Characterization of the latex clearing protein of the poly(cis-1,4-isoprene) and poly(trans-1,4-isoprene) degrading bacterium *Nocardia nova* SH22a

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Summary

*Nocardia nova* SH22a is an actinobacterium capable of degrading the polyisoprenes poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene). Sequencing and annotating the genome of this strain led to the identification of a single gene coding for the key enzyme for the degradation of rubber: the latex clearing protein (Lcp). In this study, we showed that Lcp<sub>SH22a</sub> – contrary to other already characterized rubber cleaving enzymes – is responsible for the initial cleavage of both polyisoprene isomers. For this purpose, *lcp<sub>SH22a</sub>* was heterologously expressed in an *Escherichia coli* strain and purified with a functional His<sub>6</sub>- or Strep-tag. Applying liquid chromatography electrospray ionization time-of-flight mass spectrometry (LC/ESI-ToF-MS) and a spectrophotometric pyridine hemochrome assay, heme *b* was identified as a cofactor. Furthermore, heme-associated iron was identified using total reflection X-ray fluorescence (TXRF) analysis and inhibition tests. The enzyme's temperature and pH optima at 30°C and 7, respectively, were determined using an oxygen consumption assay. Cleavage of poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene) by the oxygenase was confirmed via detection of carbonyl functional groups containing cleavage products, using Schiff’s reagent and electrospray ionization mass spectrometry (ESI-MS).

Keywords: gutta-percha; microbial rubber degradation; *Nocardia nova* SH22a; poly(*cis*-1,4-isoprene) rubber; poly(*trans*-1,4-isoprene) rubber
Introduction

Polyisoprenes were used to craft various items, such as rubber balls, since 1600 B.C. A study dealing with the ancient Mesoamerican processing of the polymer even describes the vulcanization with archaic methods (Hosler et al. 1999). When communication technology advanced centuries later, attention was drawn to vulcanized polyisoprenes again as they showed the ability to insulate wires. At this time, gutta-percha, containing the trans-isomer of polyisoprene, was even more important than the cis-isomer (Tully 2009). This can be attributed to the fact that gutta-percha is almost biologically inert.

Nowadays, gutta-percha has been widely replaced by synthetic materials. In contrast to that, the extensive use of mostly poly(cis-1,4-isoprene) rubber materials has led to the accumulation of rubber waste material and the investigation of the bacterial degradation of the polymer. For studies on the bacterial degradation of poly(cis-1,4-isoprene), numerous strains were isolated due to their capability to degrade the polymer (reviewed in Yikmis and Steinbüchel 2012). These studies often aimed at the development of biotechnical recycling methods for polyisoprenes. During this time, the question arose as to the existence of microorganisms capable of also degrading poly(trans-1,4-isoprene). Warneke et al. (2007) succeeded in isolating bacteria which were able to degrade the trans-isomer of polyisoprene. The isolation of six poly(trans-1,4-isoprene) degrading strains belonging to the genus Nocardia (Nocardia nova SH22a, Nocardia nova SEI2b, Nocardia nova SEII5a, Nocardia nova L1b, Nocardia takedensis WE30 and Nocardia jiangxiensis SM1) showed that poly(trans-1,4-isoprene) is not biologically inert (Warneke et al. 2007). In contrast to the degradation of poly(cis-1,4-isoprene), the bacterial degradation of poly(trans-1,4-isoprene) is, however, a very slow process taking approximately one month to decompose 0.2 % (w/v) of material at 30°C in flask scale experiments.
Interestingly, all strains showing the ability to degrade the trans-isomer were also able to efficiently degrade poly(cis-1,4-isoprene), but not vice versa. In order to identify putative genes for this observation, the genome of Nocardia nova SH22a was sequenced (Luo et al. 2014). Together with the results of a previous study (Luo et al. 2013) that inter alia covers the establishment of a transformation protocol for strain SH22a and led to the isolation of transposon mutants, a catabolic pathway for poly(trans-1,4-isoprene) was postulated (Luo et al. 2014). It was shown that the degradation pathways for both polymers resemble each other and share common steps. Furthermore, these studies revealed that the genome of N. nova SH22a harbors one lcp homologous gene probably coding for a latex clearing protein. It is similar to other Lcps from different actinomycetes (Birke et al. 2015, Hiessl et al. 2012, Watcharakul et al. 2016), which catalyze the first step in poly(cis-1,4-isoprene) degradation: the oxidative cleavage of the double-bond. Other genes coding for oxygenases that were previously also reported to be involved in rubber degradation like RoxA (Braaz et al. 2004), RoxB (Birke et al. 2017), and LatA (Kasai et al. 2017) were not identified in the genome of N. nova SH22a. Luo et al. (2014) assumed that the Lcp of N. nova SH22a is responsible for the cleavage of gutta-percha and natural rubber as well. In a recent study, the Lcp of Nocardia sp. NVL3 was investigated, but this study focused on the poly(cis-1,4-isoprene) cleavage by the enzyme (Linh et al. 2017). In this study, the ability of strain NVL3 to degrade gutta-percha was not reported.

The aim of the present study was the characterization of the latex clearing protein from N. nova SH22a. In addition, its involvement in the degradation of poly(cis-1,4-isoprene) and also in the degradation of poly(trans-1,4-isoprene) is revealed.

Materials & Methods
Bacterial strains, growth conditions, media. *Escherichia coli* strains Mach1™ T1 and C41 (DE3) were used for cloning and expression of *lcpSH22a*. Cells were grown in lysogeny broth medium (Bertani 1951). If the *E. coli* strain was transformed with either the pJET1.2::*lcpSH22a* or the pET23a(+)::*lcpSH22a* vector, 100 µg/ml ampicillin were added. For solid media, 1.8% (w/v) of agar was added. *E. coli* cells were incubated at 37°C and agitated at 150 rpm, if applicable.

Chemicals, oligonucleotides. Chemicals were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Oligonucleotides were acquired from Eurofins Genomics GmbH (Ebersberg, Germany). Synthetic poly(cis-1,4-isoprene) (CAS no. 104389-31-3) and synthetic poly(trans-1,4-isoprene) (CAS no. 104389-32-4) were cryomilled as described previously (Hiessl et al. 2012, Warneke et al. 2007). Latex as a source of natural rubber (CAS no. 9006-04-06) (Neotex Latz) was obtained from Weber & Schaer GmbH & Co. KG (Hamburg, Germany) and centrifuged at 10,000 × g for 10 min to remove ammonia. The solid top latex layer and the bottom ammonia layer were discarded. The liquid middle latex layer was diluted with 0.2 M BisTris buffer (pH 7) to a 20% (v/v) latex solution and applied for oxygen consumption assays as described before (Hiessl et al. 2014).

Purification of *LcpSH22a*. Genomic DNA of *N. nova* SH22a was isolated using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The oligonucleotides lcp_SH22a_fw (5’-AAAACATATGCACCACCACCACCAC GAACCGTGGACGTGGTCACCGTCC-3’) and lcp_SH22a_rev (5’-AAAAGGATCCTCACCGGTTGGTCTGCGGATTTC-3’) served as primers to amplify *lcpSH22a* from genomic DNA of *N. nova* SH22a using Phusion
Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). Thereby, NdeI and BamHI sites were generated, and the coding sequence for an N-terminal His6-tag was added. The His6-tag replaced the twin-arginine translocation signal sequence. The enzymatic cleavage of the twin-arginine translocation signal sequence between the amino acids 32 and 33 (…TARA-EPWT…) was predicted using the TatP software (Bendtsen et al. 2005). The PCR product was purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific) and cloned into pJET1.2/blunt according to the manufacturer’s instructions. Competent cells of E. coli Mach1™ T1 (Hanahan 1983) were transformed with pJET1.2::His6-lcpSH22a. The plasmid was purified by gel extraction and digested using restriction enzymes NdeI and BamHI (Thermo Fisher Scientific). Afterwards, the digested lcpSH22a with NdeI and BamHI restriction sites was purified using gel extraction and cloned into pET23a(+) (Thermo Fisher Scientific) previously digested by NdeI and BamHI. Competent E. coli C41 (DE3) cells were transformed with the resulting plasmid pET23a(+):His6-lcpSH22a. A pre-culture of E. coli C41 (DE3) pET23a(+):His6-lcpSH22a was grown overnight. The following day, main cultures of E. coli C41 (DE3) pET23a(+):His6-lcpSH22a were inoculated, induced with 0.4 µM isopropyl-β-D-thiogalactopyranoside (IPTG) at an OD600 of 0.4, and incubated for 18 h at 20°C. Cells were harvested by centrifugation at 3,345 × g for 15 min at 4°C and frozen at -20°C until further processing. A French press (Aminco, Silver Spring, MD, USA) was used to disrupt the cells, which were resuspended in buffer (40 mM imidazole, 100 mM Tris-HCl, 500 mM NaCl, pH 7). The insoluble fraction was separated from the soluble fraction by centrifugation for 45 min at 21,000 × g and 4°C. Immobilized metal affinity chromatography (IMAC) was performed employing an Äkta Prime Plus system (Amersham Biosciences, Amersham, England) connected to a HisTrap FF column (GE Healthcare Life Sciences, Uppsala, Sweden). The chromatography was performed with a flow rate of 1 ml/min. A HiPrep 26/10 Desalting column (GE Healthcare Life Sciences) was employed to exchange the imidazole containing elution buffer for 0.2 M Tris buffer (pH 7) using a flow rate of 5 ml/min. A VivaSpin 6
concentrator (Sartorius AG, Göttingen, Germany) with a 10 kDa molecular weight cutoff was used to concentrate His<sub>6</sub>-tagged L<sub>cpSH22a</sub>. The final protein concentration was determined by the Bradford assay (Bradford 1976). SDS-PAGE (Laemmli 1970) was done to control the purity of the enzyme.

The oligonucleotides lcpSH22a fw_STREP (5′-AAACATATG TGGAGCCACCCGCAGTTCGAAAAAGAAAACCTGTACTTCCAATCCGAAACCGTGAC GTGGTCACCG-3′) and lcp_SH22a rev (see above) served as primers to amplify the coding sequence for an N-terminally Strep-tagged L<sub>cpSH22a</sub>. NdeI and BamHI restriction sites were generated, and the twin-arginine translocation signal sequence was removed as well. A TEV-protease-recognition site was also introduced to remove the tag if necessary (this feature was ultimately not used in this study). Basically, cloning and biosynthesis of L<sub>cpSH22a</sub> with <i>E. coli</i> C41 (DE3) pET23a(+)::Strep-lcp<sub>SH22a</sub> were performed as described above. A French press (Aminco, Silver Spring, MD, USA) was used to disrupt the cells, which were resuspended in buffer W without ethylenediaminetetraacetic acid (EDTA) from the Strep-tag kit (IBA GmbH, Göttingen, Germany). Purification with the Strep-tag kit was done following the standard protocol. The Strep-tagged Lcp was used for TXRF analysis to exclude interference of metal ions with the His<sub>6</sub>-tag. Sequences of the plasmids were analyzed using the Mix2Seq kit from Eurofins Genomics GmbH (Ebersberg, Germany).

**Temperature and pH optima.** Enzyme activity was determined with an oxygen consumption assay as described elsewhere (Hiessl et al. 2014). In brief, 20 µl of a 20% (v/v) latex solution and 100 µg His<sub>6</sub>-tagged L<sub>cpSH22a</sub> were mixed ad 1000 µl with 0.2 M BisTris buffer (pH 7). The oxygen consumption was measured using a Dual Digital 20 oxygen electrode (Rank Brothers Ltd., Cambridge, England) under different conditions. To determine the temperature optimum of L<sub>cpSH22a</sub>, the incubation chamber of the oxygen electrode was connected to a
water bath to control the temperature, and the enzyme activity was determined in duplicate. Furthermore, the pH optimum was investigated in duplicate by performing the oxygen consumption assay in Britton-Robinson universal buffer (Britton and Robinson 1931) with varying pH values from 5 to 10. In order to calculate the relative activities, the highest enzyme activity was set to 100%.

**Inhibition studies.** For inhibition studies, Lcp$_{SH22a}$ [10 µg/ml] was incubated with 10 mM chelators (EDTA, disodium 4,5-dihydroxy-1,3-benzenedisulfonate, phenanthroline, 2,2’-bipyridyl) for 30 min on ice prior to the activity measurements with the oxygen consumption assay. Ethylxanthate was applied at a concentration of 2 mM. Chelators were solved in 0.2 M BisTris buffer (pH 7). The inhibitors were used to determine if the cofactor was Fe$^{2+}$ (phenanthroline, 2,2’-bipyridyl), Fe$^{3+}$ (disodium 4,5-dihydroxy-1,3-benzenedisulfonate), Cu$^{2+}$ (ethylxanthate), or another bivalent cation (EDTA).

**TXRF analysis.** Strep-tagged Lcp$_{SH22a}$ was analyzed with total reflection X-ray fluorescence (TXRF) to identify a potential metal cofactor using an S2 PICOFOX system (Bruker Nano GmbH, Berlin, Germany) using a low power X-ray tube with a molybdenum anode and an energy-dispersive, Peltier-cooled silicon drift detector XFlash (Bruker Nano GmbH). Therefore, 100 µl of Lcp$_{SH22a}$ solved in 0.2 M BisTris buffer (pH 7.0) were mixed with 100 µl of the internal arsenic standard [10 µg/ml] and dried by evaporation. For the analysis, 5 µl were placed on cleaned and contamination-free sample discs (quartz glass). The chosen excitation settings were 50 kV and 750 µA. Moreover, a signal integration over 1000 s was used for triplicate measurements of Lcp$_{SH22a}$ and BisTris buffer (control). Data evaluation was carried out using the software SPECTRA version 6.1.5.0 (Bruker Nano GmbH).
Absorption spectrum and pyridine hemochrome determination. The absorption of His$_6$-tagged Lcp$_{SH22a}$ was measured at wavelengths from 260 to 900 nm with the UV-2600 UV/Vis spectrophotometer (Shimadzu, Kyoto, Japan). The heme type was specified by analysis of the pyridine hemochrome spectra (Berry and Trumpower 1987).

LC/ESI-ToF-MS analysis. The liquid chromatography/electrospray ionization-time of flight-mass spectrometry (LC/ESI-ToF-MS) analysis of Lcp$_{SH22a}$ was performed to determine the heme type of Lcp$_{SH22a}$ as described by Oetermann et al. (2018). For this analysis, Lcp$_{SH22a}$ was solved at a concentration of 10 µM in 10 mM ammonium acetate. The same concentrations were used for the controls (Lcp$_{1VH2}$, myoglobin and cytochrome c).

Production and detection of rubber degradation products. As a larger amount of His$_6$-tagged Lcp$_{SH22a}$ was needed for the enzymatic production of oligo-isoprenoid molecules with carbonyl groups, the enzyme production process was scaled up and carried out in auto-induction medium as described elsewhere (Andler and Steinbüchel 2017). Nevertheless, protein purification was performed as described above (IMAC) to guarantee the use of a pure protein for in vitro experiments. Oligo-isoprenoid molecules were produced by incubating 30 ml Tris buffer (pH 7) and 0.5% (w/v) poly(cis-1,4-isoprene) or poly(trans-1,4-isoprene) with 15 µg/ml of His$_6$-Lcp$_{SH22a}$, which was added again every 24 h. Oligo-isoprenoid molecules were subsequently extracted by adding an equal volume of pentane and vigorously shaking the samples. Afterwards, the aqueous phase and the organic phase were separated by centrifugation at 3,345 × g for 10 min at 4°C. The organic phase containing extracted oligoisoprenoid molecules was transferred to a falcon tube and evaporated. Extraction was repeated
twice to maximize the yield of oligo-isoprenoid molecules. The extracted oligo-isoprenoid molecules with carbonyl groups were mixed with 2 mg Girard’s reagent T, 500 µl ethanol, and 40 µl 50% (v/v) formic acid (Ibrahim et al. 2006). After sonication for 10 min, the mixture was incubated at 65°C for 1 h. Samples were diluted 1:10 with methanol and triplicates were analyzed by ESI-MS with a Finnigan LXQ system (Thermo Fisher Scientific). In addition to the ESI-MS analysis, 1 ml of the supernatant of the *in vitro* experiments was used to detect oligoisoprene aldehydes with 25 µl of Schiff’s reagent.

**Results**

**Characterization of Lcp**<sub>SH22a</sub>. We purified His<sub>6</sub>-tagged Lcp<sub>SH22a</sub> and Strep-tagged Lcp<sub>SH22a</sub> to electrophoretic homogeneity for the characterization of the enzyme (Fig. 1). The temperature and pH optima of Lcp<sub>SH22a</sub> were determined. Lcp<sub>SH22a</sub> resembles other Lcps like Lcp<sub>VH2</sub> (Hiessl et al. 2014) and Lcp<sub>Rr</sub> (Watcharakul et al. 2016) possessing a temperature optimum at about 30°C (Fig. 2). In addition, the pH optimum of Lcp<sub>SH22a</sub> was found to be at 7. Under these conditions, the specific activity of Lcp<sub>SH22a</sub> was determined to be 0.03 µmol/min×mg.

The use of different chelating agents was with the purpose of inhibiting Lcp<sub>SH22a</sub> in order to gain information about the metal cofactor of the enzyme. A strong inhibition occurred with 2,2’-bipyridyl and phenanthroline as chelating agents, lowering the enzyme’s activity to 15% and 19% when compared with Lcp<sub>SH22a</sub> in the absence of chelating agents, respectively. An incubation with EDTA prior to the oxygen consumption assay also resulted in a reduced activity (25%). The effects of disodium 4,5-dihydroxy-1,3-benzenedisulfonate and ethylxanthalte were comparatively lower resulting in activities of 45% and 46%, respectively. Thus, ferrous iron was determined as a cofactor by these inhibition studies. The metal cofactor was also determined by TXRF analysis. In this analysis, iron (0.668 mg/l) was
detected as the major metal component. Only insignificant amounts of zinc (0.014 mg/l) were detected. The absorption spectrum was measured and revealed a characteristic peak at 412 nm (Fig. 3). As this peak was identified as the Soret band of heme-containing proteins in other Lcps (Birke et al. 2015, Oetermann et al. 2018, Watcharakul et al. 2016), assays to characterize a putative heme group of Lcp_{SH22a} were carried out. A pyridine assay confirmed the presence of heme b, which was evident due to an increase of the absorption at 556 nm (data not shown). Moreover, an LC/ESI-ToF-MS analysis was carried out to confirm the results of the above-mentioned experiments (Fig. 4). A non-covalently bound heme b with a molecular weight of 616.2 g/mol was detected for Lcp_{SH22a} as well as for Lcp1_{VH2} and myoglobin controls.

**Degradation of polyisoprenes.** Purified Lcp_{SH22a} was added to both structural isomers of polyisoprene in Tris buffer. The products obtained by the incubation of Lcp_{SH22a} with both polyisoprene isomers were measured with ESI-MS. Oligo-isoprenoid molecules with carbonyl groups were coupled to Girard’s reagent T and different molecular weights were detected. The molecular weights represent *cis*-oligo-isoprenoid molecules with a range of n = 4 (299 g/mol) to n = 37 (1423 g/mol), where n is equal to the number of isoprene units of the oligo-isoprenoid molecules (Fig. 5A). We also showed the *in vitro* formation of oligo(*trans*-1,4-isoprenes) with Lcp_{SH22a}. Interestingly, only products corresponding to oligo(*trans*-1,4-isoprene) with n = 2 (231 g/mol) and n = 4 (299 g/mol) were detected (Fig. 5B). Other peaks detected in the analysis were also detected in the negative controls, while peaks corresponding to oligo-isoprenoid molecules with carbonyl groups were not detected in the negative controls (incubation of polyisoprenes without enzyme, extraction with pentane and coupling with Girard’s reagent T). However, it was not possible to assign molecules to these peaks. Moreover, oligoisoprene aldehydes were detected in the supernatants of the *in vitro* assays for
the cleavage of both polymers using Schiff’s reagent, while no aldehydes were detected in the negative controls (no enzyme added to the polyisoprene).

Discussion

In this study, we characterized the Lcp of *N. nova* SH22a. This actinomycete is able to degrade not only poly(*cis*-1,4-isoprene), as occurring in natural rubber, but also poly(*trans*-1,4-isoprene), as occurring in gutta percha. Hence, Lcp_{SH22a} could be responsible for the cleavage of both polymers as was previously assumed by Luo et al. (2014). As stated above, purification protocols for Strep-tagged and His_{6}-tagged Lcp_{SH22a} were established, and the optimal catalysis conditions with respect to temperature and pH were determined. Under the conditions (30°C, pH 7), Lcp_{SH22a} had a specific activity of 0.03 μmol/min×mg for the *cis*-isomer. When compared with the specific activity of Lcp_{VH2}(1.3 μmol/min×mg, Hiessl et al. 2014), this activity is lower. However, as the growth rate of *N. nova* SH22a with poly(*cis*-1,4-isoprene) is also lower than the growth rate of *Gordonia polysoprenivorans* VH2, this observation is consistent with the organisms phenotypes.

Furthermore, the cofactor of the novel Lcp of *N. nova* SH22a was characterized in more detail in our experiments. Using chelating agents prior to the enzyme assay, we determined a strong inhibition by 2,2’-bipyridyl, phenanthroline and EDTA. In conclusion, Fe^{2+} is most likely to be the catalytically relevant form of Lcp_{SH22a} and responsible for the binding of oxygen. This was previously also reported for Lcp_{K30} by Ilcu et al. (2017) after analyzing the electron paramagnetic resonance of an Lcp from a *Streptomyces* strain. Moreover, the TXRF analysis showed that iron is the metal component of the heme. As the spectrum of Lcp_{SH22a} revealed a Soret band typical for heme proteins, the heme was analyzed by LC/ESI-ToF-MS analysis. From the LC/ESI-ToF-MS analysis, we learned that Lcp_{SH22a} has a heme b cofactor. This
cofactor was also reported for Lcps from *Streptomyces* sp. K30 (Birke et al. 2015), *Rhodococcus rhodochrous* RPK1 (Watcharakul et al. 2016) and *G. polyisoprenivorans* VH2 (Oetermann et al. 2018).

In order to elucidate the poly(*trans*-1,4-isoprene) degradation capabilities of Lcp$_{SH22a}$, the enzyme was used in our *in vitro* degradation experiments (for a reaction scheme, see Fig. 6). A broad spectrum of molecules corresponding to oligoisoprenes was detected when the ESI-MS analysis was applied to the degradation products of the *cis*-isomer. This demonstrates the endo-cleavage mechanism of the enzyme, which was also observed for Lcp of *Nocardia farcinica* S3 before (Ibrahim et al. 2006). However, when analyzing the degradation products of poly(*trans*-1,4-isoprene), only molecular weights corresponding to two oligoisoprenes were detected: 231.25 m/z correlates to $n = 2$; 299.00 m/z correlates to $n = 4$, while e.g. 291.17 m/z does not correlate to any oligo-isoprenoid, but was also detected in the negative control. The occurrence of oligoisoprene aldehydes was also confirmed for the *in vitro* degradation of poly(*trans*-1,4-isoprene) by staining with Schiff’s reagent. A cleavage of poly(*trans*-1,4-isoprene) by any other Lcp was not reported before. We found two probable explanations for these observations. The first explanation addresses the cleavage mechanism of Lcp$_{SH22a}$. It may be possible that the cleavage of poly(*trans*-1,4-isoprene) differs from the cleavage of poly(*cis*-1,4-isoprene). According to our analysis, Lcp$_{SH22a}$ cleaves both polymers. Nonetheless, the substrate specificity is probably different for the polymers, and Lcp$_{SH22a}$ may use a different cleavage mechanism to catalyze the oxidative cleavage of the *trans*-isomer. However, after predicting a crystal structure for Lcp$_{SH22a}$ using Swiss-Model (Biasini et al. 2014), almost no difference to the active site of the only crystalized Lcp of *Streptomyces* sp. K30 (Ilcu et al. 2017) was evident, and it has not been recorded that *Streptomyces* sp. K30 is able to degrade poly(*trans*-1,4-isoprene). Continuing the analysis of the crystal structure, the only difference between the amino acid residues, that are involved in the catalysis process
(actively or passively by stabilizing the heme ligand) of the Lcps, was the orientation of a lysine residue (Lys168 of LcpSH22a). This residue was shown to be involved in the heme association to LcpK30 (IICu et al. 2017). Nevertheless, this different orientation of the lysine residue was identified in various other Lcps (e.g. Lcp2VH2, Lcp of Streptomyces coelicolor A3(2)). According to the prediction of the heme association to these Lcps using SwissDock (Grosdidier et al. 2011), the heme position may slightly vary in some Lcps. In conclusion, the orientation of the lysine residue and the resulting differences in the active site are not responsible for the ability of Lcps to also cleave poly(trans-1,4-isoprene), because neither G. polysoprenivorans VH2 nor S. coelicolor A3(2) are able to cleave the trans isomer of the polymer. Furthermore, we can neither confirm nor deny whether the capability of LcpSH22a to cleave poly(trans-1,4-isoprene) can be attributed to the protein structure.

The second explanation for the detection of less oligo-isoprenoid molecules in the ESI-MS analysis, addresses the solubility of poly(trans-1,4-isoprene). In various publications, mostly dealing with the removal of gutta-percha from fillings in dentistry (Gördüysus et al. 1997, Magalhães et al. 2007, Wourms et al. 1990), a low solubility of the polymer is reported. Eventually, the degradation products have to be applied to the ESI-MS analysis in an aqueous solution. Because of the properties of poly(trans-1,4-isoprene), it is likely that only small oligo(trans-1,4-isoprenes) are soluble, and larger oligo(trans-1,4-isoprenes) are – although being produced – not transferred to the analysis. Nevertheless, oligo(trans-1,4-isoprenes) with n = 3 were not detected, which might be due to the catalysis mechanism of LcpSH22a that has yet to be revealed.

In the end, we cannot completely exclude that no other enzyme participates in the cleavage of both polymers, but lcpSH22a is definitely involved in the degradation of both polyisoprenes. Thus, LcpSH22a is unique among the so far characterized latex clearing proteins. However, the question remains whether this Lcp is the only reason why N. nova SH22a and other Nocardia
strains are able to degrade gutta-percha. After the initial cleavage of the polyisoprenes, oxidized oligoisoprenes are imported into the cells by mammalian cell entry (Mce) transporters. In the transposon mutant *N. nova* OC14-14-5, a specific transporter for the uptake of oligo(*trans*-1,4-isoprenes) was identified (Luo et al. 2014). This mutant was able to grow with poly(*cis*-1,4-isoprene) as a sole source of carbon and energy, but not with poly(*trans*-1,4-isoprene). The transposon inserted in one of fourteen gene clusters coding for an Mce transporter. So, this transporter is specific for the uptake of oligo(*trans*-1,4-isoprenes). Mce transporters were also shown to be involved in the uptake of oligoisoprenes in *G. polyisoprenivorans* VH2 (Hiessl et al. 2012). Therefore, Mce transporters are also important for the notable ability of *N. nova* SH22a to degrade gutta-percha.

In summary, the investigation of gutta-percha degradation remains challenging. Here, we report the characterization of a novel latex clearing protein, which is involved in the oxidative cleavage of poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene). Although the biochemical properties (optimal catalysis conditions, cofactor, heme type) of the oxygenase did not differ from other Lcps, we have demonstrated the new capability of Lcp$_{SH22a}$ to degrade poly(*trans*-1,4-isoprene) by the detection of oligo(*trans*-1,4-isoprenes) with ESI-MS and Schiff’s reagent.
350 **Acknowledgments**

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References


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Fig. 1. Images of SDS-polyacrylamide gels illustrating the purity of His$_6$-tagged Lcp$_{SH22a}$ and Strep-tagged Lcp$_{SH22a}$. (M) PageRuler prestained protein ladder (Thermo Fisher Scientific, Waltham, MA, USA), (1) 4 µg proteins from the elution fraction of the IMAC (A), (2) 4 µg proteins from the elution fraction of the Strep-tag affinity chromatography (B).

Fig. 2. Results of the determination of temperature and pH optima of His$_6$-Lcp$_{SH22a}$. For the determination of the temperature optimum (A), 20 µl of a 20% (v/v) latex solution and 100 µg His$_6$-tagged Lcp$_{SH22a}$ were mixed ad 1000 µl 0.2 M BisTris buffer (pH 7) at different temperatures. The pH optimum (B) of Lcp$_{SH22a}$ was investigated using Britton-Robinson buffer (Britton and Robinson 1931) instead of BisTris buffer. The oxygen consumption was measured, and the relative activities were calculated.

Fig. 3. Absorption spectrum of His$_6$-Lcp$_{SH22a}$. Local maxima typical for heme proteins were identified at 412 nm (Soret band) and 544 nm (Q band).

Fig. 4. Results of the LC/ESI-ToF-MS analysis. The molecular weight of non-covalently bound heme $b$ is indicated for Lcp$_{1VH2}$, Lcp$_{SH22a}$ and myoglobin. Heme $c$ of cytochrome $c$ was not detected as it is covalently bound to the enzyme and not separated from the enzyme during the procedure.
Fig. 5. Result of the ESI-MS analysis for oligoisoprene molecules obtained from the *in vitro* production with LcpSH22a. 30 ml of Tris buffer (pH 7) with 0.5% (w/v) polyisoprene particles (size 63 – 500 µm) were incubated at 30°C and agitated at 150 rpm for 10 d. 15 µg/ml of His6-tagged LcpSH22a were added every 24 h. Produced oligo(cis-1,4-isoprenoid) (A) and oligo(trans-1,4-isoprenoid) (B) molecules were extracted with pentane and coupled to Girard’s reagent T for the MS analysis. The intervals of 34 m/z correspond to the mass of one isoprene unit (68 g/mol) because of the coupling of two Girard’s reagent T-molecules to one oligoisoprene molecule.

Fig. 6. Reaction schemes of the polyisoprene degradation. For the *in vitro* degradation only the initial cleavage of the polyisoprene is performed. In order to show degradation, oligoisoprene aldehydes are coupled with Girard’s reagent T for the ESI-MS analysis.
Fig. 6

Latex clearing protein

Aldehyde dehydrogenase

Acyl-CoA ligase

β-oxidation

β-oxidation