI was ligated to *Bgl*II linearized DNA of pIJ702 resulting in plasmid pSubR which was transformed into mutant 6/1 of *Streptomyces* sp. K30 and conferred also the capability to form clear zones on NR latex. Subsequently, this 4.8-kbp *Bam*HI restriction fragment was ligated to pBluescript SK$^-$ DNA and transformed into *E. coli*. This 4.8-kbp fragment and the adjacent regions on the 8-kbp fragment were completely sequenced, and three reading frames, which are related to rubber degradation, were identified (Figure 1).

ORF1 comprised 1191 bp and encodes a putative protein of 397 amino acids with a theoretical mass of 42 755 Da and a pI of 6.26. The SignalP V2.0 analysis program identified a putative signal sequence of 32 amino acids and a characteristic signal peptidase processing site (Ala−X−X−Ala). A putative ribosome binding site was detected 9 bp upstream from the putative start codon ATG. A comparison of the translated ORF1 sequence with known sequences in databases (Blast program) revealed a high similarity (50% identical aa) to a putative secreted protein from *S. coelicolor* A3(2), and it was referred to as *lcp* (latex clearing protein). Interestingly, this streptomycete like strain K30 was able to form clear zones on NR latex agar plates. Only minor homologies were found to other hypothetical proteins with unknown function from *S. coelicolor* A3(2), *Cytophaga hutchinsonii* and *Leptospira interrogans* which all comprised about 400 amino acids. A sequence alignment of these five protein sequences revealed several conserved motifs (Figure 2), but no functional domains could be identified.

ORF2 comprised 2322 bp and encodes a putative protein of 774 amino acids with a theoretical mass of 81 987 Da and a pI of 7.67. A putative ribosome binding site was detected 12 bp upstream from the putative start codon ATG. A signal sequence of 35 amino acids could be identified. The translated sequence revealed high homology (40% identical aa) to a putative oxidoreductase beta subunit from *S. avermitilis*, whereas only minor homologies were found to oxidoreductases from *S. coelicolor* A3(2). It was therefore referred to as *oxiB*. Homologies were also found to corresponding subunits or domains of isoquinoline-1-oxidoreductase and aldehyde dehydrogenases from different species. A third ORF and putative gene (*oxiA*) for the Fe−S-binding alpha subunit of the enzyme was identified downstream of ORF2 on the 8-kbp *Sau*3A fragment. *OxiA* comprised 474 bp and encodes a putative protein of 158 amino acids with a theoretical mass of 17 039 Da and a pI of 4.68. A putative ribosome binding site was detected 10 bp upstream from the putative start codon GTG. Homologies to putative alpha subunits of molybdenum hydroxylases from different species

Figure 1. Molecular organization of the 8-kbp *Sau*3A fragment from plasmid pR harboring *lcp* and adjacent genes. *nagk*, *N*-acetyl glutamate kinase; *lcp* (ORF1), (latex clearing protein; putative secreted protein); *oxiB* (ORF2), oxidoreductase β-subunit; *oxiA* (ORF3), oxidoreductase α-subunit; *eno*, enolase (2-phosphoglycerate dehydratase**);** *mem*, putative membrane transporter.

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S.sp.30          1   ----MTGGALGAVGALGAATPALARPLWTWSPSASVAGTGVGVDPEYVWDEEADPVLAAV
S.coelicolor1    1   MENLSRRGVLSLGVALGLVGVADGAKAWAWPSADSVAGAGTGADPEYVWDSATDPLMAAL
S.coelicolor2    1   ---------------------------MPHTEKSMDALRRSG---------DELADAVVATL
L.interrogans    1   ----------------------------MQNFIKYRSIT---------DVYADQVIQDY
C.hutchinsonii   1   MMSLTRIQYPSPGFSQHWKEPAGKNMLKKISVLPTAADITNFNTLYMSCDTQADQVVVDL

S.sp.30         57   IDRGEVPAVN--ALLKQWTRNDQALPGGLPGDLREFMEHARRMPSWADKAALDRGAQFSK
S.coelicolor1   61   LENGSVPSIN--TAMAGWVNNADPLPGGFPAELTAHLRQVNRLPSWADRTKLTRAADFNR
S.coelicolor2   27   FERGEVGTFN--ALMRYVSTTGQDLPDGLPGVAREYLRVTGTPPDWVDWAEMERARLFFI
L.interrogans   23   FQSDKKDKLRSLALFNILTQNFGPIPTELPSYFKEYFEKTSILPEWADLKKIQIAERVFA
C.hutchinsonii  61   FEG--LGHKKGHAILHALLEHGPDAVPEMPDSLRTLYTGSLNVPAWVDEKLLQAGAACCQ

S.sp.30        115   TKGIYVGALYGLGSGLMSTAIPRESRAVYYSKGGADMK-------DRIAKTARLGYDIGD
S.coelicolor1  119   RRDTYLFMLYGLGSGIMSTVIPREARSVYWSAGGADMQ-------DRAAKTFTFGYDLSQ
S.coelicolor2   85   DNNVHISTALSFASMPACYLVPHVARLLSATHGLNYPS-------KRMAETGQFTVYLMR
L.interrogans   83   TYGPQILMILCCKSLPMAYTCGNGAEVLVYTGRLVEENGSTQKVFRRLMETTQFVVSVLK
C.hutchinsonii 119   RAGLFSLVTLRNYSLMGGYESAAINKPLIFTGALKAGP------AKRLSDTLAFWVDVTG

S.sp.30        168   LDAYLPHGSMIVTAVKTRMVHAAVRHLLPQSPAWSQTSGGQKIPISQADIMVTWHSLATF
S.coelicolor1  172   LKAFEPTGQFVVTANKTRLVHAAVRHLLPRSPHWTSG-ADQDIPISAADILVTFHSLGTY
S.coelicolor2  138   PDAFESGGRFVPAAQKVRLLHAAIRHHLKREDRWDTD--TLGVPICQEDMIGGQMFFSLL
L.interrogans  143   EGGLSQGGEGIRAAQKVRLMHASIRHFIFESNQWKEE---WGKPINQQDMAGTLQSFSSL
C.hutchinsonii 173   TDAMKPAAVGFNAAIRVRIIHAMARHYTRKSPGWSDE--QWGIPVNHGDMVATYLGFSLV

S.sp.30        228   VMRKMKQWGVRVNTADAEAYLHVWQVSAHMLGVSDEYIPATWDAANAQSKQVLDPILAHT
S.coelicolor1  231   VRSKLIEWKVPFPAADQEAFLHSWQVALHLLGVPDEYIPKTWAAAEAQSAQVLTPILAPT
S.coelicolor2  196   VLDSLHRLGIHMSAEGADAYYYAWRVVGAMLGVDQAAVPATLDEARRFLDLYMLRHMGPS
L.interrogans  200   ILEGLAFSGITLDEEEKDSYIHLWKVTGHILGVLPELSPDTYEDAYALGLSIFQDQRRKS
C.hutchinsonii 231   FLEGLYMQGFRPSSQEVSGIFHLWKYIGYLLGIPHQLLPDTEEQAIQTLYKWTMTQPPAD

S.sp.30        288   PEGEALTEVLLG------IVAELDAG-LTRPLIGAFSRYTLGGEVGDMIGLAKQPVLERL
S.coelicolor1  291   TEGIKLAEELLG------LTAQIDLG-VTRGFLNEFVRYVLSDEVGDWLGLRRDPVAAAL
S.coelicolor2  256   EEGAHLTRQLID------LYEEVVPGTLFDPVVSALIRYLVGDTCADWLDVPR-TTWDTL
L.interrogans  260   EAGKILAKSLLD------FMEYMVPGNLLDGVPLYFLQKYLGKENCEILELPW-EEKEIL
C.hutchinsonii 291   ADTIALAHALMHAPLTATFPEKNWQKKLLVRIYLGFNYYYLGRHACRRMALPS-------

S.sp.30        341   IATAWPLLVAFREGLIPLPAVPAVLWTLEEALRKFVLLFLSEGRRIAIDIPDVQPPVLNR
S.coelicolor1  344   VRTAWPAYIKFREGLS--PIMPGTFYLVDQFVRALAMLFLNKGSSGATTP--ITIPTGNR
S.coelicolor2  309   VKAAPHLLGVLETIEDRSPLGAWALDRLGHLTTALELSSLTRGRVMHYAIPEQLRKEYGV
L.interrogans  313   GEILEKVITGFDETLSENDHFQKLAAHFSSKLILGMDNFYYKGKKAKFEIPPSLKGNWGV
C.hutchinsonii 343   --------TCFKFLPYLSACINRMNEYRIGSSEKRRERAIKKGRKAQVEVTDAFLKSTGF

S.sp.30        400   ------------------
S.coelicolor1  400   PG----------------
S.coelicolor2  369   SGAAARTRRWTPPPPTVS
L.interrogans  372   ------------------
C.hutchinsonii 396   GKSVQK------------
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Figure 2. Multiple sequence alignment of the *lcp* encoded protein with related proteins exhibiting highest homology. The ClustalX program was used for generation of the alignment. Residues similar in at least four sequences are shaded in gray; residues identical in at least four sequences are shaded in black. *S.* sp. 30, *Streptomyces* sp. K30 *lcp*; S. coelicolor1, *Streptomyces coelicolor* A3(2) putative secreted protein (accession number NP_630852), hyp. S. coelicolor2, *Streptomyces coelicolor* A3(2) hypothetical protein (accession number NP_624877); L. interrogans or C. hutchinsonii, *Leptospira interrogans* or *Cytophaga hutchinsonii* hypothetical proteins, respectively (accession numbers NP_711343 and ZP_00119984).

could be found (Figure 3). All of these enzymes are members of the xanthine oxidase family using a molybdopterin cofactor and possessing two [2Fe-2S] clusters

The construction of a *knock-out* mutant of *lcp* in *Streptomyces* sp. K30 was not successful hitherto; unfortunately only very low transformation and conjugation frequencies were achieved with this newly isolated strain although intensive efforts were made to increase transfer rates of foreign DNA.

Heterologous Expression of Lcp in *S. lividans* TK23. Gene *lcp* was amplified with oligonucleotides PSPNter and PSPCter and cloned into the *Bgl*II site of pIJ702, resulting in pIJ702::*lcp*. Amplification included 300 bp upstream and 100 bp downstream of the start and stop codon of *lcp*, respectively. *S. lividans* TK23 was able to form clear zones on NR latex agar plates not only with plasmids pR (Figure 4) or pSubR but also with pIJ702::*lcp* (data not shown). In addition, mutants 1/1, 4/1, and 6/1 of *Streptomyces* sp. strain

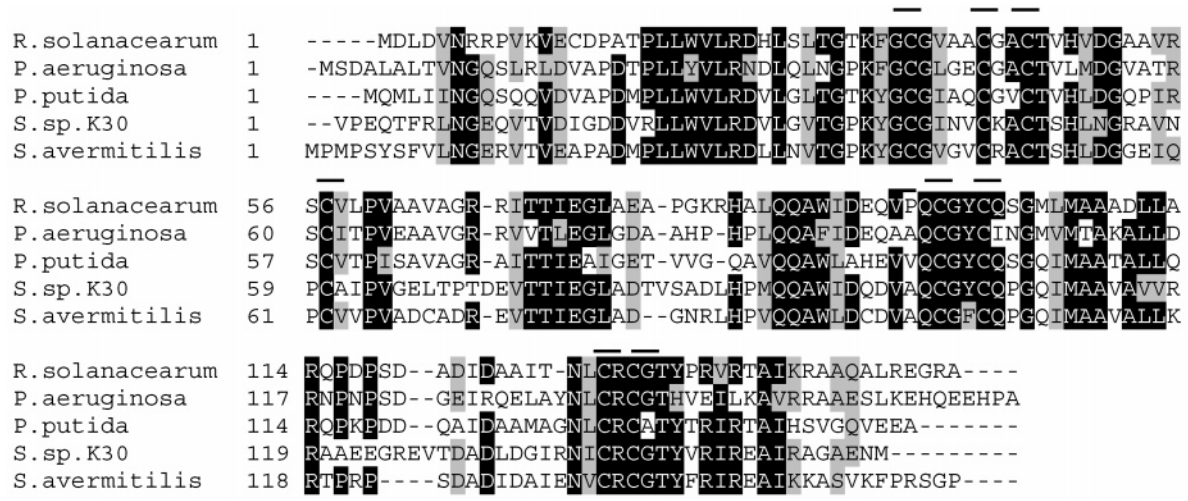

Figure 3. Multiple sequence alignment of the *oxiA* translational product with related proteins exhibiting hi504ghest homology. The program CLUSTALX was used for generation of the alignment. Residues similar in at least four sequences are shaded in gray; residues identical in at least four sequences are shaded in black. Eight conserved cysteine residues with iron−sulfur cluster binding function are highlighted by bars. R. solanacearum, *Ralstonia solanacearum* putative isoquinoline-1-oxidoreductase (accession number NP_520010); P. aeruginosa, *Pseudomonas aeruginosa* putative aldehyde dehydrogenase (accession number NP_251068); P. putida, *Pseudomonas putida* putative isoquinoline-1-oxidoreductase (accession number NP_744626); S. sp. K30, *Streptomyces* sp. K30 putative oxidoreductase; S. avermitilis, *Streptomyces avermitilis* putative oxidoreductase (accession number NP_821691).

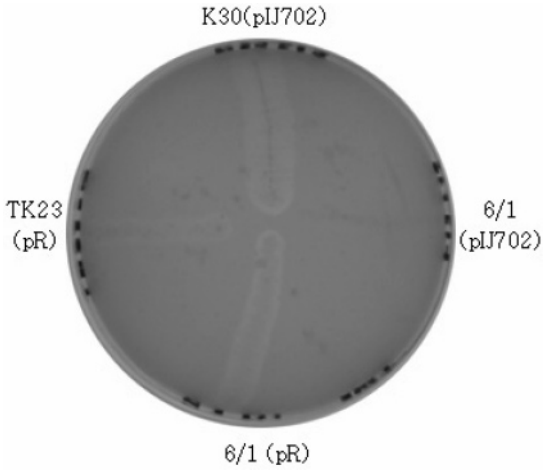

Figure 4. Growth of different *Streptomyces* strains on latex overlay-agar plates and clear zone formation. Latex overlay-agar plates were prepared as described in materials and methods and incubated for 5 days at 30 °C. Thiostreptone (25 μg/mL) was used for plasmid maintenance. Top, *Streptomyces* sp. K30 (pIJ702); right, *Streptomyces* sp. K30 6/1 (pIJ702); bottom, *Streptomyces* sp. K30 6/1 (pR); left, *Streptomyces lividan*s TK23 (pR).

K30 could be complemented not only with pR but also with pIJ702::*lcp*. The results of these two sets of experiments strongly suggest that *lcp* is coding for the enzyme responsible for clear zone formation or that it is at least an essential constituent of a protein complex forming clear zones by cleavage of poly(*cis*-1,4-isoprene). Clear zone formation was also observed with transformants of *Streptomyces antibioticus* harboring pR. No clear zone formation was observed with transformants of *S. griseus* harboring pR or pSubR (pIJ702::*lcp* was not investigated). Clear zone formation of *S. lividans* TK23 (pR) was slightly stronger than of *S. lividans* TK23 (pIJ702::*lcp*) indicating a positive effect of *oxiAB* on clear zone formation.

Growth on Latex Gloves and IR. Bacterial strains, mutants, and transformants with altered clear zone formation on NR latex agar plates were also grown on solution cast films of IR and on latex gloves (Tables 1 and 2). The protein contents of the control strain *S. lividans* TK23 and of the latex negative mutant 6/1 of *Streptomyces sp*. K30 increased only marginally during incubation on the rubber substrates indicating the inability of these strains to grow on IR. In addition, no modifications of the glove material could be observed (Figure 5B). In contrast, cultures of the clear zone forming bacteria *A. missouriensis* and *Streptomyces* sp. K30 showed a significant increase in protein content. Furthermore, the glove material was deteriorated, and holes could be found in the material incubated for 12 weeks with these two strains. Cultures of strain TK23 harboring plasmid pR or pIJ702::*lcp* also showed a significant increase in protein content after incubation on IR or gloves. However, the glove material was less deteriorated in comparison with the material recovered from cultures of strain K30, and after 12 weeks of incubation, hole formation was in a very initial state if rubber was the only carbon source (data not shown). In contrast, if 0.5% (w/v) glucose was added as cosubstrate, cultures of strain TK23 harboring plasmid pR or pIJ702::*lcp* deteriorated the glove material severely, and hole formation could be

Table 2. Determination of Protein Contents after 12 Weeks of Incubation on IR and Latex Gloves in MSM[a]

strain (plasmid)	rubber material	protein conc. [μg/mL] after inoculation	protein conc. [μg/mL] after 12 weeks of cultivation
TK23	IR	4	9
TK23 (pR)	IR	6	54
TK23	gloves	6	10
TK23 (pR)	gloves	7	80
TK23 (pIJ::*lcp*)	gloves	4	51
S. sp. K30 6/1 (pIJ)	IR	7	21
S. sp. K30 6/1 (pR)	IR	6	23

[a] A 100 mL culture was harvested and lyophilized. Subsequently, 5 mL water and 2 mL of 1 M NaOH were added to the samples, and they were boiled in a water bath for 30 min. Protein concentration was measured photometrically.

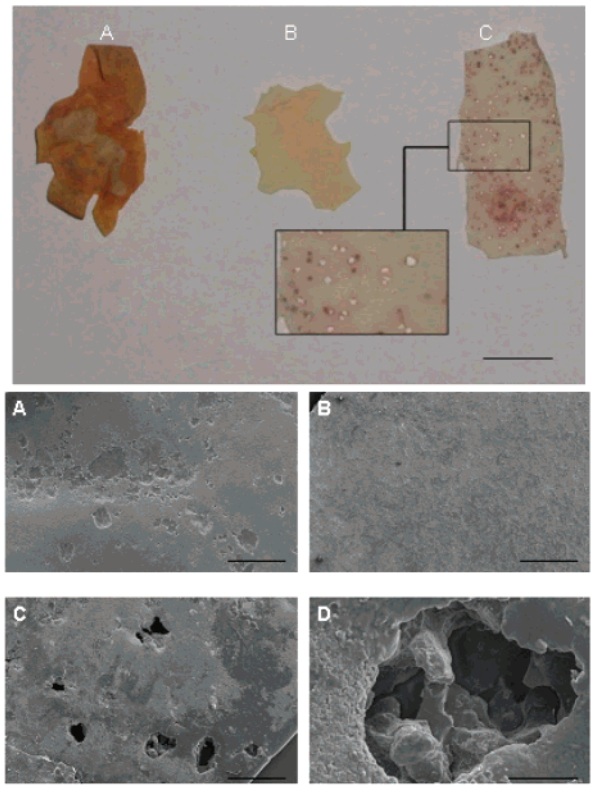

Figure 5. Deterioration of latex gloves after incubation with some *Streptomyces* strains. Pieces of latex glove were incubated in MSM medium with various strains of *Streptomyces* for 12 weeks. Photograph (top) and scanning electron micrographs (A−D) were taken. A, *Streptomyces* sp. K30; B, *Streptomyces lividans* TK23 (pIJ702); C and D, *Streptomyces lividans* TK23 (pIJ702::*lcp*). For the two *Streptomyces lividans* strains, 0.5% glucose and thiostreptone (25 μg/mL) were added to the medium. Bars, 1 cm (photograph), 1 mm (A−C), and 200 μm (D).

observed (Figure 5C,D). With cells of the latter, the holes in the glove material appeared larger but not so numerous than the pits occurring after inoculation with the wild-type strain K30 (Figure 5A). A weight loss of the latex glove material of 5.1% (w/w) was recorded after incubation with *S. lividans* TK23 (pIJ702::*lcp*), whereas gloves cultivated with *S. lividans* TK23 barely showed weight reduction (0.9%, w/w).

GPC Analysis of the Degradation Products. GPC analysis of the residual polyisoprene after 12 weeks of incubation was performed. The polymer of the non inoculated control and of *Streptomyces* sp. K30 6/1 and *S. lividans* TK23 harboring pIJ702 (the latter strain was cultivated in the presence of 0.5% glucose in addition to the polyisoprene) showed no change in molecular weight of the 800 kDa polyisoprene, which was used as starting material (Figure 6A). In contrast, the height and the peak area of the 800 kDa polyisoprene peak decreased after incubation with *Streptomyces* sp. strain K30 and a tailing could be observed (Figure 6B). Interestingly, the residual polyisoprene from cultures of *S. lividans* TK23 harboring pIJ702::*lcp* showed an additional peak of about 12 kDa (Figure 6C).

The purple color produced after staining the degradation product of TK23 pIJ::*lcp* with Schiff's reagent provided evidence that oligomeric isoprene degradation products containing aldehyde groups were produced and accumulated

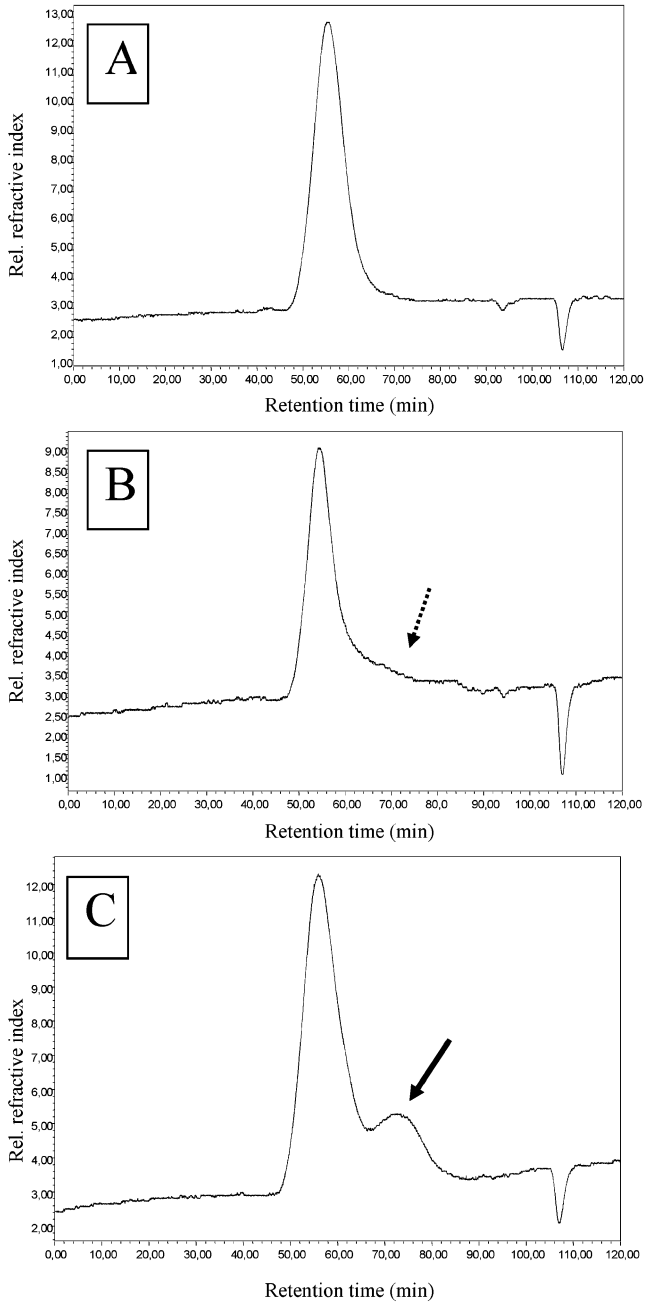

Figure 6. Changes of molecular weight of poly(*cis*-1,4-isoprene) after incubation with strains of *Streptomyces*. GPC elution profiles for the residual polymers after incubation of synthetic polyisoprene with *Streptomyces lividans* TK23 (pIJ702) (A), *Streptomyces* sp. K30 (B), and *S. lividans* TK23 (pIJ702::*lcp*) (C) for 12 weeks. The dotted arrow points to the tailing of the 800 kDa peak; the black arrow points to the 12-kDa degradation product.

(12 kDa peak). No aldehyde groups could be detected with IR incubated with *Streptomyces* sp. K30 or *Streptomyces lividans* TK23 (pIJ702) (Figure 7).

Inhibitory Effect of Tungsten on Clear Zone Formation on NR Latex and Accumulation of Aldehydes. Experiments with enzyme mediator systems have demonstrated that radicals could disintegrate polyisoprene rubber material;[14,15] therefore, radical scavenging substances were analyzed for effects on clear zone formation. *S. lividans* TK23 harboring plasmid pR was grown on latex overlay agar plates containing in addition 0.5% (w/v) glucose and inhibitors at concentrations as indicated below. A complete inhibition of

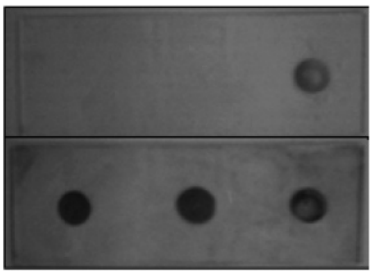

Figure 7. Occurrence of aldehyde groups in partially degraded polyisoprene. The residual polymers were stained with Schiff's reagent (upper row) after incubation of synthetic polyisoprene with *S. lividans* TK23 (pIJ702) (left), *Streptomyces* sp. K30 (center), and *S. lividans* TK23 (pIJ702::*lcp*) (right). For this, 30 μL of polymer dissolved in $CHCl_3$ was spread on a glass slide and stained with Schiff's reagent (top row). The lower row represents the spots after additional staining with Sudan black (0.05%, w/v, in 96% EtOH) to visualize hydrophobic substances.

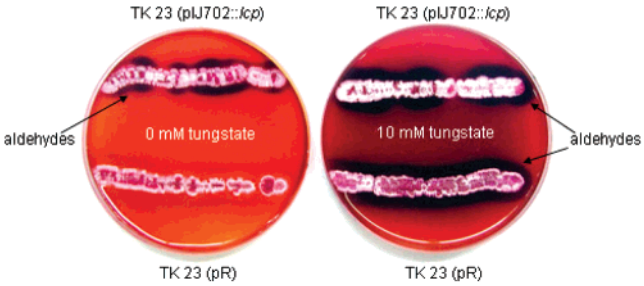

Figure 8. Inhibition of aldehyde catabolism by tungsten. Different *Streptomyces* strains were incubated on latex overlay-agar plates containing 0.5% glucose for 10 days and stained for aldehydes with Schiff's reagent. *S. lividans* TK23 (pIJ702::*lcp*), upper row; *S. lividans* TK23 (pR), lower row. The right agar-plate contained 10 mM tungstate.

clear zone formation on NR latex occurred with 1 μM tungstate, 0.3 mM butylated hydroxytoluene (BHT), 0.2 mM α-tocopherol, or 0.4 mM allopurinol. Surprisingly, no inhibitory effect could be observed with *Streptomyces* sp. K30, even if a 25-fold higher concentration of the inhibitors was used. The strong inhibitory effect of tungstate on clear zone formation is astonishing; other heavy metal ions such as cobalt did not show such effects even if they were used at 100-fold higher concentrations.

Aldehydes could be detected only around colonies of the recombinant strain TK 23 harboring pIJ702::*lcp* growing on latex overlay agar plates and not around colonies of the recombinant strain TK23 harboring pR. The recombinant strain TK 23 (pSubR), harboring *lcp* and *oxiB* only, also accumulated aldehydes, indicating, that *oxiA* is necessary for a functional active molybdenum hydroxylase. However, strain TK 23 harboring pR also accumulated aldehydes on latex overlay plates containing 10 mM tungstate (Figure 8). No aldehydes could be detected on overlay plates incubated with *S. lividans* TK23 harboring pIJ702 (data not shown).

Discussion

To understand the microbial rubber degradation at the molecular level, it is necessary to identify and characterize the genes and proteins involved in this process. All streptomycetes and related actinomycetes, which were able to deteriorate rubber material, show the phenotype of clear zone formation on NR latex plates.[9] Investigations of growth on rubber material with mutants lacking this ability, showed the involvement of the latex clearing protein Lcp in degradation of natural and synthetic rubber. Clear zone formation of *S. lividans* TK23 harboring pIJ702::*lcp* on NR latex overlay plates demonstrated that Lcp is responsible for the clear zone formation, whereas OxiA and OxiB are not essential for polyisoprene cleavage in this strain. However, the latter proteins are probably involved in further catabolism of compounds released from Lcp.

Sequence identities of Lcp from *Streptomyces* sp. strain K30 of about 50% to a putative secreted protein (SCO6780) of *S. coelicolor* A3(2) indicate a similar function of these proteins in both organism. *S. coelicolor* A3(2) is able to form clear zones on latex overlay plates and to deteriorate rubber material (data not shown). Interestingly, downstream of SCO6780 in *S. coelicolor* A3(2) many genes involved in fatty acid metabolism are located (for example fatty acid CoA racemase, acyl CoA dehydrogenase, and a fatty oxidation protein) with unclear function in rubber degradation. Sequence alignment of the translational product of *lcp* with hypothetical proteins from *S. coelicolor*, *L. interrogans*, and *C. hutchinsonii* (Figure 2) revealed some conserved motifs, but signal sequence prediction showed no characteristic signal sequences; therefore, it is unlikely that these enzymes are secreted in the other organisms. Interestingly, a prenyl synthetase gene is located downstream of the hypothetical protein (SCF73.11c) homologous to *lcp* in *S. coelicolor* A3(2). Because of the fact that genes responsible for polymer synthesis and genes responsible for polymer degradation are often located in clusters, it may be speculated, that this protein is involved in the intracellular degradation of ologoisoprenoids or polyprenyls such as ubiquinones, menaquinones, or carotenoids. Conserved histidine residues H188 and H193 might be involved in iron binding, but the enzymatic function of the gene product remained still unclear. The putative oxidoreductase, encoded by *oxiAB* located downstream of *lcp*, possibly possesses the function of an aldehyde dehydrogenase (inferred from alignment data). This is interesting, because an oxidative cleavage of the carbon double bond of the polyisoprene necessarily leads to aldehyde groups, which have to be further oxidized. The oxidoreductase complex (OxiAB) consists of a large subunit (OxiB) with the possible function of binding the molybdopterin cytosine dinucleotide cofactor and of a small subunit (OxiA), responsible for the iron−sulfur cluster center. A third subunit or domain, harboring FAD, is absent, similar to few molybdenum hydroxylases such as the aldehyde oxidoreductases from *Desulfovibrio gigas* (MOP)[21] or *D. desulfuricans* (MOD),[22] or the isoquinoline 1-oxidoreductase (IorAB) from *Brevundimonas diminuta* 7.[23,24] Two conserved motifs (Moco III and Moco I; Figure 9), presumed to be involved in binding the molybdopterin cytosine dinucleotide-cofactor, are present in the sequence. Remarkably, OxiB shows the same sequential order of motifs (Moco III is located upstream of Moco I) like in IorB and Adh from *Acetobacter polyoxogenes*[25,26] and not the opposite arrangement as in most other molybdenum hydroxylases. The alignment of OxiA revealed eight conserved cysteine residues (Figure 3). Therefore, OxiA

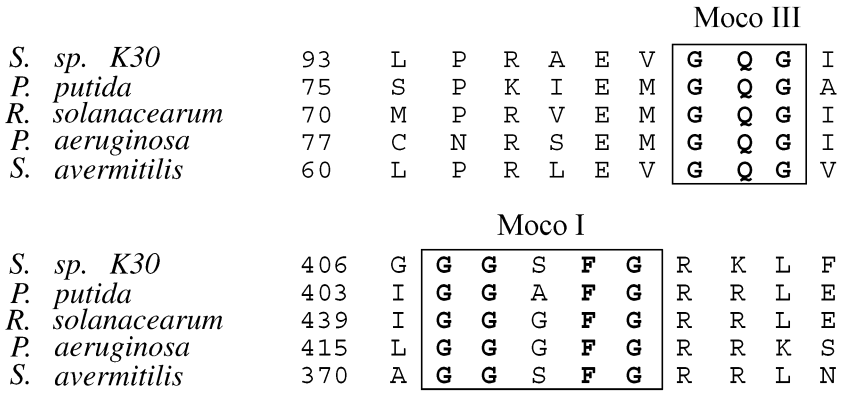

Figure 9. Multiple sequence stretch alignment of the *oxiB* translational product with homologous proteins. The program ClustalX was used for generation of the alignment. Residues, which are completely conserved, are shown in bold letters. P. putida, *Pseudomonas putida* putative isoquinoline-1-oxidoreductase (accession number NP_744626); R. solanacearum, *Ralstonia solanacearum* putative isoquinoline-1-oxidoreductase (accession number NP_520010); P. aeruginosa, *Pseudomonas aeruginosa* putative aldehyde dehydrogenase (accessin number NP_251068); S. sp. K30, *Streptomyces* sp. K30 putative oxidoreductase; S. avermitilis, *Streptomyces. avermitilis* putative oxidoreductase (accession number NP_821691). Conserved residues with putative molybdopterin cofactor contacting function are pointed out by Moco III and Moco I.

probably contains two [2Fe-2S] centers as cofactors, which is a typical feature of iron−sulfur cluster binding consensus sequence of bacterial ferredoxins and which is similar to all eukaryotic xanthine dehydrogenases and the prokaryotic molybdenum hydroxylases MOP, MOD, and IorA.[27] Molybdenum-containing hydroxylases are known for their ability to oxidize various aliphatic or aromatic aldehydes. Remarkably, the retinal oxidase from rabbit belongs to this group of enzymes,[28] indicating that aldehydes with an isoprenoid structure are used as substrates by this kind of enzymes.

GPC analysis shows the accumulation of an oligomeric degradation product with an average molecular mass of 12 kDa after cultivation of *S. lividans* TK23 (pIJ702::*lcp*) with synthetic polyisoprene (Figure 6). Positive staining with Schiff's reagent and molecular mass indicate an oligoisoprenoide of about 180 isoprene units containing aldehyde groups (Figure 8). Occurrence of intermediates comprising aldehyde groups was previously shown during degradation of rubber by the clear zone forming bacteria *Micromonospora aurantiaca* W2b and by *Gordonia polyisoprenivorans* strain VH2, *Gordonia westfalica* and *Mycobacterium fortuitum* strain NF4.[4] Cultivation of *Streptomyces* sp. K30 with polyisoprene shows a tailing of the 800 kDa polyisoprene to lower values but no accumulation of distinct degradation products. This indicates a rapid further consumption and degradation of these substances by the strain, and products containing aldehyde groups were therefore barely accumulated. They were only accumulated if the subsequent enzyme in the degradation pathway (aldehyde dehydrogenase, e.g., OxiAB) was absent in the recombinant strain or inhibited by tungstate (see below).

Because the retention time of the original peak resulting from the employed polymeric material did not change during degradation, endocleavage must occur as the initial step of rubber degradation by S. *lividans* TK23 (pIJ702::*lcp*). However, random endocleavage did obviously also not occur, otherwise the size of the cleavage products would be less defined. In the case of exocleavage, the retention time of the partially degraded polymer would continuously increase, and intermediates with approximately 12 kDa with a low range of dispersity should not be generated. The occurrence of the about 12 kDa intermediates may be due to the molecular confirmation of the poly(*cis*-1,4-isoprene) molecules in the solid material. Maybe, the 12 kDa degradation product is an intermediate of random endocleavage of the polymer (Pers. communication: Frank Reineke). This has to be considered, if only a small amount of the rubber material is accessible for enzymatic attack.

Due to missing homologies of Lcp in databases it is difficult to identify an enzymatic function/mechanism of the protein. It could be speculated that the protein possesses a binding site to polyisoprene. The hydrophobicity plot of Lcp revealed a hydrophobic region (amino acid positions: 300−400) in the C-terminal region of the protein, which might be responsible for binding the rubber material (data not shown).

The strong effect of tungstate on clear zone formation suggested the involvement of a molybdenum containing enzyme in rubber degradation. The detection of aldehydes on latex overlay plates indicated that rubber cleavage is initiated by the gene product of *lcp* resulting in the formation of aldehydes. The recombinant *S. lividans* TK23 (pIJ702::*lcp*) is not able to further utilize the accumulated aldehydes. The next degradation step may be the oxidation of the aldehydes by OxiAB. The genes of this enzyme are located downstream of *lcp* on plasmid pR. If tungstate containing overlay plates inhibited molybdenum dependent oxidoreductases, as indicated by occurrence of aldehydes on tungsten containing overlay agar plates, strain *S. lividans* TK23 (pR) is accumulating aldehydes (Figure 8).

A consensus motif (RRRFL[29]) in the signal peptide of OxiB indicated a twin arginine translocator secretion pathway for this enzyme. This Tat pathway is known for at least partially folding of the proteins prior to export, which might be essential for a functional active OxiAB. The absence of transmembrane structures in the N terminal region of the protein indicated an extracellular location of the enzyme. To our knowledge, this would be the first molybdenum hydroxylase with an extracellular location.

For the first time, genes involved in clear zone formation on natural rubber latex were identified, and the putative rubber degradation pathway, which is up to now only based

on data obtained from the analysis of degradation products,[12−14] was confirmed by cloning of genes, analysis of sequence data of the involved proteins, and heterologous expression studies. In the future, the latex clearing protein Lcp must be purified and characterized to reveal the reaction mechanism of this enzyme acting on polyisoprene and to employ this protein for biotechnological applications, for example for conversion of rubber waste material.

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