รายงานวิจัยฉบับสมบูรณ์

Final Research Report

Natural rubber degradation:

Optimum conditions, gene and protein characterization of newly isolated

Rhodococcus rhodochrous RPK1

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โครงการวิจัยนี้ได้รับทุนสนับสนุนจากมหาวิทยาลัยสงขลานครินทร์

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ชื่อโครงการวิจัย การย่อยสลายยางธรรมชาติ: สภาวะที่เหมาะสม การศึกษายีนและโปรตีน ของเชื้อ *Rhodococcus rhodochrous* RPK1 ที่คัดแยกได้ใหม่

Natural rubber degradation: Optimum conditions, gene and protein characterization of newly isolated *Rhodococcus rhodochrou*s RPK1

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บทคัดย่อ

การศึกษานี้สามารถคัดแยกแบคทีเรีย *Rhodococcus rhodochrous* สายพันธุ์ RPK1 ที่มีประสิทธิภาพสูงในการย่อยสลายยางวัลคาไนซ์ จากกลุ่มเชื้อย่อยสลายยางที่แยกได้จากบ่อบำบัดน้ำเสียของโรงงานยาง เมื่อทำการคัดแยกโปรตีน Latex clearing protein (Lcp_{Rr}) จากแบคทีเรียชนิดนี้ และวิเคราะห์ลำดับดีเอ็นเอพบ Open reading frame ขนาดใหญ่ที่กำหนดการสร้างโปรตีน Lcp เมื่อทำการบ่งชี้ชนิดและศึกษาคุณลักษณะเฉพาะของโปรตีน Lcp_{Rr} โดยทำการคัดแยกยีนที่ทำหน้าที่เปลี่ยนรหัสการสังเคราะห์โปรตีนที่ทำให้ไอโซพรีนแตกตัว โคลน และทำการแสดงออกใน *Escherichia coli* นั้น เมื่อทำให้บริสุทธิ์ โปรตีน Lcp_{Rr} มีกิจกรรมจำเพาะ ที่อุณหภูมิ 30 องศาเซลเซียส เท่ากับ 3.1 U/mg โดยสามารถย่อยสลายไอโซพรีนสายยาว ให้เป็นโมเลกุลของไอโซพรีนสายสั้นที่มีปลายเป็นคีโตนและ แอลดีไฮด์ ได้ โดยมีค่าพีเอชที่เหมาะสมสำหรับการทำงานของโปรตีน Lcp_{Rr} เท่ากับ 8 ซึ่งมีค่าสูงกว่าค่าพีเอชของเอนไซม์ย่อยสลายยางชนิดอื่นคือ 7 เมื่อวิเคราะห์ Lcp_{Rr} ด้วย UVvis spectroscopy พบว่าโปรตีนชนิดนี้มีการดูดกลืนแสงที่ช่วงความยาวคลื่นจำเพาะของไซโทโครม และยังพบลักษณะเด่นคือสามารถดูดกลืนแสงในช่วงความยาวคลื่นที่ยาวกว่าโปรตีนย่อยสลายยางชนิดอื่น และยังไม่เคยมีรายงานมาก่อน นอกจากนี้ยังพบว่าโปรตีน Lcp_{Rr} เป็นโปรตีนที่มีฮีมชนิดบีเป็นโคแฟกเตอร์ (*b*-type heme) เมื่อทำการวิเคราะห์จาก 1) การวิเคราะห์โลหะ 2) การสกัดด้วยสารตัวทำละลาย 3) การวิเคราะห์ด้วยวิธี Bipyridyl และ 4) การตรวจวัดด้วย mass-spectrometry

ในการศึกษาครั้งนี้ชี้ให้เห็นถึงประสิทธิภาพในการย่อยสลายถุงมือยางวัลคาไนซ์ของ *Rhodococcus rhodochrous* สายพันธุ์ RPK1 และยังแสดงให้เห็นถึงคุณลักษณะเฉพาะของโปรตีน Lcp_{Rr} ซึ่งแตกต่างจากโปรตีนย่อยสลายยางชนิดอื่นที่เคยมีรายงานมาก่อนหน้า

คำสำคัญ: การย่อยสลายทางชีวภาพ ยางธรรมชาติ
ลาเท็กเคลียริ่งโปรตีนบริสุทธิ์ แบคทีเรียย่อยสลายยาง โรโดคอคคัส

Abstract

In this research study, a novel rubber degrading bacterium identified as a *Rhodococcus rhodochrous* RPK1 was discovered from the potential rubber degrading consortium isolated from a rubber waste pond at a rubber latex processing factory. A new potential Latex clearing protein (Lcp_{Rr}) was identified and characterized. DNA sequence analysis revealed one large open reading frame encoding for Lcp protein in *R. rhodochrous*. A gene of *R. rhodochrous* RPK1 that coded for a polyisoprene-cleaving latex clearing protein (Lcp_{Rr}) was identified, cloned, expressed in *Escherichia coli* and purified. Purified Lcp_{Rr} had a specific activity of 3.1 U/mg at 30°C and degraded poly(*cis*-1,4-isoprene) to a mixture of oligoisoprene molecules with terminal keto and aldehyde groups. The optimum pH of Lcp_{Rr} was higher (pH 8) than for other known rubber-cleaving enzymes (≈ pH 7). UVvis spectroscopic analysis of Lcp_{Rr} revealed a cytochrome-specific absorption spectrum with an additional feature at long wavelengths that has not been observed for any other rubber-cleaving enzyme. The presence of one *b*-type heme in Lcp_{Rr} as a co-factor was confirmed by (i) metal analysis, (ii) solvent extraction, (iii) bipyridyl assay and (iv) detection of heme-*b* specific m/z values via mass-spectrometry.

This study points the potential of *Rhodococcus rhodochrous* RPK1 to degrade vulcanized rubber glove and revealed the substantial differences in the active sites of Lcp_{Rr} proteins to other rubber degrading proteins.

Keywords: Biodegradation, Natural rubber, Lcp, Rubber degrading bacteria, Rhodococcus

Rationale and Background

During this century, environmental issues have been a focus of law, especially waste management, which has been of particular concern. There has been a growing interest in renewable resources using a base of biodegradable products. An alternative material widely used in many applications is natural rubber, which is a highly valuable source with superior physical properties. One of the major problems with rubber products is that they cannot be recycled and even though it is a natural product, it is very difficult to degrade either biologically or chemically. As a consequence of these difficulties, huge amounts of waste rubber materials are accumulating around the world, on the landfill and in the ocean, and are one of the causes of environmental pollution (Shah et al., 2013). The International Rubber Study Group (IRSG) has reported that the total world rubber consumption is increasing annually up to a million tons, leading to further organic rubber waste problems (IRSG, 2016).

In addition modified rubber products contain various antioxidants, accelerator residues, stearic acid and other compounding ingredients from production processes cause leaching to environments and can cause the health problem in humans. One example is plasticizers, which is suspected to be an endocrine-disrupting activity that interferes with the reproductive system and normal development of animals and humans (Wang et al., 2003; Wang et al., 2004; Xu et al., 2005; Pan et al., 2009). Degradation of plasticizer, rubber vulcanization additive and other compounds are also concerned with the rubber degradation process with regard to biotechnological applications in rubber waste treatment. (Wever and Verachtert, 1997; Wang et al., 2003; Wang et al., 2004; Li et al., 2005; Xu et al., 2005).

One possible approach to deal with such waste problems is burning, deposits in landfill or bury in the soil. However, these methods can produce further environmental pollution and health problems (Fatta et al., 1999). Thus, a better way to control this problem is to induce microbial transformation of rubber and their derivatives or plasticizer into useful products or even to completely degrade into CO_2 as a growth substrate (Bode et al., 2001; Braaz et al., 2004; Linos et al., 2000a; Rose and Steibüchel, 2005; Tsuchii et al., 1985; Tsuchii and Takeda., 1990). More recent experiments have revealed that the compounding ingredients of rubber products such as curing agents or antioxidants serve as growth substrates during the degradation process (Pan et al., 2009).

However, the biodegradation of rubber products in the environment is a result of microbial strategies for an effective availability of solid and hydrophobic substrate. Due to the million units of rubber have high molecular weights with their chemical weakness for microbial attack, being the C=CH bond the long closely aligned hydrocarbon chains make the rubber extremely hydrophobic, and difficult to degrade. However, it is thought that after the initial attachment and preliminary degradation, some bacteria can excrete detergent-like molecules that tend to separate the hydrophobic rubber chains, and open up access to more double bonds. This is especially, true for cross-linked structures such as rubber gloves that have been used as a typical rubber representative product and as a model for these experiments. However, in many cases the bacteria isolated with an ability to begin to attack the C=CH bonds and to degrade the rubber with an accumulation of products and substrates with aldehyde and ketone groups are unable to complete the process (Shah et al., 2013). To characterize these single bacteria, our experiments used rubber agar plates, which showed that at least two different strategies were involved, one resulted in the production of clearing zones and the other did not. Those that formed a clearing zone on rubber agar plates exhibited a slightly faster growth rate, and required cultivation for a period of an additional month (Bröker et al., 2004). Bacteria such as *Streptomyces* sp*.,* a non-clearing zone producing strain, required a longer incubation period to obtain a reasonable cell mass (Rose et al., 2005).

One of a major approach is to study the rubber degrading enzymes and their corresponding genes. So far, there is very little known about biochemically characterization of rubber cleaving protein. Only two types of rubber-cleaving enzymes have been investigated, one is the rubber oxygenase RoxA from *Xanthomonas* sp. 35Y (Tsuchii and Takeda., 1990; Braaz et al., 2004) and it has been found only in Gram-negative bacteria (Birke et al., 2013). Extracellular dioxygenase RoxA secreted by *Xanthomonas* sp. 35Y, is characterized by its biochemistry, and can be classified as *c*-type dihaemdioxygenase. This enzyme function to cleave poly(*cis*-1,4-isoprene) into a C_{15} compound with a terminal keto and aldehyde group (12-oxo 4,8-dimethyl-trideca-4,8-diene-1-al, ODTD) as the one major product (Braaz et al., 2005; Schmitt et al., 2010; Birke et al., 2012; Birke et al., 2013). The other rubber cleaving enzyme is a protein designated as latex clearing protein (Lcp) (Rose et al., 2005). It shares no significant sequence homology with RoxA, with cytochrome *c* peroxidases or with dihaeme 7,10-diolsynthases (Estupiñán et al., 2015), and it is present in Gram-positive rubber degrading bacteria and other Gram-positive Actinobacteria. Lcp was first described for *Streptomyces* sp. K30

(Rose et al., 2005). Lcp of *Gordonia polyisoprenivorans* VH2 and *Streptomyces* sp. K30, two well-studied Gram positive rubber degraders, oxidatively cleave poly(*cis*-1,4-isoprene) to products of different sizes of cleavage products (C_{20}-C_{35}) with the terminal keto and aldehyde end groups as in RoxA-generated ODTD (Ibrahim et al., 2006; Birke and Jendrossek, 2014; Hiessl et al., 2014). There have been different published reports of the cofactor and metal-contents of the Lcps from *Streptomyces* sp. K30 and of *G. polyisoprenivorans* VH2, and at present there are only two biochemically characterized Lcp proteins (Hiessl et al., 2014; Birke and Jendrossek, 2014).

This research provides the use of a newly isolated bacterium strain RPK1 to degrade rubber effectively, taxonomic analysis revealed that isolate RPK1 was a member of the genus Rhodococcus, a taxon that had not previously been identified as having the ability to utilize rubber as a sole source of carbon and energy, but that is well known for its members to have a high potential for the biodegradation of recalcitrant compounds (Martínková et al., 2009). Biochemical and biophysical characterization of the purified recombinant Lcp protein of *Rhodococcus rhodochrous* strain RPK1 revealed some properties not previously described for any other rubber-degrading enzyme.

Objectives

1. To investigate rubber degradation by the isolated bacterium.
2. To identify the corresponding genes of the selected key bacterium that play important roles in the rubber degradation process and characterize the biochemistry of purified rubber-cleaving enzyme.

A bacterial consortium that was derived from a rubber waste factory has shown a substantial disintegration of vulcanised rubber gloves that were used as a sole carbon and energy source in MSM medium over a period of 30 days. Throughout the process the most dominant species that remained attached to the surface was *Rhodococcus rhodochrous* RPK1. Others that were attached were also found growing in the liquid in addition to other different bacteria that were presumably feeding off the derived metabolites. Due to the rubber-degrading strain *Rhodococcus rhodochrous* RPK1 plays an important role in the rubber degradation process, and acts as a dominant species remaining attached to the rubber surface throughout the experiments, while the others were fed off after long incubation periods. Thus, the identification of the key bacteria of the effective consortium was performed in this study. Multiple evidence showed that the rubber degrading protein,

namely the Latex clearing protein (Lcp) is responsible for the *lcp* gene in Gram positive rubber degrading bacteria, and many *lcp* genes in rubber degrading species have been described (Linos et al., 2000a & 2002b; Braaz et al., 2005; Rose and Steinbüchel, 2005; Yikmis et al., 2008; Yikmis and Steinbüchel, 2012a; Yikmis and Steinbüchel, 2012b). Although only a little knowledge of *R. rhodochrous* genomes is accessible, the biochemistry of polyisoprene cleavage by Lcp_{Rr}, the types of protein and functions of the cofactors involved are concealed.

It is well known that the initial microbial attack on rubber depends on the ability to produce and secrete the rubber-cleaving enzymes into the environment. Recently, only two types of rubber-cleaving enzymes have been discovered. One is the rubber oxygenase RoxA, that was first isolated from *Xanthomonas* sp. 35Y (Tsuchii and Takeda., 1990; Braaz et al., 2004), and so far this enzyme has been found only in Gram-negative bacteria (Birke et al., 2013). RoxA of *Xanthomonas* sp. 35Y is a *c*-type diheme dioxygenase, and cleaves poly(*cis*-1,4-isoprene) into a C_{15} compound with a terminal keto and aldehyde group (12-oxo-4,8-dimethyl-trideca-4,8-diene-1-al, ODTD), as the major products (Braaz et al., 2005; Schmitt et al., 2010; Birke et al., 2012; Birke et al., 2013). The other rubber cleaving enzyme is a protein designated as a latex clearing protein (Lcp) (Rose and Steinbüchel, 2005). It shares no significant sequence homology with RoxA, with cytochrome *c* peroxidases or with diheme 7, 10-diol synthases (Estupiñán et al., 2015), and it is present in Gram-positive rubber degrading bacteria, such as *Streptomyces* sp. K30 (Rose and Steinbüchel, 2005) and other Actinobacteria. *G. polyisoprenivorans* VH2 and *Streptomyces* sp. K30, two well-studied Gram-positive rubber degraders, oxidatively cleave poly(*cis*-1,4-isoprene) to the different sizes but with the same keto and aldehyde end groups as in RoxA-generated ODTD (Ibrahim et al., 2006; Birke and Jendrossek, 2014; Hiessl et al., 2014).

Currently, there have been many reports published for the cofactor, and metal-contents of the Lcps from *Streptomyces* sp. K30 and of *G. polyisoprenivorans* VH2 (Hiessl et al., 2014; Birke and Jendrossek, 2014), however at present there are only two biochemically characterized Lcp proteins.

In this study, the dominant strain in consortium was isolated as rubber degrading strains. Taxonomic analysis revealed that one strain was a member of the genus Rhodococcus, a full taxon that had not been previously identified as having the ability to utilize rubber as the sole source of carbon and energy but it had been well known for having a high potential for the biodegradation of recalcitrant compounds (Martínková et

al., 2009). Biochemical and biophysical characterization of the purified recombinant Lcp protein of *Rhodococcus rhodochrous* strain RPK1 demonstrated some unexpected properties which are not previously described for any other rubber-degrading enzymes, in addition their properties were shared with the other characterized Lcp proteins.

Materials and Methods

Enrichment and isolation of rubber-degrading microorganisms

Sediment from a rubber waste pond at a rubber latex processing factory in Thailand (Namom rubber factory, Namom, Songkhla) was used as an inoculum to enrich the rubber-degrading microorganisms in a Mineral Salts Medium (MSM) supplemented with pieces of rubber gloves as a sole source of carbon and energy. After two weeks of incubation at 30°C, 10% of volumes (without pieces of rubber) were transferred to a fresh medium and were incubated for an additional month. Substantial disintegration of the new rubber pieces became visible was indicated that the active rubber-degrading microorganisms were present as a consortium. Several bacterial strains were isolated from this enriched culture by repeated purification by streaking on NB and LB agar plates. Each isolate was subsequently tested for its ability to degrade rubber in liquid MSM with rubber pieces as a carbon source. One isolate designed as isolate RPK1, remained attach to the rubber surface throughout the experiments. This isolate might play an important role in the biodegradation process with a strong rubber-degrading activity thus it was selected for this study.

Bacterial strains, plasmids and culture conditions

Rubber degrading bacteria *R. rhodochrous* strain RPK1 was grown with a nutrient broth (NB) medium or in mineral salts medium (MSM, 9 g/L Na_2HPO_4 x 12 H_2O, 1.5 g/L KH_2PO_4, 1 g/L NH_4NO_3, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.02 g/L $CaCl_2$ x 2 H_2O, 1.2 mg/L Fe(III) ammonium citrate with solid rubber pieces or with rubber latex milk and were then inubated at 30°C. Pieces (1 cm x 1 cm) of heat-sterilized vulcanized rubber (0.6% [wt/vol]) (Siam Top Glove Co., LTD), were added to the sterile MSM for the enrichment and growth of *R. rhodochrous*. Plasmid-haboring recombinant *E. coli* strains were grown with LB medium at 22°C or 37°C in the presence of the appropriate antibiotics. Polyisoprene latex was kindly provided by Weber and Schaer, Hamburg (Germany) and was washed for 3 washing steps in 0.1% (wt/vol) Nonidet P40 before used for purification of Lcp_{Rr}, recombinant *E. coli* cells were grown in LB medium supplemented with 0.1% (wt/vol) L-rhamnose at 22°C. Utilization of carbon sources was examed on mineral salts agar with separately filter-sterilized carbon sources at these following final concentrations (sugars, sugar alcohols and sugar acids at 0.5% [wt/vol],

sodium acetate [0.25%, wt/vol], sodium benzoate [0.1%, wt/vol]). Volatile compounds (alkanes) were applied by adding a quantity of 100 µL to a sterile filter paper placed in the lid of a petri disk. The plates were sealed with parafilm and were then incubated separately at 30°C.

Cloning and heterologous expression of *lcp*$_{Rr}$, and determination of the 16S rRNA gene sequence of the isolate RPK1

The *lcp*$_{Rr}$ gene was amplified using the chromosomal DNA from *R. rhodochrous* strain RPK1 as DNA template and the oligonucleotides Lcp$_{Rr}$-complete_for and Lcp$_{Rr}$-complete_rev as the PCR primers. Takara Primestar DNA polymerase was used as the proof-reading polymerizing enzyme. The DNA sequence of the product was determined, and it had been then deposited under the accession no. KU140417. Alternatively, the coding sequence of mature Lcp$_{Rr}$ was amplified from chromosomal DNA, using Lcp$_{Rr}$-mature-PstI_for and Lcp$_{Rr}$-mature-HindIII_rev as the primers. The DNA products were purified by agarose gel electrophoresis, and were then cleaved with restrictive enzymes e.g. PstI and HindIII. The cleaved products were ligated into plasmid pUC9::*lcp*$_{K30}$ that had been cleaved by the same restriction enzymes. The coding sequence for strep-tagged lcp$_{Rr}$ was cut using HindIII and NdeI, and was subsequently ligated into the expression plasmid p4782.1 and transformed to competent *E. coli* JM109 cells.

A part of the 16S rRNA gene of the isolate RPK1 was PCR-amplified using the primers 16S-universal_for and 16S-universal_rev. The DNA sequence of the resulting PCR product was determined (1412 bp), and demonstrated a strong similarity to the 16S rRNA genes of several *Rhodococcus* sp. strains. The 16S rRNA gene sequence of the isolate RPK1 was determined after PCR amplification using the primers (16S-Rr-complete_for and 16S-Rr-complete_rev), that were specific for the known 16S rRNA gene sequences of *R. rhodochrous* strains reported in the NCBI data base, and it is now available under the accession no KU140418.

Purification of Lcp$_{Rr}$, Lcp$_{K30}$ and of RoxA$_{Xsp}$

Purification of the rubber oxygenase of *Xanthomonas* sp. 35Y (RoxA$_{Xsp}$) and latex clearing protein Lcp$_{K30}$ was performed as described previously (Birke et al.,

2015). Both of $RoxA_{Xsp}$ and Lcp_{K30} were used for comparison of some properties, sharing with the Lcp_{Rr} proteins (Schmitt et al., 2010; Hiessl et al., 2014).

Lcp_{Rr} was purified as followed: eight individual 600 mL LB cultures in 3 L Erlenmeyer flasks were inoculated each with 0.02 volumes of a seed culture of *E. coli* JM109 harboring the plasmid p4782.1::*lcp*$_{Rr}$ that had been grown with the same medium. It was important that the presence of L-rhamnose (0.1%, wt/vol) at the beginning in the main cultures was enhanced the yield of the expressed Lcp_{Rr} protein. Cells of the main culture were then harvested by centrifugation after approximately 20 hours of growth at 22°C and were immediately used for protein purification. The cell pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.7, containing 150 mM sodium chloride (KPN, 2 mL KPN/g cell wet weight). A soluble cell extract was prepared by two French press steps, and subsequent centrifugation at 40,000 g for 40 min. The supernatant (about 60 mL) was directly applied to a 10 mL Strep-Tactin HC gravity flow column that had been equilibrated with KPN buffer. The column was washed with at least five volumes of KPN buffer before the Lcp_{Rr} protein was eluted by about 30 mL of 5 mM desthiobiotin dissolved in KPN. Lcp_{Rr}-containing fractions were combined, and desalted by running through a G25 Sephadex (26/160) Hiprep desalting column (53 mL bed volume), that had been equilibrated with 1 mM potassium phosphate (KP) buffer, pH 7.0 and subsequently concentrated to 1-2 mL using ultrafiltration (10 kDa cut-off). Remaining impurities were removed by chromatography on a Superdex 200 column (16/600, equilibrated with 1 mM KP, pH 7) at a flow rate of 1 mL/min. Combined Lcp_{Rr}-containing fractions were ultrafiltrated (10 kDa cut-off) and were then concentrated to approximately 1.5 mL. Aliquots of the purified Lcp_{Rr} protein were stored on ice for about three days (Lcp_{K30} up to one week) or shock-frozen with liquid nitrogen, and stored at -70°C for further study.

Determination of the cytochrome type of Lcp_{Rr}

The heme type of Lcp_{Rr} was determined by the bi-pyridyl assay as described by Hiessl et al. (2012). Purified $RoxA_{Xsp}$, cytochrome *c* (horseheart, type III, Sigma, St. Louis, USA) (both *c*-type cytochromes) and hemoglobin (*b*-type) (bovine, Sigma, St. Louis, USA) were used as the controls for known *c*-type and *b*-type cytochromes, respectively. The respective protein stock solution of about 25 µL (4-8 mg/mL) was added to 975 µL solution A (100 mM sodium hydroxide, 20% (vol/vol) pyridine, 0.3 mM potassium ferricyanide). Subsequently, 2-5 mg sodium dithionite were added, and the

spectrum of the reduced cytochrome was recorded. The absorption maxima of the resulting α-bands were characteristic for *b*-type (556 nm), and *c*-type (550 nm) cytochromes. Bi-pyridyl-heme complexes of α-type cytochromes absorb at 584-588 nm. Additional assays for determination of the heme type were performed using extraction of heme by acidic acetone, and a matrix assisted laser desorption ionization time of flight (MALDI-ToF) analysis as previously described (Birke et al., 2015).

Assay of Lcp activity

An HPLC-based assay for Lcp_{Rr}-derived polyisoprene degradation products, was used for most routine assays: poly(*cis*-1,4-isoprene) latex was diluted with 100 mM KP buffer, pH 7, to 0.2% (assay volume 0.7 mL), and was incubated in the presence of the purified Lcp protein for 2 hours at a temperature as indicated (for routine assay at room temperature [23°C]). For inhibition studies, the corresponding compound was added and gently solubilized in the reaction mix before the enzyme was added (final inhibitor concentration 1 mM). The products were extracted with 1 mL ethyl acetate (in a 2 mL Eppendorf tube), dried, and dissolved in 100 µL methanol. Aliquots were applied to an RP8 HPLC column (12 x 4 mm, 5 µm particle size, 0.7 mL/min) with water (A), and methanol (B) as mobile phases. The concentration of B was increased from 50% (vol/vol) to 100% (vol/vol) within 15 min; products were detected at 210 nm. The C_{35} product peak (at about 23 min) was used for quantification and compared to a control without inhibitor. Alternatively, activity of Lcp_{Rr} was assayed by determination of the rate of oxygen consumption in an OXY-4 mini apparatus (PreSens, Regensburg, Germany) as described previously (Birke et al., 2015). Triplicates and controls without Lcp_{Rr} or with heat-inactivated Lcp_{Rr} were recorded simultaneously. A stability assay was performed by incubation of the purified Lcp_{Rr} protein in the assay buffer at 37°C for variable time periods. The remaining activity of the protein was determined as described above.

Other techniques for protein determination

The concentration of protein solutions was determined by the bicinchoninic acid (BCA) method. The concentrations of the purified rubber-cleaving enzymes were determined from the molar extinction coefficients of Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$: Lcp_{Rr}, $\varepsilon_{407} = 9.5 \times 10^4\ M^{-1}\ cm^{-1}$, Lcp_{K30}, $\varepsilon_{412} = 8.0 \times 10^4\ M^{-1}\ cm^{-1}$, and $RoxA_{Xsp}$, $\varepsilon_{406} = 2.06 \times 10^5$

M^{-1} cm^{-1}. Separation of proteins was performed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) under reducing (2-mercaptoethanol) conditions. The metal content of the purified Lcp protein was determined using inductively coupled plasma-MS (ICP-MS). Fuchsin staining of polyisoprene degradation products was performed, by addition of a 1% Fuchsin solution (0.5 g Fuchsin, 12.5 ml acetic acid, 2.5 g $Na_2S_2O_3$, 0.2 ml HCl (37%) and 37.5 ml H_2O) to the Lcp assay mixture. Staining of the cells for Poly-3-hydroxybutyrate [P(3HB)] and polyphosphate was performed as described previously (Yikmis and Steinbüchel, 2012a).

Results and discussions

Screening, isolation and identification of rubber-degrading bacteria in consortium

Rubber-degrading bacteria strain RPK1 was isolated from the rubber degrading consortium, which substantially disintegrated pieces of rubber gloves when incubated together in MSM. A liquid culture of inoculum flask was taken as a series period (0, 6, 12, 18, 24, 30 days of incubation) for bacterial cultivation on a rubber latex agar plate. Several bacterial strains were presented from this enrichment medium especially, a yellow colony which was presented at all interval periods as shown in Fig. 1. The colony presented throughout the experiment was repeated purified by streaking on rubber latex agar plates. Each isolate was subsequently tested for rubber-degrading ability in liquid MSM with rubber pieces as a carbon source. One isolate, designated as isolate RPK1, was found in all of the incubation periods and acted like a dominant strain showed a high rubber-degrading activity in liquid culture as observed by a strong disintegration of the rubber pieces within four weeks.

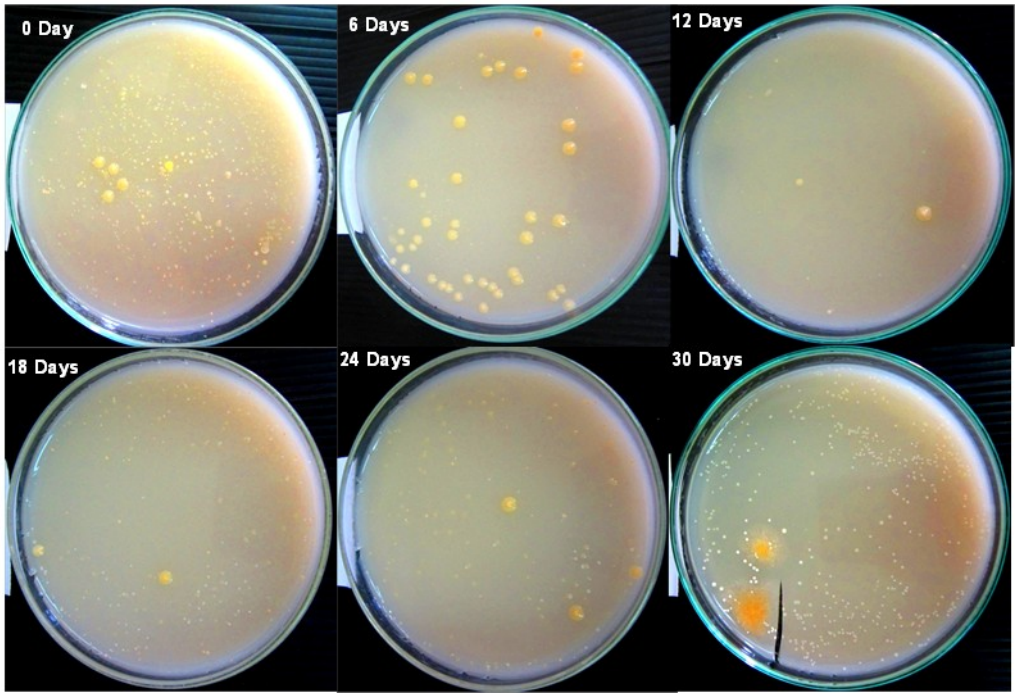

Fig. 1 The cultivation of rubber-degrading bacteria from rubber degrading bacterial consortium on rubber latex agar plate at different times.

Isolate RPK1 did not form clearing zones on an opaque polyisoprene latex mineral salts agar, whereas known clear zone formers bacteria such as *Xanthomonas* sp. 35Y (Tsuchii et al., 1990), or the *Streptomyces coelicolor* strain 1A (Jendrossek et al., 1997) formed relatively large clearing zones. Isolate RPK1 has been interested because of the development of the remarkable intense orange to red color of their colonies on NB agar after prolonged incubation (Fig. 2A).

Microscopic examination of the cells showed the presence of non-motile cells. Depending on the growth phase, the cells were coccoid (cells from late stationary phase), rod-shaped (cells from early and late log phase) or long rods (up to 1 x 5 µm), partially branched and star-like in exponentially growing cultures (Fig. 2B, 2C, 2D, 2E). Isolate RPK1 was catalase positive and was stained positive by the Gram-staining procedure. It grew well at 43°C (but not over 45°C) and also tolerated in the presence of 3% NaCl (in NB). Strain RPK1 was able to accumulate Nile red stainable storage compounds such as polyhydroxyalkanoates (PHAs) or triacylglycerols and to synthesize polyphosphate granules. RPK1 utilized complex media (NB, LB medium) and grew on MSM with D-mannitol, fructose, acetate, benzoate or octane as a single carbon source. Glucose, sucrose, gluconate, pentane, petroleum or pyridine could not be utilized for growth. Polymers such as polyhydroxybutyrate (PHB), casein or starch were also not utilized by RPK1. These characteristics, in combination with the red color of the colonies, and the variable morphology of the cells, suggested that isolate RPK1 could be a member of the genus Rhodococcus.

To verify this assumption, the DNA-sequence of the PCR-amplified 16s rDNA (accession No. KU140418) was determined. The 16S rDNA was 99.7% and 99.2% identical to *Rhodococcus* MK3027 and *R. rhodochrous* MTCC11081, respectively. According to the biochemical and morphological data, the isolate RPK1 was classified in a member of the species *R. rhodochrous*. It differed from rubber degrading *Xanthomonas* sp. 35Y (Tsuchii et al., 1990, Braaz et al., 2004), *Streptomyces* sp. K30 (Rose et al., 2005), and other rubber degrading streptomycetes (Jendrossek et al., 1997) by the inability to produce clearing zones on opaque polyisoprene latex agar. Previously, bacteria with strong rubber-degrading activity without a clearing zone formation were isolated and identified as *Gordonia polyisoprenivorans* or *Gordonia westfalica* (Bröker et al., 2008).

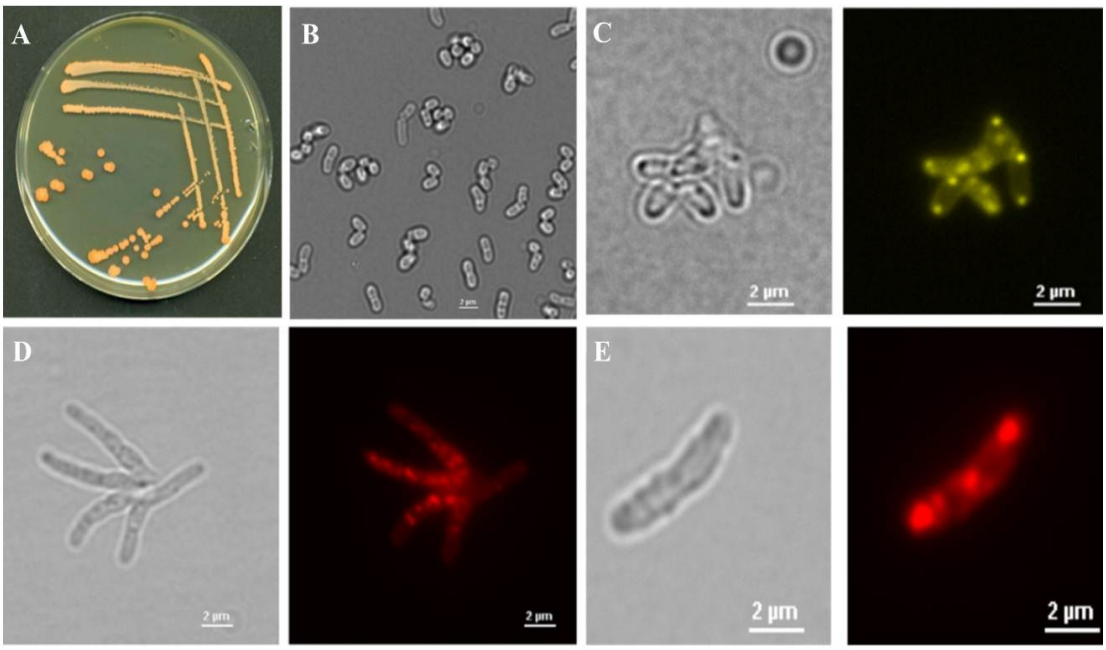

Fig. 2 Features of *R. rhodochrous* RPK1. (A) Formation of red-coloured colonies of *R. rhodochrous* RPK1 when grown NB agar; (B) morphology of stationary of *R. rhodochrous* RPK1 cells in bright field microscopy; (C) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with DAPI using DAPI-polyphosphate-specific emission filters); (D) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with Nile red); (E) *R. rhodochrous* RPK1 cells when grown on NB medium supplemented with acetate (bright field and fluorescent image staining with Nile red).

Identification of the gene for the latex clearing protein in *R. rhodochrous* RPK1

Reccently, the tested rubber degrading Actinobacteria have been at least one gene coding for a so-called latex clearing protein (Lcp) that is supposed to be responsible for the initial oxidative attack of the polyisoprene carbon backbone (Rose et al., 2005; Hiessl et al., 2012; Yikmis et al., 2012; Nanthini, 2015). Remarkably, non-latex-clearing zone formers such as *G. polyisoprenivorans* also have *lcp* genes (Broker et al., 2008). Alignment of the amino acid sequences of Lcp proteins of the different species revealed the conserved regions within the Lcp amino acid sequence (Hiessl et al., 2014). Moreover, the *lcp* genes in the genomes of *Rhodococcus* strain MTCC11081, *Rhodococcus* sp. MK3027 and *Rhodococcus* sp. ARG-BN062 were identified hypothetically by a screening

of the published genome sequences for the presence of *lcp* sequences. The deduced amino acid sequence of these hypothetical Lcp proteins is including the DUF2236 domain, containing the central part of most, but not all Lcp proteins (Hiessl et al., 2014). Two oligonucleotides based on the upstream and downstream region of the *lcp* genes of these *Rhodococcus* strains were generated (Lcp_{Rr}-PstI_for and Lcp_{Rr}-HindIII_rev), and a PCR reaction was performed with chromosomal DNA of *R. rhodochrous* strain RPK1. A 1.5 Kbp DNA fragment was obtained and its DNA sequence was determined (accession number KU140417). Analysis of the DNA sequence revealed one large open reading frame of 1227 bp coding for a peptide of 408 amino acids (45.2 KDa). The deduced amino acid sequence showed strong similarities to postulated Lcp proteins of *R. rhodochrous* and of several *Rhodococcus* sp. strains (81 to 99 % identical amino acids). A high degree of similarities was also detected to many other putative Lcp proteins of genome-sequences bacteria, including that of many Streptomycetes and other Actinobacteria. The amino acid sequence of Lcp_{Rr} was compared with that of the only two other biochemcially characterized Lcp proteins, a 70% (76%) identity (similarity) and a 57% (66%) identity (similarity) were determined to Lcp_{VH2} of *G. polyisoprenivorans* (Bröker et al., 2008) and to Lcp_{K30} of *Streptomyces* sp. K30 (Rose et al., 2005), respectively (Fig. 3). A 30 amino acid long sequence of the N-terminus of Lcp_{Rr} was predicted to code for a signal peptide enabling secretion of the protein. The molecular mass of the predicted mature protein was 42.2 KDa.

		Identity [%]		
		Lcp_{Rr}	**Lcp_{K30}**	**Lcp_{VH2}**
Similarity [%]	**Lcp_{Rr}**	100	57	70
	Lcp_{K30}	66	100	52
	Lcp_{VH2}	76	60	100

Fig. 3 An identity and similarity values of the purified and biochemically characterized Lcp proteins

Expression and purification of Lcp_{Rr}

The DNA sequence coding for the Lcp_{Rr} signal peptide was replaced by a Strep-tag coding sequence and the modified gene was cloned under control of an L-rhamnose-dependent promoter into p4782.1 vector and subsequently transformed to *E. coli* JM109. The Lcp_{Rr} protein was purified by two subsequent chromatographic steps from soluble cell extracts that had been prepared from the combined cells of a 5-L of *E. coli* (p4782.1::lcp_{Rr}) culture after grown in a LB medium supplemented with 0.1% L-rhamnose at 22°C for 20 h. A yield of approximately 7.7 mg purified Lcp_{Rr} protein (5.3 mg/mL in BCA assay) was obtained after concentration of the Superdex 200 eluate through ultrafiltration. The Lcp proteins of S*treptomyces* sp. K30 (Lcp_{K30}) and rubber oxygenase RoxA of *Xanthomonas* sp. strain 35Y ($RoxA_{Xsp}$) were also purified and were then used for comparison purposes. All purified proteins were separated by SDS-PAGE and checked for the purity. As shown in Fig. 4, Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$ were almost homogenous (≥ 97%). Lcp_{Rr}, similar to Lcp_{K30}, migrated at slightly higher apparent molecular masses (50 and 47 kDa) as deduced from the gene sequences (43 and 42 kDa, respectively). Most remarkably, concentrated Lcp_{Rr} had a brownish color. This was in sharp contrast to the red color of concentrated solutions of Lcp_{K30} or of $RoxA_{Xsp}$ and suggested substantial differences of the Lcp_{Rr} in comparison to the other rubber-cleaving proteins.

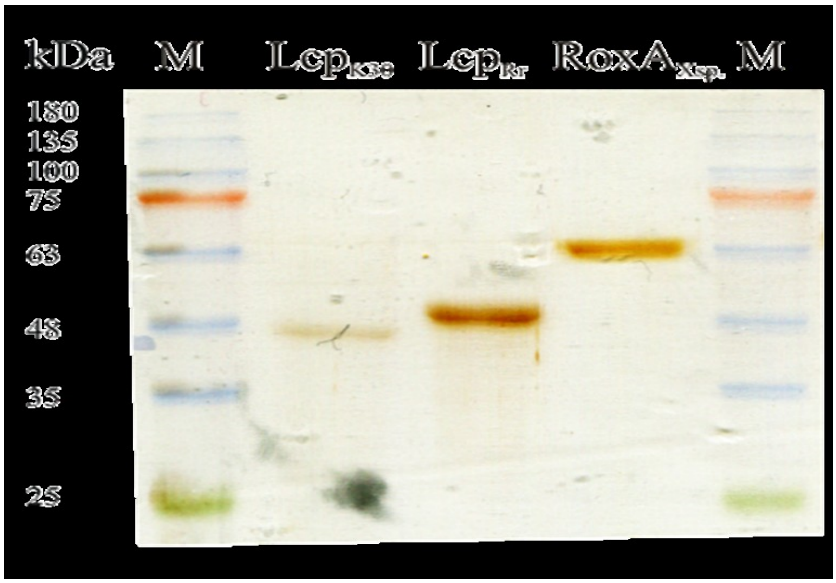

Fig 4 SDS-PAGE of the purified Lcp_{Rr}, Lcp_{K30} and RoxA. Purified Lcp_{Rr}, Lcp_{K30} and $RoxA_{Xsp}$ proteins were separated by reduction of SDS-PAGE and subsequently staining with silver. KDa values of marker proteins are indicated.

Biochemical properties of the Lcp_{Rr}

The purified Lcp_{Rr} protein was investigated for rubber cleaving activity using the oxygen consumption and the HPLC-based rubber cleavage product assay. The oxygen consumption assay confirmed that the Lcp_{Rr} cleaved poly(*cis*-1,4-isoprene) latex in an oxygen-dependent manner; specific activities of 0.9 U/mg and of 3.1 U/mg were determined for Lcp_{Rr} at pH 8 and at 23°C and 30°C, respectively. Variable data were determined for the specific activity of Lcp_{Rr} at 37°C, possibly because of a decreasing stability of the Lcp_{Rr} protein at higher temperatures. The HPLC (Fig. 5A) and Fuchsin assay (Fig. 5B) revealed that Lcp_{Rr} produced the same mixture of polyisoprene cleavage products (C_{20} and higher oligo-isoprenoids with terminal keto and aldehyde groups), that had been determined for Lcp_{K30}. 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD), was only detectable in trace amounts for Lcp_{Rr} or for Lcp_{K30} but was the main product of the $RoxA_{Xsp}$-derived rubber cleavage products. Determination of the activities of purified Lcp_{Rr} at different pH values using the HPLC-based product assay, showed a pH optimum around pH 8 (Fig. 7), which was about one pH unit higher than the pH optimum, that had been previously determined for $RoxA_{Xsp}$ or for Lcp_{K30} and Lcp_{VH2} (Braaz et al., 2004; Hiessl et al., 2014; Birke and Jendrossek, 2014). The stability of both the Lcp preparations decreased upon incubation in buffer at 37°C (Fig. 6B). In accordance with these results, the concentration of rubber degrading products in an *in vitro* latex cleavage assay with Lcp_{Rr} or Lcp_{K30} increased for only 4-8 hours (Fig. 6A). $RoxA_{Xsp}$, on the other hand, was much more stable and continuously produced ODTD molecules for up to 70 h (Schmitt et al., 2010).

Lcp_{Rr} is a *b*–type cytochrome and remarkable difference from LcpK30

The purified Lcp_{Rr} had a brownish colour whereas the Lcp_{K30} was red. Lcp_{Rr} (and Lcp_{K30}) as isolated, both proteins showed similar absorption maxima at 407 (412) nm and at 535 (544) nm that are typical for heme containing proteins in the oxidised state. However, the purified Lcp_{Rr} had an additional broad absorption maximum around 645 nm. The absorption band at 645 nm was absent in Lcp_{K30} and in other biochemically characterized RoxA proteins such as $RoxA_{Xsp}$ and $RoxA_{Cco}$ (Birke et al., 2013). When the Lcp preparations were chemically reduced by the addition of sodium dithionite, the absorption bands at 407 (412) nm and 535 (544) nm were shifted to 428 (430) nm and 560

(562) nm. A comparison of the reduced spectra of both Lcp proteins showed differences in the Q-bands (500 – 600 nm). Apparently, Lcp_{Rr} is far less pronounced in this region than Lcp_{K30}. Nevertheless, these data corresponded to the Soret and Q-bands that are typical for heme-containing proteins and strongly indicated that Lcp_{Rr} is a heme-containing protein. The band around 645 nm, however, was not changed by the addition of dithionite. To confirm that Lcp_{Rr} is a heme-containing protein and to determine its heme type, a metal analysis and a spectral analysis by the heme-bipyridyl assay were performed. 6.5 µg Fe/mL Lcp_{Rr} protein solution (5.3 mg protein/mL) were determined. This corresponded almost perfectly with a stoichiometry of one atom Fe per one Lcp_{Rr} molecule. It was of interest that low amounts of copper (2.8 µg/mL) were also identified and corresponded to 0.36 atoms Cu per one Lcp_{Rr} molecule. Zinc was detected at the detection limit (0.1 µg/mL) and Nickel was below the detection limit (<0.1 µg/mL); other metals (vanadium to zinc tested) were also not detected in significant amounts. Divalent cations such as magnesium or calcium were not present (below the detection limit of 0.1 µg/mL) (Watcharakul et al., 2016). An absorption maximum of 556 nm was determined using the bipyridyl assay for Lcp_{Rr} and for haemoglobin that was used as a *b*-type cytochrome control protein. These results indicated the presence of a *b*-type heme in Lcp_{Rr}. In contrast to the covalently linked *c*-type cytochromes, the heme groups of the *b*-type cytochromes are not covalently linked to the peptide chain and can be therefore extracted by an acid solvent extraction (Morrison and Horie, 1965). Acid solvent extraction of the purified Lcp_{Rr} yielded a coloured supernatant and a non-coloured precipitate. In contrast, solvent extraction of the *c*-type cytochromes such as $RoxA_{Xsp}$ or of other commercially available cytochrome *c* enzymes yielded a non-coloured supernatant and a red precipitate which is corresponding with the covalent attachment of porphyrin to the polypeptide. MALDI-ToF analysis of the purified Lcp_{Rr} resulted in the identification of ions with *m/z* values of 616 (data not shown) which is typical for heme *b* (Yang et al., 2013). All these results indicated that, Lcp_{Rr} is a *b*-type cytochrome similar to Lcp_{K30} (Birke et al., 2015). In addition, MALDI-ToF analysis of Lcp_{Rr} also revealed an ion species with *m/z* values of 619 besides that of 616 which could correspond to a verdo-heme (Andreoletti et al., 2009). As the activity of the purified Lcp_{Rr} rapidly and substantially decreased during storage, the heme species with *m/z* value of 619 could represent a heme degradation product of the inactivated Lcp_{Rr} (Watcharakul et al., 2016).

Lcp_{Rr} is accessible for external ligands

Previous studies on the rubber oxygenase, RoxA had revealed that the active heme site in RoxA had only one axial amino acid ligand. The other axial ligand was a dioxygen molecule that was stably bound to heme in a Fe^{3+}——O_2− transition state. The oxygen molecule in RoxA could be partially removed by the addition of imidazole, thereby moving the negative charge from the oxygen molecule to the iron atom (Fe^{2+}). This charge transfer resulted in a small visible change of the UVvis spectrum as observed by an increase of the absorption of the Q-bands at 549 nm (Birke and Jendrossek, 2014; Hiessl et al., 2014; Schmitt et al., 2010). When imidazole was added to the dithionite-reduced RoxA, substantial increases in the Soret and Q-bands were determined compared to the reduction of RoxA bands without imidazole (Birke et al., 2015; Schmitt, 2012). The increase in absorption was interpreted as the result of the binding of the imidazole molecule to the (now) free sixth (axial) coordination site of the heme iron. An analog experiment with purified Lcp_{K30} was performed and showed that there was a significant difference between both Lcp proteins: addition of imidazole to the dithionite-reduced Lcp_{K30} had no effect on the UVvis spectrum and there was no detectable increase of the Q-bands. This indicated that the 6^{th} coordination site of the heme apparently was not accessible for imidazole and the Lcp_{K30} protein was present in a "closed state". Binding of the substrate (polyisoprene) would therefore require a conformational change of the Lcp_{K30} structure. In contrast, addition of imidazole to the dithionite-reduced Lcp_{Rr} protein resulted in a substantial increase of the Soret- and Q bands and this can be explained by the binding of imidazole to the reduced heme. Similar results were obtained when both the Lcp proteins were treated with mercaptoethanol: no change of the UVvis spectrum was determined for Lcp_{K30} while the prominent changes were detected for the Lcp_{Rr} protein. In conclusion, the Lcp_{Rr} seems to rest in a different conformation in their as isolated states. Whereas the Lcp_{K30} was in a six-fold coordinated "closed" state, the heme group of Lcp_{Rr} was readily accessible to external ligands and substrates, and this indicated a five-fold coordinated "open" state. Further evidences can be found in the UVvis spectra of five-fold coordinated myoglobin in the oxidised (met myoglobin) and reduced (desoxy-myoglobin) state. The UVvis spectra of the latter proteins showed similarities to the corresponding spectra of Lcp_{Rr}, particularly in the region of the less pronounced Q-bands of the reduced Lcp_{Rr} compared to Lcp_{K30} as well as in the 645 nm region in the oxidised state (Schenkman et al., 1997). The presence of the 645 nm absorption band in Lcp_{Rr} might be also explained

by a charge transfer phenomenon of a charged residue/ion in close neighbourhood to the heme group in Lcp_{Rr} and in its absence in Lcp_{K30} (Pond et al., 1999). Unfortunately, only the RoxA structure (Seidel et al., 2013) but no Lcp structure was available to obtain direct support for our assumption.

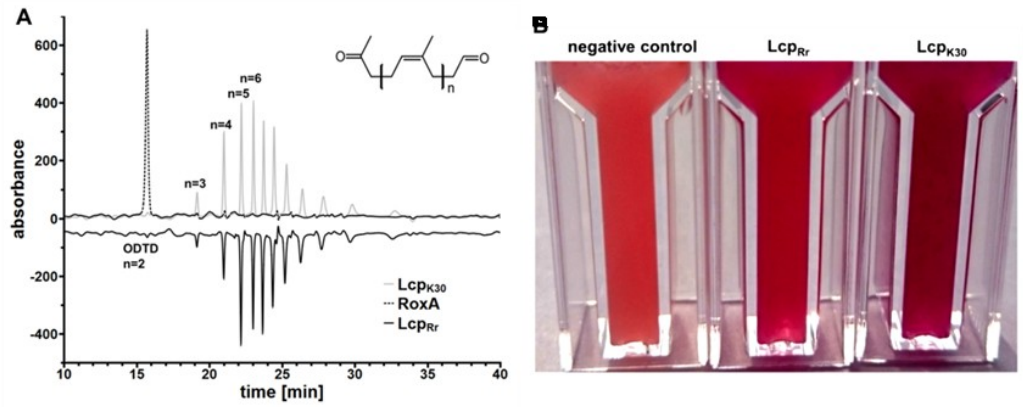

Fig. 5 Activity assays of $Lcp_{Rr.}$ (A) HPLC-product assay. Purified Lcp_{Rr} after size exclusion chromatography was used in the assays. Graphs for purified Lcp_{K30} and purified $RoxA_{Xsp}$ are given for comparison. (B) Detection of aldehyde products of Lcp-degraded polyisoprene by Fuchsin assay. Polyisoprene latex in KP buffer, pH 8 was incubated with 4 µg/mL of the purified Lcp_{Rr}, 4 µg/mL of the purified Lcp_{K30} or without enzyme (control) incubated at 30°C for 1 h. Development of pink color after addition of Fuchsin solution confirmed the formation of carbonyl groups in degradation products by Lcp_{Rr} and Lcp_{K30}

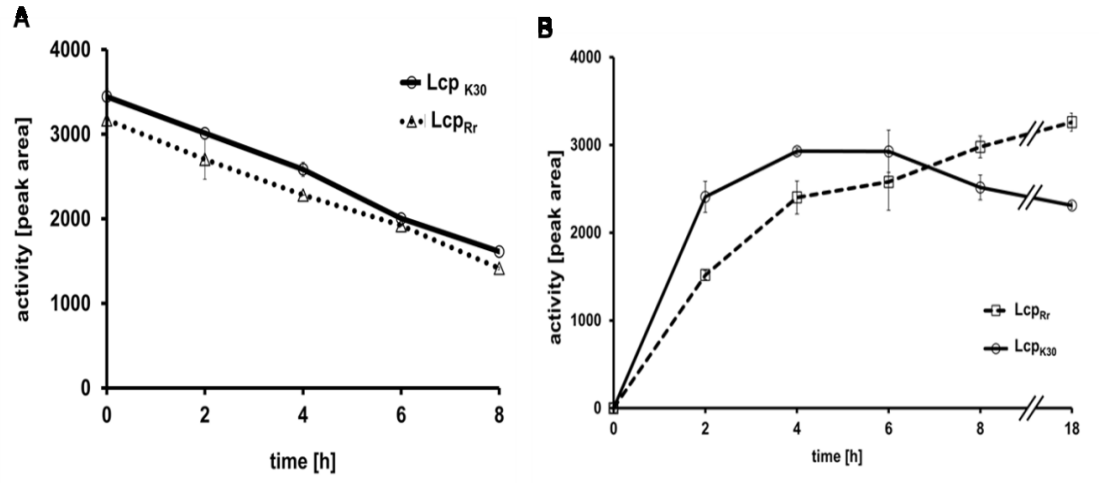

Fig. 6 Stability of Lcp_{K30} and Lcp_{Rr} and product formation. Lcp proteins were incubated in the presence of polyisoprene latex for 0 to 8 h at room temperature and the amount of the products was determined by HPLC (A). Lcp proteins were incubated at 37°C for up to 18 h before the standard activity assay was performed (B).

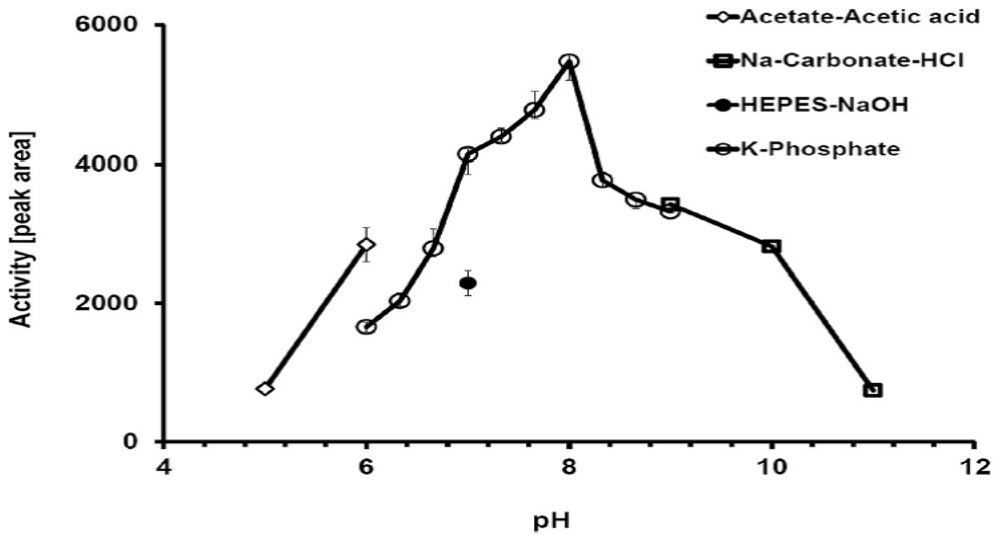

Fig. 7 pH optimum of the Lcp_{Rr}. The pH optimum was determined using the HPLC-based product assay in a pH range of 5 to 11 using acetate buffer (pH 5 - pH 6, diamonds), phosphate buffer (pH 6 - pH 9, open circles), carbonate buffer (pH 9 – pH 11, squares), or HEPES (pH 7, closed circle). Assays were performed with two biological and two technical replicates. Error bars indicate standard deviation

Lcp_{Rr} is insensitive to most chelating inhibitors.

Metal-dependent proteins are often inhibited by chelating compounds. Therefore, a variety of known chelator compounds were tested for an effect on the activity of Lcp_{Rr} using the HPLC-based activity assay. EDTA, tiron, or phenanthroline had no significant effect on its activity (Fig. 8). Ethyl xanthogenate partially inhibited Lcp_{Rr} by about 40% similar to $Lcp_{K30,}$ but different from Lcp purified from *G. polyisoprenivorans* (Lcp_{VH2}) that completely inhibited Lcp_{VH2} at 2 mM xanthogenate (Hiessl et al., 2014). The only compound that had a strong effect on the activity of the Lcp_{Rr} was the metal chelator diethyl dithiocarbamate (82% inhibition, Fig. 8). However, as in the case of diethyl dithiocarbamate had no effect on the UVvis spectrum of Lcp_{Rr} and this excludes a direct effect of the inhibitor to the heme site Carbon monoxide, which completely inactivated by RoxA, had no effect on the UVvis spectrum of Lcp_{Rr} as isolated and this finding was in agreement with the presence of an oxidized (Fe^{3+}) heme center. Carbon monoxide had no inhibitory effect on polyisoprene cleavage during the HPLC-based product assay when sufficient oxygen was also present in the assay mixture. However, when Lcp_{Rr} was incubated in a carbon monoxide-saturated and oxygen-free buffer before added to an oxygenated polyisoprene latex assay solution, a lag phase of Lcp_{Rr} dependent oxygen consumption was observed in the oxygen consumption assay. However, the polyisoprene-cleaving activity recovered within 20 to 30 min during incubation and exposure of the assay solution to air. The same result was obtained when Lcp_{K30} was exposed to carbon monoxide. Addition of carbon monoxide to dithionite-reduced Lcp_{Rr} or Lcp_{K30} had (minor) visible effects on the UVvis spectra as revealed by a small increase of the α-band of Lcp_{Rr} and Lcp_{K30} upon addition of carbon monoxide. The effect of carbon monoxide on the UVvis spectrum of Lcp was reversible by addition of a dioxygen atmosphere and indicated that the binding of carbon monoxide to the chemically reduced heme group in Lcp was reversible. This is in contrast to $RoxA_{Xsp}$, which binds carbon monoxide irreversibly and completely inhibits the activity of RoxA without recovery. An apparent consequence of our data is that the hemes of Lcp proteins undergo a Fe^{3+} to Fe^{2+} reduction and the reduced Lcp proteins presumably are sensitive to carbon monoxide.

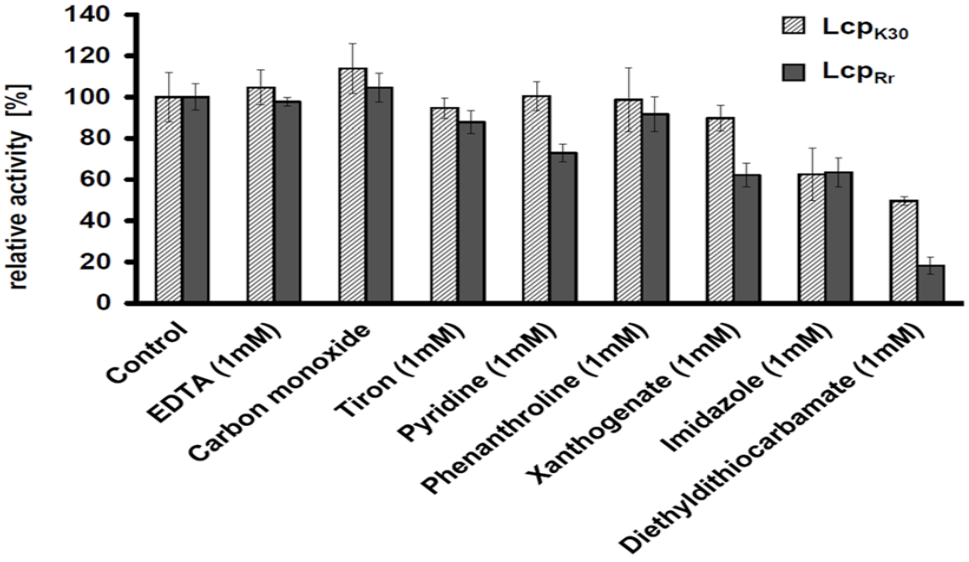

Fig. 8 Inhibition of Lcp_{Rr} by potential inhibitors

lcp genes are frequently present in the genomes of Actinobacteria (Yikmis et al., 2012a) and many rubber degrading species have been described in this group (Jendrossek et al., 1997; Linos et al., 2000a; Linos et al., 2002; Arenskötter et al., 2001; Imai et al., 2011; Nanthini et al., 2015). Most of the rubber degrading Actinomycetes such as *Streptomyces* sp. K30 (Rose et al., 2005), *Streptomyces coelicolor* 1A, and many others are able to perform clearing zones on opaque polyisoprene latex agar. However, several of the most potent rubber degraders do not produce clearing zones and apparently need close contact to the degraded rubber material. Two well known rubber degrading *Gordonia* species e.g. *G. polyisoprenivorans* and *G. westfalica* and also the strain used in this study e.g. *R. rhodochrous* RPK1 belong to this group of non-clearing zone formers. One might speculate that Lcp proteins of non-clearing zone formers constitute a group having an open conformation with free access to the active site and that the other Lcp proteins that confer clearing zone formation ability to the Lcp-producing strains have a closed form. Prototype of the first group would be Lcp_{Rr} while the prototype of the latter would be Lcp_{K30}. It will be necessary to biochemically investigate more Lcp proteins and to elucidate the structure of Lcp proteins as well as to find more evidences for or against this hypothesis.

Conclusions

This study extends the list of rubber-degrading non-clearing zone formers by latex clearing proteins (Lcp) to the genus Rhodococcus (besides Gordonia). Isolation and characterization of the Lcp protein of *R. rhodochrous* RPK1 in this study shows that the current studies, Lcp proteins differ in some spectroscopic features and/or in spatial arrangements of their metal ions/cofactors and indicate the presence of two or even more subgroups of Lcp proteins. It will be necessary to study more Lcp proteins to reveal the complete variability of rubber degrading enzymes present in rubber-degrading organisms.

Suggestions for future research

1. This research is the first identification of the chemical and biophysical characteristics of the purified Lcp protein of the *Rhodococcus rhodochrous* strain RPK1. It will be necessary to biochemically investigate more Lcp proteins and to elucidate the structure of the Lcp proteins to find more evidences for or against this hypothesis.

2. This research is only relevant with regard to Lcp from the *Rhodococcus rhodochrous* strain RPK1. The representative of key bacteria in this rubber degradation process was investigated its biochemical properties and was solved some structure to understand of the degradation mechanisms. However, there has been insufficient research about the rubber-degrading enzymes of other microorganisms. It is also necessary to investigate more Lcp proteins from others strains, in order to reveal the variability of rubber degrading enzymes present in rubber-degrading organisms. Further research should include an optimization of the suitable conditions to enhance the activity of enzymes, so that maximum rubber degradation can be achieved.

3. Biotechnological processes can be designed to benefit from microbial degradation of rubber to produce useful products, rather than using chemical and physical processes. This might include using genetic engineering to develop more efficient rubber degrading strains. However, biotechnological specialists must consider a variety of different methods and designs.

The number of biodegradation processes available for rubber waste is still very limited, and microbial recycling is probably the most investigated option, although it is not yet in commercial use. However, the biotechnological method is the most promising solution to the future increases in rubber waste.

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