Importance of the latex-clearing protein (Lcp) for poly(cis-1,4-isoprene) rubber cleavage in Streptomyces sp. K30

Meral Yikmis¹ & Alexander Steinbüchel¹,²

¹Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster D-48149, Münster Germany
²King Abdulaziz University, Jeddah, Saudi Arabia

Keywords
Biopolymer, knock out lcp mutant, lcp (latex-clearing protein), natural rubber latex, poly(cis-1,4-isoprene) rubber degradation, secretion, Streptomyces.

Correspondence
Alexander Steinbüchel, Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Correnstrasse 3, D-48149 Münster, Germany.
Tel: +49-251-8339821; Fax: +49 251 8338388; E-mail: steinbu@uni-muenster.de

Funded by the Deutsche Forschungsgemeinschaft (STE-386/10-1).

Received: 2 November 2011; Revised: 3 November 2011; Accepted: 10 November 2011

MicrobiologyOpen 2012; 1(2): 13–24
doi: 10.1002/mbo3.3

Abstract
Streptomyces sp. strain K30 induces the formation of an extracellular Lcp (latex-clearing protein) during poly(cis-1,4-isoprene) degradation. To investigate the function of this enzyme in Streptomyces sp. strain K30, the lcp gene was disrupted. This was the first time that the screening for a knock out lcp mutant of Streptomyces sp. strain K30 was successful. The resulting mutant Streptomyces sp. K30lcp2Km exhibited reduced growth in liquid mineral salts media containing poly(cis-1,4-isoprene) as the sole carbon and energy source. Additionally, there was no detectable Lcp activity on latex overlay agar plates. When Lcp from Streptomyces sp. strain K30 was heterologously expressed in strains TK23 and TK24 of Streptomyces lividans and a strain of S. erythraea with plasmid pH6021::lcp, the recombinant strains acquired the ability to cleave synthetic poly(cis-1,4-isoprene), confirming the involvement of Lcp in initial polymer cleavage. Specific anti-LcpK30 IgGs were employed in Western blot analysis to detect the secretion of Lcp in the supernatant. We have conducted an important experiment to demonstrate Lcp activity using the supernatant of these Lcp-expressing strains in vitro. All three strains obviously secreted a functional Lcp, as indicated by the formation of halo. Functional testing of Lcp with different plasmids in Escherichia coli strains and Pseudomonas strains was, however, not successful.

Introduction
Actinomycetes play a major role in the degradation of natural rubber (NR), while some other bacteria and fungi are also known to attack rubber (Kumar et al. 1983). Microorganisms capable of degrading NR cannot degrade synthetic rubbers other than synthetic isoprene rubber (Linos and Steinbüchel 1998). The latex-clearing protein (Lcp) from the rubber-degrading bacterium Streptomyces sp. strain K30 is involved in the initial cleavage of poly(cis-1,4-isoprene), yielding isoprenoid aldehydes and ketones (Rose et al. 2005). Lcp homologues have so far been detected in all investigated clear zone forming rubber-degrading bacteria.

The microbial degradation of natural and synthetic poly(cis-1,4-isoprene) rubber is currently being intensively investigated (Rose et al. 2005; Rose and Steinbüchel 2005), and two different strategies for the degradation of isoprene rubber have been unraveled thereby distinguishing two different groups of rubber-degrading bacteria (Peczynska-Czoch and Mordarski 1988).

Members of the first group form translucent halos when cultivated on solid media containing dispersed latex particles, indicating the excretion of rubber-cleaving enzymes. Mycelium-forming actinomycetes such as Actinoplanes, Micromonospora, and Streptomyces species belong to this group. The second group comprises mycolic acid containing Actinobacteria belonging to the genera Gordonia, Mycobacterium, and Nocardia. These bacteria do not form translucent halos, but they grow adhesively on the surface of rubber particles in liquid culture, and they represent the most potent rubber-degrading bacterial strains (Arenskötter et al. 2004). Xanthomonas sp. strain 35Y is the only known rubber-degrading bacterium that does not belong to the actinomycetes but is a Gram-negative bacterium (Jendrossek and Reinhardt 2003).

A rubber oxygenase RoxA, which is synthesized during growth on NR latex by Xanthomonas sp. 35Y, was
Importance of the Lcp

M. Yikmis & A. Steinbüchel

Figure 1. Hypothetical pathway of poly(cis-1,4-isoprene) degradation by Streptomyces sp. strain K30.

Rose et al. (2005) identified the lcp gene encoding a latex clearing protein from Streptomyces sp. strain K30. The clear zone forming phenotype was used to identify clones harboring the lcp gene from Streptomyces sp. strain K30 by phenotypic complementation of a clear zone negative mutant. The 1191-bp structural gene was preceded by a putative signal sequence and restored the capability of forming clear zones on NR latex agar plates in the mutant. Like RoxA, also Lcp is secreted into the extracellular medium leading to the formation of translucent halos on NR latex. However, both proteins share no sequence homologies. The putative translation product of lcp exhibited strong homologies (50% aa identity) to a putative secreted protein from S. coelicolor strain A3 (Bagdasarian and Timmis 1982), which is another clear zone forming strain (Rose et al. 2005). Sequence analysis of Lcp and characterization of mutants of Streptomyces sp. strain K30 showed secretion of Lcp via the twin-arginine translocation (Tat) pathway (Yikmis et al. 2008; Thomas et al. 2001).

Because expression of functional Lcp in recombinant Escherichia coli strains or in recombinant γ-Proteobacteria such as Pseudomonas putida was not successful, expression of
recombinant Lcp in other bacteria belonging to the genus *Streptomyces* sp., was performed. In this study, we show a system optimized for the expression of recombinant Lcp and the microbial degradation of rubber by these strains. Three actinomycetes strains, *S. lividans* TK23, TK24, and *Saccharopolyspora erythraea*, were able to produce clear zones on rubber overlay agar plates upon transfer of the wild-type lcp gene to these strains. Furthermore, we have conducted an important experiment to demonstrate Lcp activity using the supernatant of these Lcp-expressing strains in vitro. All three strains obviously secreted a functional Lcp, as indicated by isolating and investigating the knock out lcp mutant, we have now confirmed evidence that Lcp is responsible for the initial rubber degradation.

**Materials and Methods**

**Bacterial strains and culture conditions**

Bacteria and plasmids used in this study are listed in Table 1. If not otherwise mentioned, cells of *Streptomyces* sp. were grown in tryptic soy broth (TSB) medium at 30°C (Merck, Darmstadt, Germany), whereas cells of *E. coli* were cultivated at 37°C in Luria Bertani broth (LB) (Sambrook et al. 1989), mineral salts medium (MSM) (Schlegel et al. 1961), or in standard I (St-I) medium (Merck). Antibiotics were applied according to Sambrook et al. (1989) and as indicated in the text. For growth experiments with natural and synthetic polyisoprene, cells were cultivated in MSM (Schlegel et al. 1961). The following carbon sources were added to liquid MSM: 0.5% (v/v) natural latex concentrate (Neotex Latz; Weber & Schaer, Hamburg, Germany) or 0.3% (w/v) synthetic poly(cis-1,4-isoprene) with an average molecular mass of 800 kDa. Liquid cultures were grown in Erlenmeyer flasks, which were incubated on a horizontal rotary shaker. Solid media were prepared by addition of agar–agar (18 g/L). Purified NR latex from *Hevea brasiliensis* was a gift from We- ter, Germany), whereas cells of *E. coli* were cultivated at 37°C in Luria Bertani broth (LB) (Sambrook et al. 1989), mineral salts medium (MSM) (Schlegel et al. 1961), or in standard I (St-I) medium (Merck). Antibiotics were applied according to Sambrook et al. (1989) and as indicated in the text. For growth experiments with natural and synthetic polyisoprene, cells were cultivated in MSM (Schlegel et al. 1961). The following carbon sources were added to liquid MSM: 0.5% (v/v) natural latex concentrate (Neotex Latz; Weber & Schaer, Hamburg, Germany) or 0.3% (w/v) synthetic poly(cis-1,4-isoprene) with an average molecular mass of 800 kDa. Liquid cultures were grown in Erlenmeyer flasks, which were incubated on a horizontal rotary shaker. Solid media were prepared by addition of agar–agar (18 g/L). Purified NR latex from *Hevea brasiliensis* was a gift from We-ber & Schaer and was used for the preparation of overlay plates as described previously (Jendrossek et al. 1997). Latex overlay agar plates were used for growth of clear zone forming strains. For this, MSM agar plates were covered with an overlay of MSM agar containing 0.2% (v/v) disperged latex concentrate.

**Protoplast formation and regeneration**

Protoplasts of *Streptomyces* sp. were prepared from cells grown in modified YEME (3%, w/v, yeast extract; 5%, w/v, Bacto peptone; 3%, w/v, malt extract; 34%, w/v, sucrose) medium (Kieser et al. 2000). R5 agar plates were used for protoplast regeneration (Kieser et al. 2000).

**Isolation, analysis, and manipulation of DNA**

Plasmid DNA was prepared from crude cell lysates by the alkaline extraction method (Kieser et al. 2000). Cells of *Streptomyces* were incubated at 37°C for lysis in presence of lysozyme (2 mg/mL) for at least 2 h. Recombinant DNA techniques in *Streptomyces* were performed as described by Kieser et al. (2000). Total DNA from *Streptomyces* was isolated by the versatile quick-prep method for Gram-positive bacteria according to Pospiech and Neumann (1995). DNA was restricted with endonucleases (Gibco/BRL, Gaithersburg, MD) as mentioned in the text under the conditions recommended by the manufacturer. All other genetic procedures and manipulations were conducted as described by Sambrook et al. (1989).

**Aldehyde staining of poly(cis-1,4-isoprene) and degradation products**

Aldehyde groups resulting from poly(cis-1,4-isoprene) cleavage during clear zone formation on NR latex overlay agar plates were stained for 20 min with Schiff’s reagent. Afterwards, the staining reagent was removed, and the slides were washed with sulfite solution. The composition of the staining solution was as follows: 2 g of fuchsin dissolved in 50 mL of glacial acetic acid, 10 g Na₂S₂O₅, 100 mL of 0.1 N HCl, and 50 mL H₂O. The composition of the sulfite solution was 5 g of Na₂S₂O₅ plus 5 mL of concentrated HCl (37–38%, v/v) in a 100-mL aqueous solution.

**DNA sequencing and sequence analysis**

DNA sequencing was carried out at the Institut für Klinische Chemie und Laboratoriumsmedizin (Münster, Germany). Obtained sequences were analyzed using Genamics Expression software (version 1.100 [http://genamics.com/expression/index.htm]). Sequence comparisons and alignments were performed using the BLAST online service available on NCBI (National Center for Biotechnology Information [http://blast.ncbi.nlm.nih.gov/Blast.cgi]), BioEdit (Hall 1998), and ClustalW (Towbin et al. 1979). Postgenome analyses were made using the KEGG (Kyoto encyclopedia of genes and genomes) database at GenomeNet (Kanehisa 1997; Kanehisa and Goto 2000; Kanehisa 2002; Kieser et al. 2000, [http://www.genome.jp]).

**Cloning and expression of Lcp**

The coding region of lcp from *Streptomyces* sp. K30 was amplified by PCR by applying primers Lcp-EcoRI_6021 and Lcp-NdeI_6021. The amplified PCR product was then cloned into the pGEM-T Easy vector, excised by restriction with EcoRI and NdeI, and ligated to EcoRI-NdeI-linearized plasmid pTJ6021 DNA. For expression analyses, the resulting plasmid, pTJ6021::lcp, was transferred to *Streptomyces* strains via protoplast transformation (Hidalgo et al. 2004). These strains

© 2012 The Authors. MicrobiologyOpen published by Blackwell Publishing Ltd.
Table 1. Bacterial strains, plasmids, and oligonucleotides used in this study.

| Strains and plasmids | Relevant characteristics | Reference |
|----------------------|-------------------------|-----------|
| **Strains**          |                         |           |
| Streptomyces sp. K30 | Wild type producing clear zones on NR latex overlay agar plates | Rose et al. 2005 |
| Streptomyces sp. K30, kp ÆKm | kp knock out mutant, clear zone negative | This study |
| Streptomyces lividans TK23 | Clear zone negative; host strain for heterologous expression | Hopwood 1983 |
| Streptomyces lividans TK23 plU6021::kp | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type kp from Streptomyces sp. strain K30 | This study |
| Streptomyces lividans TK24 | Clear zone negative; host strain for heterologous expression | Hopwood 1986 |
| Streptomyces lividans TK24 plU6021::kp | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type kp from Streptomyces sp. strain K30 | This study |
| Saccharopolyspora erythraea | Wild type, clear zone negative; host strain for heterologous expression | DSMZ 40517 |
| Saccharopolyspora erythraea plU6021::lcp | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type kp from Streptomyces sp. strain K30 | This study |
| Pseudomonas putida | Wild type, clear zone negative; host strain for heterologous expression | DSMZ 291 |
| Pseudomonas putida KT2440 | Clear zone negative; host strain for heterologous expression; pWW0-, r-, m+; spontaneous mutant from P. putida mt-2 | Bagdasarian et al. 1982 |
| Pseudomonas putida KT2440 pBBR1MCS2::LcpHis6 | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type His- tagged kp protein from Streptomyces sp. strain K30 | This study |
| Pseudomonas putida KT2440 pBBR1MCS2::LcpHis6 | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type His- tagged kp protein from Streptomyces sp. strain K30 | Bagdasarian et al. 1981 |
| Pseudomonas putida KT2440 pBBR1MCS2::LcpHis6 | Clear zone negative; host strain for heterologous expression; pWW0-, r-, m+; spontaneous streptomycin resistant mutant from P. putida mt-2 | This study |
| Pseudomonas putida KT2440 pBBR1MCS2::LcpHis6 | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type His- tagged kp protein from Streptomyces sp. strain K30 | This study |
| Pseudomonas putida KT2440 pBBR1MCS2::LcpHis6 | Producing clear zones on natural latex overlay agar plates; host strain for heterologous expression harboring wild-type His- tagged kp protein from Streptomyces sp. strain K30 | This study |
| Escherichia coli Top10 | Donor strain | Stratagene |
| Escherichia coli ET112567 | Nonmethylating plasmid donor strain | Flett and MacNeil 1992 |
### Table 1. Continued

| Strains and plasmids | Relevant characteristics | Reference |
|----------------------|--------------------------|-----------|
| **Plasmids**         |                          |           |
| pET23a::lcp          | pET23a harboring the wild-type *kp* from *Streptomyces* sp. strain K30 | This study |
| pBBR1MCS2            | Broad host-range promoter-probe vector, pBBR1MCS2 | Kovach et al. 1995 |
| pBBR1MCS2::LcpHis6   | Shuttle vector harboring the wild-type His-tagged *kp* protein from *Streptomyces* sp. strain K30 | This study |
| pJ653                | Broad host-range promoter-probe vector, pJ653 | Blatny et al. 1997 |
| pJ653::lcpHis6       | Shuttle vector harboring the wild-type His-tagged *kp* protein from *Streptomyces* sp. strain K30 | This study |
| pGEM-T Easy          | E. coli TA cloning vector; *Ap* | Promega |
| pJ6021               | High-copy-number plasmid expression vector; contains a thiostrepton-inducible promoter, *p*~*tipA*~ from *Streptomyces* lividans 66 | Takano et al. 1995 |
| pJ6021::kp           | pJ6021 harboring wild-type *kp* from *Streptomyces* sp. strain K30 | This study |
| pJ702                | Plasmid contains the tyrosinase gene and thiostrepton resistance (*tsr*) gene | Rose et al. 2005 |
| pJ702::lcp           | pJ702 harboring wild-type gene and the native promoter region of *kp* isolated from *Streptomyces* sp. strain K30 | This study |
| pJ702::kp            | pJ702 harboring the wild-type *kp* from *Streptomyces* sp. strain K30 | Rose et al. 2005 |
| **Oligonucleotides** |                          |           |
| PSPNter              | CCAGATCTCGCGAGGACGATCCCGCG | Rose et al. 2005 |
| PSPCter              | CCAGATCTGTCGTCGAGG       | Rose et al. 2005 |
| Hyala_FW_Ncol        | AATCTAGAAAAAATTTTTGTTAACTTTAGAGGATATATACATATGAAACAAGAAACCCCTTTATCAGGCC | This study |
| Hyala_RW_Ncol        | AACCATGGGCGCCACCGCAATTTTCCGC | This study |
| pqpQ88-for,Sal       | ATATGTCGACCTAAAAATGGAGTCACTAAGACAAACGAGAAACCTTTATCAGGCCATG | This study |
| pqpQ88-rev,Sbf       | ATATGAGTCCACCCACCATACACCATGCTCGCGAGGGTTCACTCCGGAAATATCAATCG | This study |
| pqpQ88-rev,NcoI      | ATATCCTCGAAGTAGATCATGAAACAACGAGAAACCTTTATCAGGCCATG | This study |
| pqpQ88-rev,Sac       | TATAGAGTCCACCCACCATACACCATGTCGCGAGGGTTCACTCCGGAAATATCAATCG | This study |
| Lcp_EcoRI_6021       | AAGAGATTCAGACCGGGGCCTTACGGGCGGATG | This study |
| Lcp_NdeI_6021        | AAAAAAACATATGCGATAAGCGCGCCCTTCCGCCCGCGCGCCCGCC | This study |
| N_Lcp                | GGATCCTTACGTACGTAGGGGAGTTCACAGGCGGTGCGTGCG | This study |
| C_Lcp                | GGATCCGGACCGGGATGACGTGCGGGCGAGTGGGCCCC | This study |
were cultivated in LB medium containing antibiotics, which were applied according to Sambrook et al. (1989), at 30°C on a rotary shaker at 180 rpm. After 48 h of incubation, the cells were harvested by centrifugation (20 min, 4°C, 4000 rpm; Megafuge 1.0R, HERAEUS SEPA TECH GMBH, Osterode, Germany). The resulting supernatant was used for further characterization by SDS-polyacrylamide gel electrophoresis (PAGE).

**Preparation of cell-free extracts**

Supernatants from 250-mL cell suspensions were concentrated by ultrafiltration (VIVASCIENCE, Satorius Group, Göttingen, Germany) to a volume of 1 mL. For further characterization, the samples were diluted in gel loading buffer (1%, w/v, SDS; 1.25%, w/v, β-mercaptoethanol; 0.25 mM EDTA; 10%, v/v, glycerol; 0.001%, w/v, bromophenol blue; 12.5 mM Tris-HCl, pH 6.8), denatured for 10 min at 95°C, and separated in a preparative SDS-PAGE gel (12%, w/v, polyacrylamide) using a PrepCell 491 apparatus (BIO-RAD, Richmond, CA).

**SDS-PAGE and Western blot analysis**

Samples were resuspended in gel loading buffer (0.6%, w/v, SDS; 1.25%, w/v, β-mercaptoethanol; 0.25 mM EDTA; 10%, v/v, glycerol; 0.001%, w/v, bromophenol blue; and 12.5 mM Tris-HCl, pH 6.8). Proteins were prepared as described by Laemmli (1970) and were stained with Coomassie brilliant blue R-250 (Weber and Osborn 1969). Proteins blotted from SDS-polyacrylamide gels onto nitrocellulose BA83 membranes (pore size, 0.2 mm; Schleicher & Schuell, Dassel, Germany) were analyzed immunologically as described by Hein et al. (1998). To determine the N-terminal amino acid sequence, the proteins were blotted from an SDS-polyacrylamide gel onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) according to the method of Towbin et al. (1979) by use of a Semidry Fast Blot B33 apparatus and were analyzed by automated Edman degradation.

**Expression of 6xHis-tagged Lcp in *E. coli* strain BL21(DE3), isolation of inclusion bodies, and generation of anti-LcpK30 antibodies**

*Escherichia coli* strain BL21(DE3) harboring plasmid pET-23a::lcp His was cultivated in LB medium at 37°C to an OD600 of 0.5, and then expression was induced by addition of IPTG 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 1000 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 centrifugation at 4°C and 25,000 g, treatment with IB wash buffer, resuspension by sonication, and centrifugation were repeated for three times. The purified IBs were dissolved in SDS denaturation buffer (Laemmli 1970). A sample, consisting of the dissolved IBs containing the extracted Lcp protein, was separated by SDS-PAGE, excised from the gel, and its identity was confirmed by MALDI-TOF analysis (Bröker et al. 2008), before it was used for generation of polyclonal antibodies in rabbits in custom by “Eurogentec” (Seraing, Belgium). Purified polyclonal rabbit anti-LcpK30 IgGs were obtained from the serum by chromatography on Protein A-Sepharose (Hjelm et al. 1972).

**Immunoblotting**

Protein detection was performed with anti-Lcp antibodies. PVDF (Polyvinylidene Difluoride) membranes with blotted proteins were placed for 1 h in skim milk (5%, w/v) to block nonspecifically binding domains. After a membrane was washed with Tris-buffered saline (TBS) Tween buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.025% [v/v] Tween 20), it was incubated in an antibody solution (antibodies diluted 1:2000 in TBS-Tween buffer; 200 cm²−1 membrane) and shaken overnight at room temperature. The membrane was washed three times for 10 min with TBS-Tween and was then incubated with secondary (alkaline phosphatase conjugated goat anti-rabbit immunoglobulin [IgG] [Sigma-Aldrich GmbH, Munich, Germany] diluted 1:2000 in TBS-Tween buffer; 200 μl cm²−1 membrane) and shaken for 1 h at room temperature. The membrane was then washed three times for 10 min with TBS-Tween buffer and stained using 5-bromo-4-chloro-3-indolylphosphate (BCIP)/nitroblue tetrazolium tablets dissolved in 10-mL H₂O (Sigma, Deisenhofen, Germany).

**Determination of mineralization**

Evidence for biodegradation of the poly(cis-1,4-isoprene) hydrocarbon chain to CO₂ was obtained by determination of CO₂ evolution during aerobic cultivation of cells in presence of poly(cis-1,4-isoprene) as the sole carbon source. Determination was carried out in tightly closed Erlenmeyer flasks by using the property of Ba(OH)₂ to precipitate CO₂ as BaCO₃. The flasks, containing 50-mL MSM, the rubber substrate [latex concentrate or poly(cis-1,4-isoprene)], and a test tube containing 15 mL of a 0.2 M Ba(OH)₂ solution, were inoculated with 0.3% (v/v) of a well-grown culture. At each measurement point, the flasks were aerated, and the test tubes were replaced by new tubes containing fresh Ba(OH)₂ solution. Consumption of carbonate by precipitation of CO₃²⁻ as BaCO₃ was determined for each period by titration with...
HCl and was compared to that of a noninoculated control. The mineralization rate was calculated as follows: mineralization (% CO$_2$) = (required amount HCl [mL] × 0.252 M)/(C content of applied amount of cis-1,4-polyisoprene [mmol]) × 2.

Results and Discussion

Heterologous expression of lcp in E. coli

We previously identified Lcp as an important gene required for rubber degradation by Streptomyces sp. strain K30 (Rose et al. 2005) and aimed to characterize the heterologous expression of the gene product in the present study. For functionally and detailed characterization of the secretion–expression of lcp from Streptomyces sp. K30, the gene lcp was amplified employing the primers Hya_FW_XbaI and Hya_RW_NcoI (Table 1), and the PCR product was subsequently cloned into the XbaI and NcoI site of pET23a yielding pET23a::lcp. Additionally, lcp was subcloned into plasmids pUC19 and pET19b. However, the expression of all these different recombinant plasmids in several E. coli strains resulted in an overproduction of an inactive Lcp protein. However, despite of applying various experimental conditions such as cultivating the cells in LB medium or MSM, the protein was not active. In addition, different incubation temperatures (37°C, 28°C, or 20°C) with high or slow shaking rates of the culture vessels were tested, however, also here, no E. coli transformant showed an active Lcp protein, which allowed further analysis (data not shown). Escherichia coli was therefore not suitable to study the expression of Lcp.

Heterologous expression of lcp in Pseudomonas

After due consideration, we constructed hybrid plasmids for gene cloning in the metabolically versatile bacterial genus Pseudomonas (Regenhardt et al. 2002). Pseudomonas putida KT2440, a saprophytic soil bacterium, which colonizes plant roots, is a suitable microorganism for the removal of pollutants and a stable host for foreign genes used in biotransformation processes (Bagdasarian and Timmis 1982; Moreno et al. 1988; Iwasaki et al. 1994; Jimenez et al. 2002). The lcp gene from Streptomyces sp. K30 was amplified employing the primers pqsBBR-for::SalI and pqsBBR-rev::SacI, and also the primers pqsBBR-for::SbfI and pqsBBR-rev::SacI (Table 1). Both PCR products were cloned into the Sall/SacI site of pBBR (Table 1) and the SbfI/SacI site of pJB, yielding pBBR1MCS2::Lcp_His6 and pJB653::Lcp_His6, respectively. Although all experiments with conditions optimized for Pseudomonas strains resulted in the overproduction of Lcp in the supernatant and successful purification by nickel chromatography of the His-tagged protein, Lcp was inactive.

High-level expression and secretion of proteins in the native form has been proven to be difficult in both hosts, E. coli and in γ-Proteobacteria such as P. putida. Escherichia coli cells are the most commonly used host cells for large-scale production of recombinant proteins, but some proteins are difficult to express in E. coli. This includes proteins with low stability (Bertani 1951), proteins that are toxic to the host, and proteins that tend to form IBs. Due to the low content of lcp in Streptomyces sp. K30, it is difficult to isolate the overproduced protein from the original producer. Therefore, we applied a new strategy.

Heterologous expression of lcp in S. lividans TK23, TK24, and S. erythraea

The transfer of lcp to and expression of Lcp in the different host strains described above had no effect on the activity of this protein. Alternative methods for the overexpression of Streptomyces proteins in engineered expression hosts of the same or related species of this genus were in the past successfully applied to the overproduction of different enzymes (Kayser and Kilbane 2001; Moreno et al. 2003, 2005; Hidalgo et al. 2004; Torres-Bacete et al. 2007; García-Hidalgo et al. 2011).

Streptomyces lividans TK23, TK24, and S. erythraea were chosen as expression hosts as the expression of Lcp activity in Gram-negative bacteria E. coli and Pseudomonas was not successful. In contrast to S. lividans TK23, the genome of S. lividans TK24 is completely sequenced and the genome has definitely no lcp homologous. Needless to say, we have analyzed TK23 for lcp homologous with PCR without evidence but the expression of Lcp in TK24 cannot be disputed, as there is no lcp homologous in the sequence of the genome.

Therefore, lcp was cloned in the E. coli–Streptomyces shuttle expression vector pIJ6021. The resulting hybrid plasmid pIJ6021::lcp was transferred to other Streptomyces strains by protoplast transformation to study its expression. Bands presenting proteins of the expected size were visible in SDS-polyacrylamide gels after separation of the concentrated supernatants of the recombinant strains of S. lividans TK23 and TK24 as well as S. erythraea (Fig. 2a). Furthermore, Western blot analysis and immunological detection employing the Lcp antibodies raised against the purified Lcp protein of strain K30 confirmed the results and showed that Lcp was indeed synthesized by the recombinant strains (Fig. 2b).

As described previously (Jendrossek and Reinhardt 2003), purified NR latex from H. brasiliensis was used for the preparation of overlay agar plates to analyze the activity of Lcp. For this, MSM agar plates were covered with an overlay of MSM agar containing 0.2% (v/v) dispersed latex concentrate. These latex overlay agar plates were used to demonstrate clear zone formation and also growth of the recombinant strains. After four to seven days cultivation of the recombinant strains of S. lividans TK23, TK24, and S. erythraea harboring the plasmid pIJ6021::lcp on NR latex overlay plates at 30°C clear zones was observed. Thioestrepton (25 μg/mL) was used for
plasmid maintenance. A recombinant strain harboring only the vector without lcp did not form clear zones. Furthermore, we have conducted an important experiment to demonstrate Lcp activity using the supernatant of these Lcp-expressing strains in vitro (Fig. 3a–c). All three strains obviously secreted a functional Lcp, as indicated by the formation of halo. This is the first time when Lcp activity using the supernatant of Lcp-expressing strains was successful. This is an important result for future works, for example, the difficult purification of Lcp.

**Deletion of Lcp from Streptomyces sp. strain K30**

An additional experiment was necessary to verify the function of lcp in rubber degradation. 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 the transfer rates of foreign DNA. In this study, we succeeded in constructing a knock out mutant.

The 1224-bp sequence comprising the entire lcp coding region including a unique restriction site for *Sma*I was located downstream of the putative start codon. For this reason, this fragment was amplified by PCR using the primers N._Lcp and C._Lcp; subsequently it was cloned into pGEM-T Easy (Table 1), which does not possess a cleavage site for *Sma*I. The resulting plasmid, pGEM-T::lcp, isolated from *Escherichia coli* TOP10 could not be digested with *Sma*I, indicating methylation at its recognition site, it was transferred to *E. coli* ET12567 lacking the DNA methylase. The plasmid DNA could then be linearized with *Sma*I, and an approximately 1000-bp *Sma*I kanamycin resistance cassette (ΩKm) was inserted at position 281 of lcp. The 2.2-kbp lcpΩKm DNA fragment was amplified by PCR, and the resulting linear DNA fragment was purified, dialyzed, and transferred to *Streptomyces* sp. K30 by protoplast transformation. Recombinant clones were selected for chromosomal integration of the lcpΩKm fragment on
Importance of the Lcp sp. strain K30. After 50 days of mineralization, the knock mutant was unable to form a published by Blackwell Publishing Ltd. sp. K30 is quite slow (seven days to form clear -1,4-isoprene) cleavage. Lcp Streptomyces sp. strain K30 was successfully generated. All disruption mutant lcp in the same sp. K30 knock out mutant to use poly(cis-1,4-isoprene) as carbon source was compared to that of the wild type in mineralization experiments. Highest mineralization of poly(cis-1,4-isoprene) was obtained with the wild-type strain Streptomyces sp. strain K30. After 50 days of mineralization, the wild-type K30 had metabolized about 1.63% of the supplied NR cultures to CO2. In contrast, the lcp knock out mutant mineralized only about 0.82% of NR to CO2 in the same period. The experiment to measure the value of metabolized rubber was repeated three times with the wild-type Streptomyces sp. K30 and the lcp disruption mutant Streptomyces sp. K30.lcpΔKm. The rubber degradation rate of the wild-type Streptomyces sp. K30 is quite slow (seven days to form clear zones on latex overlay agar plates), strains such as TK23 and TK24 show similar results; hence, we consider the difference to be significant.

**Complementation of Streptomyces sp. K30.lcpΔKm**

The genetic complementation of the lcp knock out mutant was analyzed in detail. Plasmid pIJ702::lcp_1, harboring the wild-type gene including the native promoter region of lcp from Streptomyces sp. strain K30, was transformed by protoplast transformation into the corresponding mutant. This plasmid restored the wild-type phenotype in the lcp knock out mutant. The recombinant strain was able to produce a clear zone on latex overlay plate and to produce aldehydes as revealed by staining with Schiff’s reagent. These results confirmed the successful complementation of the lcp knock out mutant with the wild-type lcp gene.

This was the first time that an lcp knock out mutant from Streptomyces sp. strain K30 was successfully generated. All previous efforts in our laboratory had failed. In contrast to the parent strain (Fig. 4c), the lcp mutant was unable to form a clear zone (Fig. 4b). Furthermore, it did not form metabolites staining with Schiff’s reagent. Moreover, mineralization experiments clearly revealed that poly(cis-1,4-isoprene) degradation was almost completely diminished in the lcp knock out mutant of Streptomyces sp. strain K30 when compared to the wild-type Streptomyces sp. strain K30. These indisputable findings confirmed that the initial cleavage of poly(cis-1,4-isoprene) is solely dependent on Lcp in Streptomyces sp. K30.
Importance of the Lcp

M. Yikmis & A. Steinbüchel

Figure 4. Analyses of lcp disruption mutants of Streptomyces sp. K30. (a) Screening for lcp disruption mutants of Streptomyces sp. strain K30 by colony PCR. Cell material from a single colony of a putative lcp disruption mutant was suspended in 50-μl TE buffer, and the suspension was then boiled for 15 min. After centrifugation, 0.5 μl was applied as template for a PCR employing the primers NLcp and CLcp (Table 1); the product was subsequently separated in a 1% (w/v) agarose gel. M, λ DNA digested with Pst I; WT, Streptomyces sp. K30 wild type; lcpΩKm disruption mutant of Streptomyces sp. K30. (b and c) Effect of the knock out lcp mutant on clear zone formation by Streptomyces sp. K30. (b) Streptomyces sp. K30 harboring lcpΩKm and (c) the wild-type Streptomyces sp. K30 were cultivated for seven days on a natural rubber (NR) latex overlay agar plate at 30°C.

It is therefore unlikely that other proteins than this are additionally involved in rubber cleavage of the poly(cis-1,4-isoprene) chain in this bacterium.

This study encourages further studies of rubber degradation in Gram-positive microorganisms. In the future, the latex-clearing protein, Lcp, must be purified to unravel the reaction mechanism of this enzyme acting on polyisoprene and to employ this protein for biotechnological applications, for example, for the conversion of rubber waste material.

Acknowledgments

Financial support for this study by the Deutsche Forschungsgemeinschaft is gratefully acknowledged (STE-386/10-1).

References

Arensköter, M., D. Bröker, and A. Steinbüchel. 2004. Biology of the metabolically diverse genus Gordonia. Appl. Environ. Microbiol. 70:3195–3204.

Bagdasarian, M. M., and K. N. Timmis. 1982. Host: vector systems for gene cloning in Pseudomonas. Curr. Top. Microbiol. Immunol. 96:47–67.

Berti, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. J. Bacteriol. 62:293–300.

Blatny, J. M., T. Brautaset, H. C. Winther-Larsen, K. Haugan, P. Karunakaean, and S. Valla. 1997. Improved broad-host-range RK2 vectors useful for high and low regulated gene expression levels in gram-negative bacteria. Plasmid 38:35–51.

Bode, H. B., A. Zeeck, K. Plückhahn, and D. Jendrossek. 2000. Physiological and chemical investigations into microbial degradation of synthetic poly(cis-1,4-isoprene). Appl. Environ. Microbiol. 66:3680–3685.

Braaz, R., W. Armbruster, and D. Jendrossek. 2005. Heme-dependent rubber oxygenase RoxA of Xanthomonas sp. cleaves the carbon backbone of poly(cis-1,4-isoprene) by a dioxygenase mechanism. Appl. Environ. Microbiol. 71:2473–2478.

Bröker, D., D. Dietz, M. Arenskötter, and A. Steinbüchel. 2008. The genomes of non clearing-zone-forming and natural-rubber-degrading species Gordonia polyisoprenivorans
and Gordonia westfalica harbor genes expressing Lcp activity in Streptomyces strains. Appl. Environ. Microbiol., 74:2288–2297.

Flett, E., Mersinias, V., and Smith, C. P. (1997). High efficiency intergeneric conjugal transfer of plasmid DNA from Escherichia coli to methyl DNA-restricting streptomycetes. FEMS Microbiol. Lett., 155, 223–239.

Garcia-Hidalgo, J., D. Hormigo, M. A. Prieto, M. Arroyo, and I. de Mata. 2011. Extracellular production of Streptomyces exfoliatus poly-(3-hydroxybutyrate) depolymerase in Rhodococcus sp. T104: determination of optimal biocatalyst conditions. Appl. Microbiol. Biotechnol.

Hall, T. 1998. BioEdit. Biological sequence alignment editor for Windows. North Carolina State University, North Carolina.

Hein, S., H. Tran, and A. Steinbüchel. 1998. Synechocystis sp. PCC6803 possesses a two component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.

Hidalgo, A., L. Betancor, R. Moreno, O. Zafra, E. Cava, R. Fernández-Lafuente, J. M. Guisán, and J. Berenguer. 2004. Thermus thermophilus as a cell factory for the production of a thermophilic Mn-dependent catalase which fails to be synthesized in an active form in Escherichia coli. Appl. Environ. Microbiol. 70:3839–3844.

Hjelm, H., K. Hjelm, and J. Sjöquist. 1972. Protein A from Staphylococcus aureus. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. FEMS Lett. 28:73–76.

Hopwood, D. A., T. Kieser, H. M. Wright, and M. J. Bibb. 1983. Plasmids, recombination and chromosome mapping in S. lividans 66. J. Gen. Microbiol. 129:2257–2269.

Hopwood, D. A. 1986. Manual of industrial microbiology. Pp. 198–203 in A. L. Demainand N. A. Solomon, eds. Gene cloning in Streptomyces spp. Vol. 129. American Society for Microbiology, Washington, D.C.

Iwasaki, K., H. Uchiyama, O. Yagi, T. Kurabayashi, K. Ishihizuku, and Y. Takamura. 1994. Transformation of Pseudomonas putida by electroporation. Biosci. Biotech. Biochem. 58:851–854.

Jendrossek, D., and S. Reinhardt. 2003. Sequence analysis of a gene product synthesized by Xanthomonas sp. during growth on natural rubber latex. FEMS Microbiol. Lett. 224:61–65.

Jendrossek, D., G. Tomasi, and R. M. Kroppenstedt. 1997. Bacterial degradation of natural rubber: a privilege of actinomycetes? FEMS Microbiol. Lett. 150:179–188.

Jimenez, J. I., B. Minambres, J. L. Garcia, and E. Diaz. 2002. Genomic analysis of the aromatic catabolic pathways from Pseudomonas putida KT2440. Environ. Microbiol. 12:824–841.

Kanehisa, M., and S. Goto. 2000. KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 28:27–30.

Kanehisa, M., S. Goto, S. Kawashima, and A. Nakaya. 2002. The KEGG database at GenomeNet. Nucleic Acids Res. 30:42–46.

Kanehisa, M. 1997. A database for post-genome analysis. Trends Genet. 13:375–376.

Kayser, K. J., and J. J. Kilbane. 2nd. 2001. New host vector system for Thermus spp. based on the malate dehydrogenase gene. J. Bacteriol. 183:1792–1795.

Kieser, T., M. J. Bipp, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. Practical Streptomyces genetics. John Innes Foundation, Norwich, U.K.

Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop, II, and K. M. Peterson. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene 166:175–176.

Kumar, G. S., V. Kalpagam, and U. S. Nandi. 1983. Biodegradable polymers: prospects, problems, and progress. JMS-Rev. Macromol. Chem. Phys. 22:225–260.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.

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

MacNeil, D. J., Gewain, K. M., Ruby, C. L., Denzeny, G., Gibbons, P. H., and MacNeil, T. (1992). Analysis of Streptomyces avermitilis genes required for avermectin biosynthesis utilizing a novel integration vector. Gene, 111, 61–68.

Moreno, R., A. Haro, A. Castellanos, and J. Berenguer. 2005. High-level overproduction of His-tagged DNA polymerase in Thermus thermophilus. Appl. Environ. Microbiol. 71:591–593.

Moreno, R., O. Zafra, E. Cava, and J. Berenguer. 2003. Development of a gene expression vector for Thermus thermophilus based on the promoter of the respiratory nitrate reductase. Plasmid 49:2–8.

Peckysnska-Czoch, W., and M. Mordarski. 1988. Pp. 219–283 in M. Goodfellow, S. T. Williams, and M. Mordarski, eds. Actinomycetes in biotechnology. Actinomycete enzymes. Academic Press, London.

Pospiech, A., and B. Neumann. 1995. A versatile quick-prep of genomic DNA from Gram positive bacteria. Trends Genet. 11:217–218.

Regenhardt, D., H. Heuer, S. Heim, D. U. Fernandez, C. Strump, E. R. Moore, and K. N. Timmis. 2002. Pedigree and taxonomic credentials of Pseudomonas putida strain KT2440. Environ. Microbiol. 4:912–915.

Rose, K., and A. Steinbüchel. 2005. Biodegradation of natural rubber and related compounds: recent insights into a hardly understood catabolic capability of microorganisms. Appl. Environ. Microbiol. 71:2803–2812.

Rose, K., K. B. Tenberge, and A. Steinbüchel. 2005. 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. Biocatal. Biotransform. 6:180–188.
Importance of the Lcp

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schlegel, H. G., H. Kaltwasser, and G. Gottschalk. 1961. Ein Submersverfahren zur Kulturwasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. Arch. Mikrobiol. 38:209–222.

Takano, E., White, J., Thompson, C. J., and Bibb, M. J. (1995). Construction of thioestrepton-inducible, high-copy-number expression vectors for use in *Streptomyces* spp. Gene, 166, 133–137.

Thomas, J. D., R. A. Daniel, J. Errington, and C. Robinson. 2001. Export of active green fluorescent protein to the periplasm by the twin-arginine translocase (Tat) pathway in *Escherichia coli*. Mol. Microbiol. 39:47–53.

Torres-Bacete, J., D. Hormigo, M. Stuart, M. Arroyo, M. P. Castillón, C. Acebal, J. L. García, and I. de la Mata. 2007. Newly discovered penicillin acylase activity of aculeacin A acylase from *Actinoplanes utahensis*. Appl. Environ. Microbiol. 73:5378–5381.

Towbin, H., T. Stahelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350–4354.

Tsuchii, A., and K. Takeda. 1990. Rubber degrading enzyme from a bacterial culture. Appl. Environ. Microbiol. 56:269–274.

Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406–4412.

Yikmis, M., M. Arenskötter, K. Rose, N. Lange, H. Wernsmann, L. Wiefel, and A. Steinbüchel. 2008. Secretion and transcriptional regulation of the latex clearing protein Lcp by the rubber-degrading bacterium *Streptomyces* sp. strain K30. Appl. Environ. Microbiol. 74:5373–5382.