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Functional Identification of Rubber Oxygenase (RoxA) in Soil and Marine Myxobacteria

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The rubber oxygenase (RoxA) of *Xanthomonas* sp. strain 35Y ($RoxA_{Xsp}$) is so far the only known extracellular *c*-type diheme cytochrome that is able to cleave poly(*cis*-1,4-isoprene). All other rubber-degrading bacteria described are Gram positive and employ a nonheme protein (latex-clearing protein [Lcp]) for the postulated primary attack of polyisoprene. Here, we identified RoxA orthologs in the genomes of *Haliangium ochraceum*, *Myxococcus fulvus*, *Corallococcus coralloides*, and *Chondromyces apiculatus*. The *roxA* orthologs of *H. ochraceum* ($RoxA_{Hoc}$), *C. coralloides* BO35 ($RoxA_{Cco}$), and *M. fulvus* ($RoxA_{Mfu}$) were functionally expressed in a Δ*roxA Xanthomonas* sp. 35Y background. All RoxA orthologs oxidatively cleaved polyisoprene, as revealed by restoration of clearing-zone formation and detection of 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD) as a cleavage product. $RoxA_{Xsp}$, $RoxA_{Mfu}$, and $RoxA_{Cco}$ were purified and biochemically characterized. The optimal temperature of $RoxA_{Cco}$ and $RoxA_{Mfu}$ was between 22 and 30°C. All RoxA orthologs as isolated showed an oxidized UV-visible spectrum. Chemical reduction of $RoxA_{Cco}$ and $RoxA_{Mfu}$ indicated the presence of two slightly different heme centers with absorption maxima between 549 and 553 nm, similar to $RoxA_{Xsp}$. Sequence analysis and modeling of the three-dimensional structures of the RoxA orthologs revealed a high degree of similarity to the recently solved $RoxA_{Xsp}$ structure and included several conserved residues, notably, W_{302}, F_{317}, and a MauG motif at about H_{517}. Lcp-like sequences were not detected in the genomes of the *Xanthomonas* sp. 35Y, *H. ochraceum*, *M. fulvus*, and *C. coralloides*. No RoxA orthologs were found in Gram-positive bacteria, and this first description of functional RoxA in Gram-negative bacteria other than *Xanthomonas* proves that RoxA is more common among rubber degraders than was previously assumed.

Natural rubber [NR; poly(*cis*-1,4-isoprene)] is a hydrocarbon polymer that has been produced by cultivating the rubber tree (*Hevea brasiliensis*) on a large scale for over 150 years. Innumerable products based on NR are known in industry and households, but the majority of the NR produced goes into the production of tires. Abrasion from tires generates a constant supply of small rubber particles that go into roadside ditches. The fact that no enrichment of rubber particles in the environment has been observed already indicates that rubber is effectively degraded in the environment. However, the fate of rubber materials in soil and the biochemical mechanism of its biodegradation are poorly understood.

Due to the high stability of carbon-carbon bonds, biodegradation of NR is a slow process compared to biodegradation of other biopolymers, such as proteins or polysaccharides, in which the polymer backbone contains heteroatoms, such as nitrogen and/or oxygen. Heteroatoms are absent in polyisoprene, apart from sulfur atoms in vulcanized rubber. The double bonds in polyisoprene are the only functional groups in NR that allow an attack by oxidizing enzymes. In fact, rubber-degrading organisms are widespread in nature (1, 2). Some rubber-degrading bacteria, such as *Xanthomonas* sp. strain 35Y (3) and many actinomycetes, produce clearing zones on opaque latex agar, while others grow adhesively on rubber without clearing zone formation. *Gordonia polyisoprenivorans* and *Gordonia westfalica* belong to the latter group (see reference 4 and references cited therein).

Rubber oxygenase A (RoxA) and latex-clearing protein (Lcp) represent two types of enzymes that share no detectable amino acid similarities but which both catalyze the primary attack of NR and cleave the hydrocarbon polymer to low-molecular-weight degradation products. Lcp was first described in *Streptomyces* sp. strain K30 and is widely distributed in rubber-degrading bacteria (5, 6), including clearing zone formers (e.g., streptomycetes) and in adhesively growing species, such as *G. polyisoprenivorans* (7). Lcp is apparently secreted via a TAT-dependent pathway (8). No cofactors are known for Lcp, and the biochemical mechanism by which Lcp catalyzes the cleavage of polyisoprene is unknown. RoxA consists of 678 amino acids and is a *c*-type cytochrome that features two binding sites for covalent attachment of heme as well as a MauG motif (9). In contrast, the Lcp amino acid sequence (397 amino acids) does not indicate the presence of metals. Both Lcp and RoxA are responsible for the cleavage of rubber to low-molecular-weight degradation products with aldehyde and keto end groups and therefore apparently catalyze a similar or even the same reaction. RoxA has been purified and studied *in vitro* (10–12). Isolated RoxA is active in aqueous environments if the enzyme substrates, rubber and dioxygen, are present and the physical conditions (pH, temperature) are appropriate. Notably, no separate cofactors are required for activity. 12-Oxo-4,8-dimethyltrideca-4,8-diene-1-al (ODTD) was identified to be the major degradation product, and a dioxygenase cleavage mechanism was shown (13).

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TABLE 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic or sequence[a]	Reference or source
Strains		
Escherichia coli S17-1	Conjugation strain	28
E. coli XL1-Blue	Transformation strain	
Corallococcus coralloides	Strain BO35, source of $RoxA_{Cco}$	T. Schäberle, University of Bonn
Myxococcus fulvus HW1	Source of $RoxA_{Mfu}$	Y. Li, Shandong University
Haliangium ochraceum	Source of $RoxA_{Hoc}$	DSMZ 14365
Xanthomonas sp. 35Y (SN5065)	Growth on poly(*cis*-1,4-isoprene) latex, clearing zone formation	3
Xanthomonas sp. 35Y-CM	Chloramphenicol-resistant mutant of strain 35Y	15
Xanthomonas sp. 35Y-CM Δ*roxA-attB* (SN4114)	Chromosomal deletion of *roxA*, *attB* at former *roxA* site, no clearing zone formation on latex agar	12
Xanthomonas sp. 35Y-CM Δ*roxA-attB*/pNH1-*roxA-attP* (in chromosome; SN4230)	Expression of RoxA from rhamnose promoter, Km^r Cm^r, clearing zone formation in the presence of rhamnose	12
Xanthomonas sp. 35Y-CM Δ*roxA-attB*/pNH1-$roxA_{Hoc}$-*attP* (in chromosome; SN5127)	Expression of $RoxA_{Hoc}$ from rhamnose promoter, Km^r Cm^r	This study
Xanthomonas sp. 35Y-CM Δ*roxA-attB*/pNH1-$roxA_{Cco}$-*attP* (in chromosome; SN5129)	Expression of $RoxA_{Cco}$ from rhamnose promoter, Km^r Cm^r	This study
Xanthomonas sp. 35Y-CM Δ*roxA-attB*/pNH1-$roxA_{Mfu}$-*attP* (in chromosome; SN5132)	Expression of $RoxA_{Mfu}$ from rhamnose promoter, Km^r Cm^r	This study
Plasmids		
pJOE6787.1	pBR322 ori, *aphII* (Km^r) *mob int* (phiC31 integrase) *attP*	J. Altenbuchner
pNH1	pJOE6787.1 with rhamnose regulation genes	12
pNH1-$roxA_{Xsp}$	Coding sequence of $roxA_{Xsp}$ under rhamnose promoter	12
pNH1-$roxA_{Xsp}$-*attP* (SN4230)	Coding sequence of $roxA_{Xsp}$ under rhamnose promoter and *attP* site	12
pNH1-$roxA_{Hoc}$-*attP* (SN4126)	Coding sequence of $roxA_{Hoc}$ under rhamnose promoter and *attP* site	This study
pNH1-$roxA_{Mfu}$-*attP* (SN5021)	Coding sequence of $roxA_{Mfu}$ under rhamnose promoter and *attP* site	This study
pNH1-$roxA_{Cco}$-*attP* (SN5084)	Coding sequence of $roxA_{Cco}$ under rhamnose promoter and *attP* site	This study
Oligonucleotides		
NdeI-roxHoc_f	GGAATTCCATATGACGACCCGAGCGAACTTAC	
roxHoc-HindIII_r	CCCAAGCTTCTACAGGGTCTTGAGGTACTC	
NdeI-roxCco_f	GGAATTCCATATGAGACTGCGATGGAGCC	
roxCco-HindIII_r	CCCAAGCTTCTACAGCGTCTTCATGTACT	
NdeI-roxMfu_f	GGAATTCCATATGCGGCTACACTGGAGTC	
roxMfu-HindIII_r	CCCAAGCTTTCAGAGCGTCTTCACGTATT	

[a] Underlining indicates the restriction enzyme sites.

Xanthomonas sp. 35Y is the only known Gram-negative rubber degrader. Attempts to isolate other rubber-degrading Gram-negative bacteria were not successful (1; unpublished data). When we screened the database for RoxA orthologs after the first cloning of the *Xanthomonas* sp. 35Y *roxA* gene ($roxA_{Xsp}$) about 10 years ago (9), no other RoxA-related sequence could be identified. However, a recently repeated BLAST search revealed several potential RoxA orthologs. Remarkably, all identified RoxA-related sequences were from members of the myxobacteria, namely, *Haliangium ochraceum*, *Corallococcus coralloides*, *Myxococcus fulvus*, and *Chondromyces apiculatus*. Rubber-degrading activities have not been described for myxobacteria or any other Gram-negative bacteria, except for *Xanthomonas* sp. 35Y. This prompted us to investigate the potential of the putative RoxA orthologs from myxobacteria for oxidative cleavage of polyisoprene.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. All strains and plasmids used in this study are given in Table 1. Recombinant *Xanthomonas* sp. 35Y Δ*roxA-attB* harboring *roxA* under the control of a rhamnose promoter was grown in modified LB medium with a reduced concentration of yeast extract (10 g/liter tryptone, 5 g/liter NaCl, 0.25 g/liter yeast extract, 1 g/liter L-rhamnose) for 3 to 4 days. The growth temperature was 30°C for expression of $RoxA_{Xsp}$ and 22°C for expression of RoxA orthologs. *H. ochraceum* was grown on DSMZ medium 246 (pH 7.3) that included beef extract (1%), peptone (1%), and artificial seawater (750 ml per liter medium). The beef extract and peptone were dissolved in 250 ml of tap water and combined with 750 ml of artificial seawater. *M. fulvus* HW1 and *C. coralloides* BO35 were grown on solid nutrient broth agar.

Cloning of *roxA* from myxobacteria. The coding sequences of the *roxA* genes of *H. ochraceum* ($roxA_{Hoc}$), *C. coralloides* BO35 ($roxA_{Cco}$), and *M. fulvus* HW1 ($roxA_{Mfu}$) were PCR amplified from the respective genomic DNAs (for primer sequences, see Table 1) and cloned into pUC9. In the case of $roxA_{Cco}$, the chromosomal template DNA was isolated from *C. coralloides* strain BO35 and the *roxA* region was sequenced *de novo*. Finally, the $roxA_{Xsp}$-coding sequence of the pNH1-*roxA-attP* plasmid was replaced by the *roxA* orthologs ($roxA_{Hoc}$, $roxA_{Mfu}$, or $roxA_{Cco}$), resulting in plasmids pNH1-$roxA_{Hoc}$-*attP*, pNH1-$roxA_{Mfu}$-*attP*, and pNH1-$roxA_{Cco}$-*attP*, respectively (Table 1). The three plasmids were conjugatively transferred from *Escherichia coli* S17-1 to *Xanthomonas* sp. 35Y Δ*roxA-attB* and were chromosomally integrated via *attP*/*attB* recombination, as previously described in detail (12). The correct integration of *roxA* orthologs and DNA sequences was confirmed by PCR and subsequent DNA sequencing of the *roxA* genes for each *Xanthomonas* sp. 35Y clone.

Purification of recombinant RoxA. Construction of tagged variants of RoxA did not result in a simplified purification process, regardless of whether a C-terminal, N-terminal, or internal tag (a His_6 or Strep tag) was chosen (unpublished result). Therefore, conventional chromatography methods were applied for purification of RoxA. *Xanthomonas* sp. 35Y Δ*roxA-attB* harboring the *roxA* ortholog of interest (Table 1) was grown in 10 to 20 individual 600-ml cultures of modified LB medium (each in a 3-liter Erlenmeyer flask) supplemented with 0.1% (wt/vol) L-rhamnose for 92 h at 22°C by continuous shaking. Cells were harvested (4°C) by centrifugation, and RoxA was purified from the culture supernatant. The cell-free supernatant was concentrated by ultrafiltration (10-kDa cutoff) and was applied to a Q-Sepharose fast-flow column (Q-FF 50/11) that had been equilibrated with 20 mM Tris-HCl (pH 8.5; flow rate, 8 ml/min). RoxA was eluted in a subsequent step gradient at ≈50 mM NaCl in equilibration buffer. Combined RoxA-containing fractions were concentrated ≈20-fold, and Tris-HCl buffer was exchanged against potassium phosphate buffer (10 mM, pH 6.8) by gel filtration on a HiPrep 26/10 column. The RoxA pool was then applied to a hydroxyapatite column (CHT5-I) that had been equilibrated with the same buffer. RoxA was eluted with a linear gradient of 10 to 200 mM potassium phosphate, pH 6.8, at ≈40 mM. RoxA fractions were pooled and concentrated via ultrafiltration (30-kDa cutoff) in the presence of 300 mM NaCl at 0 to 4°C (on ice). High ionic strength prevented precipitation of RoxA. Purity was tested by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by determination of the quotient of the absorption at 406 nm and that at 280 nm, which was ≈1.35 for pure RoxA, 1.48 for $RoxA_{Cco}$, and 1.27 for $RoxA_{Mfu}$. Purified RoxA was frozen in liquid nitrogen and stored at −70°C.

Determination of RoxA activity. RoxA was assayed by determination of the polyisoprene cleavage product ODTD via high-pressure liquid chromatography (HPLC). The assay mixture contained 5 μg of the respective purified RoxA ortholog, rubber latex (0.2% [wt/vol] emulsion), and potassium phosphate buffer (100 mM; pH 4.5 to 9.0) in a total volume of 1 ml. In some experiments, artificial seawater consisting of 28.1 g NaCl, 0.77 g KCl, 1.6 g $CaCl_2 \cdot 2H_2O$, 4.8 g $MgCl_2 \cdot 6H_2O$, 0.11 g $NaHCO_3$, and 3.5 g $MgSO4 \cdot 7H_2O$ (all components were dissolved in 1 liter water) was additionally present in the assay buffer. The reaction was performed at the indicated temperatures for 18 h in a 15-ml Falcon tube. The mixture was extracted with 1.5 ml ethyl acetate, and 1.0 ml of the extract was dried, dissolved in 100 μl methanol, and then subjected to HPLC analysis (20 μl per sample). An RP8 HPLC column (12 by 4 mm; particle size, 5 μm) was used with water (solution A) and methanol (solution B) as the mobile phases. The concentration of solution B was increased starting from 50% (vol/vol) to 100% (vol/vol) at 20 min at a flow rate of 0.7 ml/min; products were detected at 210 nm. ODTD eluted after 15.3 ± 0.4 min. Purified ODTD was used as the standard. Mixtures without RoxA served as controls.

Clearing zone assay. RoxA activity was semiquantitatively estimated by streaking recombinant *Xanthomonas* sp. 35Y Δ*roxA*::*attB* that contained the different *roxA* orthologs (Table 1) onto previously described solid mineral salts agar plates (3) supplemented with 0.05% (wt/vol) yeast extract and 0.1% (wt/vol) L-rhamnose and with an opaque overlay agar (≈8 ml) of purified polyisoprene latex (0.25% rubber [dry wt/vol in agar]). Polyisoprene latex was obtained from Weber and Schaer, Hamburg, Germany, and was purified by washing 3 times with an aqueous solution of 0.1% (wt/vol) Nonidet P-40. *Xanthomonas* sp. 35Y Δ*roxA*::*attB* harboring chromosomally anchored wild-type *roxA* under rhamnose control (pNH1-*roxA-attP*) and *Xanthomonas* sp. 35Y Δ*roxA* served as positive and negative controls, respectively. *Xanthomonas* sp. 35Y wild type was used as a supplementary control. The plates were incubated at room temperature (≈22°C) or at 30°C. The intensity of clearing zone formation semiquantitatively indicated RoxA activity and consumption of the ODTD produced by the strain.

Other methods. Heme staining was performed after separation of RoxA-containing fractions by SDS-PAGE and subsequent assay for pseudoperoxidase activity of the RoxA heme groups, as described previously (11). The concentrations of the purified RoxA proteins were determined using the molar absorption coefficient of RoxA at 406 nm (ε_{406}; 2.07×10^5 M^{-1} cm^{-1}). RoxA structures were visualized and modeled using the SWISS MODEL server (14). The genomic DNA of *H. ochraceum* and *M. fulvus* was prepared by standard methods.

Nucleotide sequence accession numbers. Annotations of the RoxA sequences from *Xanthomonas* sp. 35Y, *H. ochraceum*, *C. coralloides*, and *C. apiculatus* have been submitted to GenBank under accession numbers KC980911 to KC980914.

TABLE 2 Identities and similarities between RoxA amino acid sequences

Protein	% similarity or identity[a]					
	$RoxA_{Xsp}$	$RoxA_{Cco}{}^{Tb}$	$RoxA_{Cco}$	$RoxA_{Mfu}$	$RoxA_{Cap}$	$RoxA_{Hoc}$
$RoxA_{Xsp}$	100	67	65	65	65	63
$RoxA_{Cco}{}^{T}$	78	100	89	85	83	75
$RoxA_{Cco}$	77	93	100	83	82	75
$RoxA_{Mfu}$	75	89	90	100	89	75
$RoxA_{Cap}$	76	88	88	92	100	74
$RoxA_{Hoc}$	75	83	82	82	80	100

[a] Percent similarity is identified by shading.
[b] $RoxA_{Cco}{}^{T}$, RoxA from *Corallococcus coralloides* type strain (DSMZ 2259).

RESULTS

Identification, cloning, and expression of RoxA orthologs in *Xanthomonas* sp. 35Y. A BLAST search (January 2013) using the *Xanthomonas* sp. 35Y RoxA-coding sequence (GenBank accession number AAP41848.1) as the template revealed the presence of several putative RoxA orthologs in the database: The amino acid sequences of COCOR_02166 from *C. coralloides* DSM 2259, of LILAB_11505 from *M. fulvus* HW1, and of A176_5502 from *C. apiculatus* DSM 436 had 65% amino acid sequence identity (75 to 78% similarity) to the $RoxA_{Xsp}$ amino acid sequence, and the amino acid sequence of Hoch_1661 from *H. ochraceum* DSM 14365 was 63% identical (75% similar) to that of $RoxA_{Xsp}$ (Table 2). All RoxA-like sequences were characterized by a high fraction of aromatic residues (Table 3). Apparently, *roxA*-like genes are present in the genomes of several species of myxobacteria.

The deduced amino acid sequences of all putative RoxA orthologs have potential signal peptides for secretion; the apparent molecular masses of the putative mature proteins were highly similar and were 73.7 kDa for $RoxA_{Hoc}$, 72.5 kDa for $RoxA_{Cco}$, 73.3 kDa for $RoxA_{Mfu}$, 73.4 kDa for $RoxA_{Cap}$, and 73.0 kDa for $RoxA_{Xsp}$ (Table 3). All RoxA orthologs (i) harbored two heme motifs (CXXCH) for covalent attachment of heme, (ii) had conserved histidine residues as axial heme ligands, (iii) contained a conserved MauG motif, and (iv) had conserved W_{302} and F_{317} residues (Fig. 1; Table 3). W_{302} in $RoxA_{Xsp}$ is located between the two hemes, and F_{317} is essential for optimal binding of dioxygen (12). In conclusion, the high similarity of the identified putative proteins suggests a structure similar to that of $RoxA_{Xsp}$ and, presumably, a biochemical function similar to that of $RoxA_{Xsp}$. To test whether the identified myxobacteria can degrade polyisoprene, *H. ochraceum* was cultivated on opaque latex agar (with 0.05% yeast extract and 0.1% polyisoprene latex supplemented with artificial seawater). Although the growth of *H. ochraceum* was poor, clearing zone formation became visible within 2 weeks and suggested that the putative *roxA* gene could have the same function for the cleavage of polyisoprene as it does in *Xanthomonas* sp. 35Y. To

TABLE 3 Properties of RoxA orthologs[a]

Attribute	$RoxA_{Xsp}$	$RoxA_{Cco}{}^{Tb}$	$RoxA_{Cco}$	$RoxA_{Mfu}$	$RoxA_{Hoc}$	$RoxA_{Cap}$
Gene length (bp)	2,037	2,022	2,022	2,025	2,031	2,016
Molecular mass (kDa)						
Mature protein	71.5	71.0	71.0	71.9	72.1	71.9
Mature protein + 2 hemes	73.0	72.5	72.5	73.3	73.7	73.4
P_i (theoretical)	7.3	6.1	6.7	7.0	4.9	6.6
% aromatic amino acids (no. of F, W, and Y amino acids)	11.4 (24, 20, 30)	11.3 (31, 16, 26)	11.3 (34, 16, 23)	11.6 (32, 16, 27)	12.7 (35, 17, 30)	11.1 (36, 15, 21)
Heme attachment (N terminus/C terminus	$CSACH_{195}/CASCH_{394}$	$CSACH_{181}/CASCH_{379}$	$CSACH_{181}/CASCH_{379}$	$CSACH_{181}/CASCH_{379}$	$CSACH_{181}/CASCH_{379}$	$CSACH_{181}/CASCH_{379}$
Axial heme ligands (N terminus/C terminus)	H_{195}/H_{394} H_{641}	H_{181}/H_{379} H_{626}	H_{181}/H_{379} H_{626}	H_{181}/H_{379} H_{626}	H_{181}/H_{379} H_{626}	H_{181}/H_{379} H_{626}
MauG motif	$PYFH_{517}$ NGSVP	$PFFH_{502}$ NGSVP	$PFFH_{502}$ NGSVP	$PYFH_{502}$ NSSVP	$PYFH_{502}$ NGSVP	$PYFH_{502}$ NSSVP
F_{317} equivalent	F_{317}	F_{302}	F_{302}	F_{302}	F_{302}	F_{302}
W_{302} equivalent	W_{302}	W_{287}	W_{287}	W_{287}	W_{287}	W_{287}
pH optimum at 22°C	6–7		6–7	7		
Optimal temp range at pH 7 (°C)	30–37		22–30	22–30	≈22[c]	
Sp act (ODTD area/μg RoxA [%])	6,700 (100)		660 (9.9)	590 (8.7)		
Soret maximum (nm)						
Oxidized	407		407	408	409[d]	
Reduced	418		418	418	418[d]	
α band (reduced) (nm)	549, 553		549, 553	551–552	550, 553[d]	
β band (reduced) (nm)	521		522	522	523[d]	
Main cleavage product	ODTD		ODTD	ODTD	ODTD[d]	
Salt tolerance	+		+	+		
Activity with the following activators/inhibitors (%):						
Ethanol solvent (1%/5%/10%)	110/140/140		120/190/140			
Squalene	75		85			
α-Tocopherol	25		35			
Carotene[e]	55		10			
Pyridine	<3		<3			

[a] Inhibitor compounds were dissolved in ethanol (final concentration, 1%, vol/vol). Blank cells indicate that the value was not determined.
[b] $RoxA_{Cco}{}^{T}$, RoxA from *Corallococcus coralloides* type strain (DSMZ 2259).
[c] From Fig. 1.
[d] The experiment was performed with a cell-free culture supernatant of the *Xanthomonas* sp. Δ*roxA* strain with chromosomally integrated pNH1-$roxA_{Hoc}$-*attP* plasmid. The red shift in UV-vis signals of the $RoxA_{Hoc}$ protein by 1 nm is probably explained by impurities of medium components.
[e] Mixture of carotenes (Roth; catalog no. 7864.1).

find evidence for this assumption, we functionally analyzed $RoxA_{Hoc}$, $RoxA_{Cco}$, and $RoxA_{Mfu}$.

Cloning and functional expression of $RoxA_{Hoc}$, $RoxA_{Cco}$, and $RoxA_{Mfu}$. Substantial expression of $RoxA_{Xsp}$ in *E. coli*, other Gram-negative bacteria, or *Bacillus subtilis* was not successful, as observed earlier for RoxA and MauG (12, 15, 16). When we attempted expression of $RoxA_{Hoc}$ in an *E. coli* strain that harbored the cytochrome *c* maturation (*ccm*) genes on pEC86 (17), growth of the recombinant *E. coli* strain stopped shortly after induction and no substantial expression was detected. We concluded that experiments aimed at the expression of $RoxA_{Cco}$ or $RoxA_{Mfu}$ in *E. coli* also might not be successful. Therefore, we decided to express *roxA* orthologs in *Xanthomonas* sp. 35Y Δ*roxA* via *attP*/*attB* recombination using the phiC31 integrase system that had successfully been developed in our lab for $RoxA_{Xsp}$ expression; this system is so far the only way to obtain reproducible expression of recombinant RoxA (12).

The $RoxA_{Hoc}$-, $RoxA_{Cco}$-, and $RoxA_{Mfu}$-encoding genes were PCR amplified, cloned, and finally, integrated into the chromosome of *Xanthomonas* sp. 35Y Δ*roxA* under rhamnose promoter control, as described in detail in Materials and Methods. Successful expression of the chromosomally anchored *roxA* orthologs was tested by growth of the recombinant strains in modified latex medium supplemented with rhamnose. Expression of active RoxA proteins was indicated (i) by the formation of clearing zones around the colonies on opaque latex-rhamnose agar (Fig. 2) and (ii) by the appearance of bands with pseudoperoxidase activity and with relative mobility in SDS-polyacrylamide gels similar to that for $RoxA_{Xsp}$ for all three cloned *roxA* orthologs (Fig. 3A). The clearing zone diameter of the $RoxA_{Mfu}$ strain was similar to that of the $RoxA_{Xsp}$ strain (control), while the clearing zones for the $RoxA_{Cco}$ and $RoxA_{Hoc}$ strains were considerably smaller. Those strains formed small clearing zones at 22°C but no or only very small clearing zones at 30°C. Therefore, we assume a considerable temperature sensitivity of the $RoxA_{Hoc}$ and $RoxA_{Cco}$ orthologs. In no case were any clearing zones formed on latex agar in the absence of rhamnose. This confirmed that RoxA expression by recombinant *Xanthomonas* sp. 35Y depends on the presence of the inducer rhamnose.

Purification and biochemical properties of $RoxA_{Mfu}$ and $RoxA_{Cco}$. *Xanthomonas* sp. 35Y RoxA and two RoxA orthologs ($RoxA_{Mfu}$ and $RoxA_{Cco}$) were purified from 10, 11, or 5 liters of cell-free culture supernatants by subsequent chromatography on Q-Sepharose and hydroxyapatite (for details, see Materials and Methods). A 32-mg quantity of $RoxA_{Xsp}$, 1.1 mg of $RoxA_{Mfu}$, and 7.7 mg of $RoxA_{Cco}$ were obtained as yields after the final chromatography and concentration step. All purified RoxA orthologs were highly pure (>98%), as revealed by SDS-PAGE analysis (Fig. 3B) and by spectral analysis (not shown).

ODTD was identified as the major *in vitro* product of oxidative cleavage of natural polyisoprene latex using the three purified RoxA orthologs ($RoxA_{Xsp}$, $RoxA_{Cco}$, and $RoxA_{Mfu}$) (Table 3). $RoxA_{Hoc}$ was not purified, but the polyisoprene-cleaving activity of $RoxA_{Hoc}$ was verified using a 10-fold-concentrated culture supernatant of a $RoxA_{Hoc}$-expressing culture, and ODTD was identified to be the main cleavage product as well. These results con-

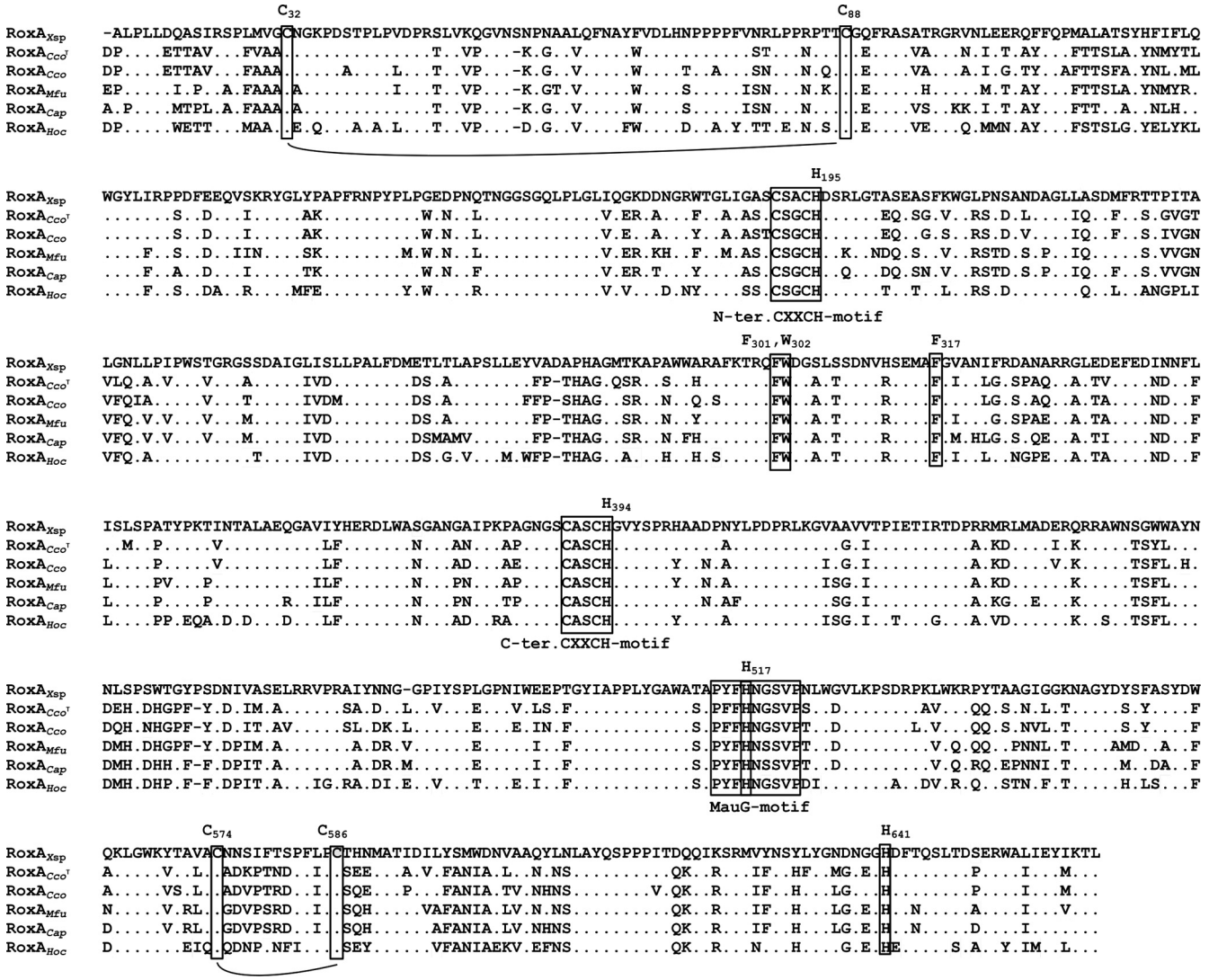

FIG 1 Alignment of amino acid sequences of RoxA orthologs. Amino acid residues of RoxA orthologs that were identical to those of $RoxA_{Xsp}$ are indicated by dots. Residues different from those in $RoxA_{Xsp}$ are given by the 1-letter amino acid code. Heme binding and MauG motifs as well as important residues in $RoxA_{Xsp}$ are emboxed. Conserved cysteine residues that contribute to disulfide bridges are connected with solid lines. Numbering of residues refers to that of $RoxA_{Xsp}$, as described in reference 9. $RoxA_{Cco}{}^{T}$, RoxA from *Corallococcus coralloides* type strain (DSMZ 2259).

firmed that $RoxA_{Mfu}$, $RoxA_{Cco}$, and $RoxA_{Hoc}$ are true rubber oxygenases (RoxAs). Purified $RoxA_{Xsp}$ had an approximately 10-fold higher specific activity than purified $RoxA_{Cco}$ and $RoxA_{Mfu}$. Determination of the pH and temperature profiles showed that the purified RoxA orthologs were active in a range from pH 5 to 9 and at 20 to 37°C. The highest activities were determined at between pH 6 and 7 and 30°C (Table 3). The thermal stability of $RoxA_{Hoc}$ was slightly lower at 30°C than at room temperature, as revealed by the reduced clearing zone formation of the $RoxA_{Hoc}$-expressing *Xanthomonas* sp. 35Y (Fig. 2) and by quantitative determination of ODTD peak areas in RoxA activity assays.

$RoxA_{Hoc}$ and $RoxA_{Mfu}$ originate from marine species, suggesting that salts could influence their activity. However, repetition of enzyme assays in the presence of different salt concentrations (0, 1, and 3% NaCl tested with or without artificial seawater) had no or only a minor influence on the formation of ODTD. RoxA orthologs of soil bacteria or of bacteria from marine habitats apparently do not differ significantly in salt tolerance. They do not depend on the presence of high salt concentrations but are not negatively affected if salts are present at considerable concentrations. Substrate analogs, such as squalene, carotene, or α-tocopherol, partially inhibited $RoxA_{Cco}$ to variable extents (only $RoxA_{Xsp}$ and $RoxA_{Cco}$ were tested with substrate analogs; Table 3). Notably, ethanol, which was used as the solvent for the inhibitor compounds, moderately increased the ODTD formation activity of RoxA in a dose-dependent manner, possibly by changing the buoyant force in the emulsion. This may simply lead to a faster concentration of latex aggregated to RoxA at the surface of the test tube, resulting in a more efficient enzyme/substrate ratio. The highest inhibitory effect on activity was, however, found for pyridine, which can act as a heme ligand. A 1 mM concentration of this compound completely inhibited the RoxA activity of all RoxA orthologs, consistent with previous investigations with $RoxA_{Xsp}$ (11).

FIG 2 Functional expression of RoxA orthologs in *Xanthomonas* sp. 35Y Δ*roxA*. *Xanthomonas* sp. 35Y wild type (*Xanth* sp. 35Y wt) and *Xanthomonas* sp. 35Y Δ*roxA* (ΔRoxA) with chromosomally integrated *roxA* orthologs from *M. fulvus* (RoxA$_{Mfu}$), *C. coralloides* BO35 (RoxA$_{Cco}$), *Xanthomonas* sp. 35Y (RoxA$_{Xsp}$), and *H. ochraceum* (RoxA$_{Hoc}$) under rhamnose control were grown on opaque latex medium that had been supplemented with L-rhamnose (0.1%) at 22°C and at 30°C. The formation and the diameter of the clearing zones semiquantitatively indicate expression of the RoxA protein and consumption of polyisoprene cleavage products. Note the reduced clearing zone formation (RoxA$_{Hoc}$) at 30°C compared to that at 22°C.

Spectral properties of RoxA orthologs. UV-visible (UV-vis) spectroscopy of RoxA$_{Xsp}$ as isolated indicated an oxidized spectrum of the heme groups. Both heme groups could be differentiated by separate absorption maxima of the α bands (549 nm and 553 nm) after chemical reduction with dithionite (11). The newly isolated RoxA preparations (RoxA$_{Mfu}$, RoxA$_{Cco}$, as well as the 10-fold-concentrated supernatant of the RoxA$_{Hoc}$-expressing culture) in the state as isolated also showed an oxidized spectrum (Soret band at 408 ± 1 nm). In the chemically reduced state, a comparison of the UV-vis properties of RoxA$_{Xsp}$ with those of RoxA of myxobacteria revealed almost identical Soret bands (at 418 nm; shown for RoxA$_{Cco}$ as an example in Fig. 4) and maxima of β bands (522 ± 1 nm) and α bands (549 ± 1 and 553 ± 1 nm) (Table 3; Fig. 4A). Only the α bands of RoxA$_{Mfu}$ were not well separated and composed a broad maximum from 551 to 552 nm, indicating different but very closely located maxima (not shown). In conclusion, the two hemes of RoxA$_{Xsp}$ and the hemes of the orthologs RoxA$_{Mfu}$, RoxA$_{Cco}$, and RoxA$_{Hoc}$ have very similar optical properties with uncommon features (11, 12) which can be seen to be characteristic for RoxA. This suggests very similar chemical environments in the immediate vicinities of the heme centers of all RoxA orthologs.

Structures of RoxA orthologs. Determination of the recently solved three-dimensional structure of the RoxA$_{Xsp}$ protein showed that the N-terminal heme has only one axial amino acid ligand (H$_{195}$) on the proximal side and that dioxygen represents

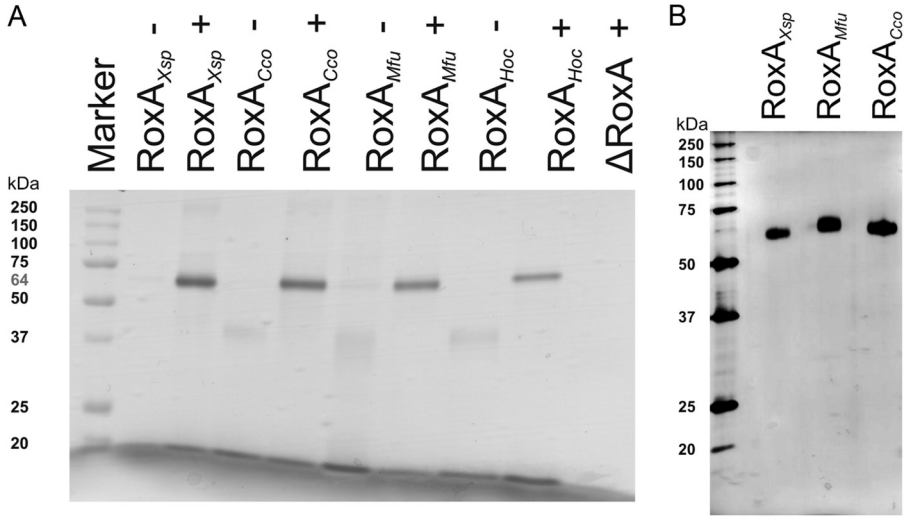

FIG 3 (A) SDS-PAGE and subsequent pseudoperoxidase staining (heme staining) of culture supernatants of *Xanthomonas* sp. 35Y Δ*roxA* harboring chromosomally integrated *roxA*$_{Xsp}$ orthologs. Each strain was grown in the absence (−) or presence (+) of the inducer L-rhamnose (0.1%). *Xanthomonas* sp. 35Y Δ*roxA* and *Xanthomonas* sp. 35Y Δ*roxA* with pNH1-*roxA*$_{Xsp}$ (RoxA$_{Xsp}$) were used as negative and positive controls, respectively. (B) Purification of recombinant RoxA orthologs. RoxA$_{Xsp}$, RoxA$_{Cco}$, and RoxA$_{Mfu}$ were purified from rhamnose-grown cultures by chromatography of concentrated cell-free culture fluid on Q-Sepharose and hydroxyapatite, as described in Materials and Methods. Aliquots of the respective RoxA pools after the hydroxyapatite step were separated by SDS-PAGE and stained with silver. Overloading of the lanes indicates the absence of significant amounts of contaminating proteins.

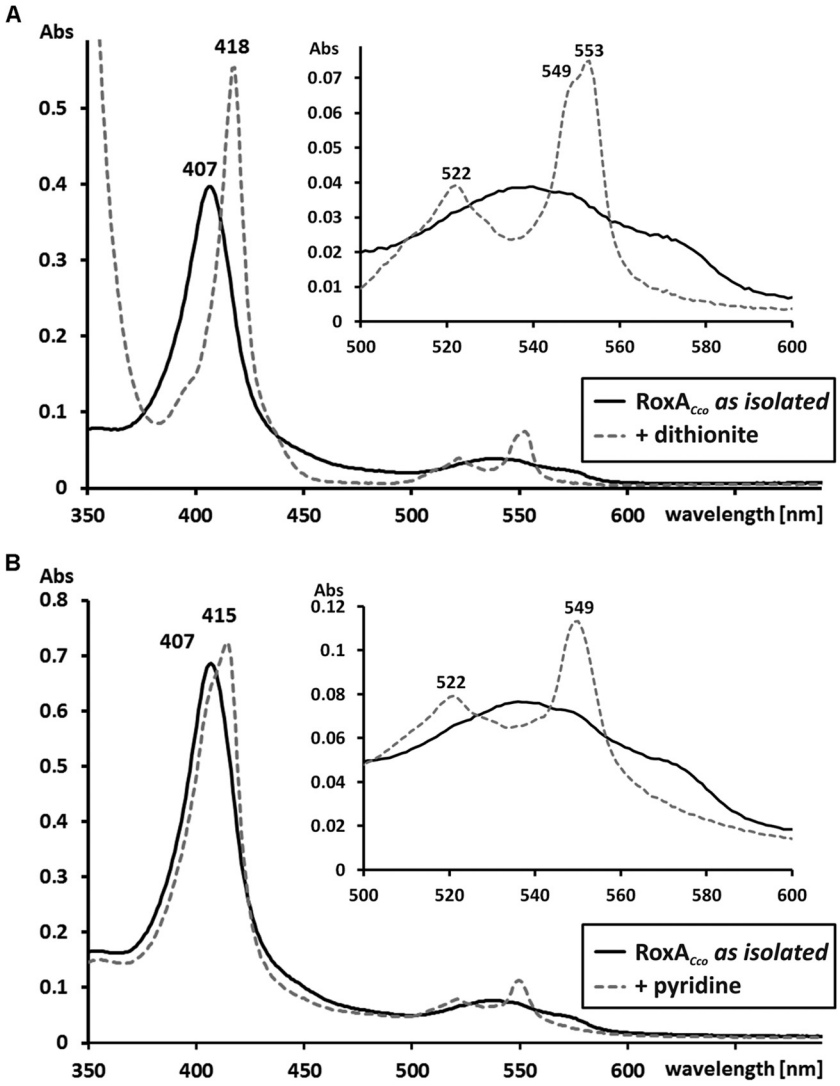

FIG 4 UV-vis spectra of purified RoxA$_{Cco}$. (A) UV-vis spectra of RoxA$_{Cco}$ as isolated (solid line) and of dithionite-reduced RoxA$_{Cco}$ (dashed line); (B) UV-vis spectrum of RoxA$_{Cco}$ as isolated (solid line) compared with the spectrum obtained after incubation of RoxA with 2 mM pyridine. The Soret band region is enlarged in the insets in both panels, and the absorption maxima of RoxA are indicated. Note that the presence of pyridine resulted in an increase of only the 549-nm α band, while in chemically reduced RoxA$_{Cco}$, both α bands (549 to 553 nm) are visible. Abs, absorbance.

the distal heme ligand (18, 19). The N-terminal heme constitutes the active site of RoxA. The other (C-terminal) heme is coordinated by two axial histidine ligands (for residue numbers, see Table 3). UV-vis and electron paramagnetic resonance (EPR) spectroscopy data suggested that a considerable part of the isolated RoxA molecules is in an Fe^{2+}-O_2 ↔ Fe^{3+}-O_2^- equilibrium at the N-terminal heme comparable to that of oxyhemoglobin (20, 21). Incubation of RoxA$_{Xsp}$ with amino acid-like molecules, such as imidazole or pyridine, that can function as axial ligands to heme resulted in a selective increase of the 549-nm α band and in inactivation of the enzyme. This can be explained by the displacement of dioxygen and binding of the ligand to Fe^{2+}. A selective increase of the 549-nm α band was also observed in the UV-vis spectrum upon incubation of RoxA$_{Cco}$ with pyridine (Fig. 4B) and indicated that the N-terminal heme of RoxA$_{Cco}$ apparently has no amino acid ligand and is present in an oxygenated state, as in RoxA$_{Xsp}$. This assumption was supported by comparing the modeled structures of RoxA orthologs shown in Fig. 5. It is evident that all structures are almost identical (root mean square deviation [RMSD] values, <1 Å). Only a minor difference was noticed for the RoxA$_{Mfu}$ structure: the major α helix (the top helix in Fig. 5) was reduced in length, and a small α helix in the enzyme center was present as a random coil. In conclusion, the structures of all RoxA orthologs, including the positions and relative orientations of the two heme centers, are highly similar and support the same biochemical function, i.e., oxidative cleavage of polyisoprene.

DISCUSSION

About 12 million tons of poly(*cis*-1,4-isoprene) are produced every year by cultivating the rubber tree (*Hevea brasiliensis*; natural rubber), and a comparable amount is produced by chemical synthesis (synthetic rubber). Polyisoprene is also produced by many plants and some fungal species (4), although the concentration of polyisoprene in the latex is much lower than that in the latex from

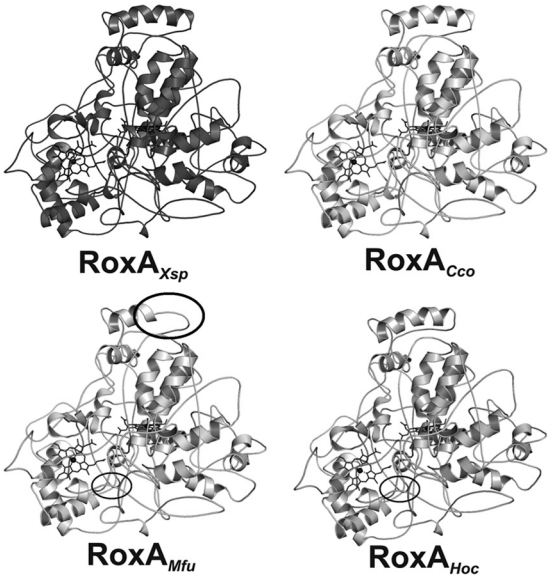

FIG 5 Similarity of the RoxA$_{Xsp}$ structure to the structures of RoxA$_{Mfu}$, RoxA$_{Cco}$, and RoxA$_{Hoc}$. Cartoons of the modeled structures of RoxA$_{Mfu}$, RoxA$_{Cco}$, and RoxA$_{Hoc}$ are shown and are compared with the known RoxA$_{Xsp}$ structure (Protein Data Bank accession number 4B2N). Areas with differences in secondary structures are indicated (circles). All modeled structures showed RMSD values of <1 Å (0.62 to 0.71 Å) compared to the RoxA$_{Xsp}$ structure.

H. brasiliensis, where the rubber solid content of the laticifers can be as high as 35% (wt/vol). For example, the common dandelion (*Taraxacum*) and lactiferous mushrooms contain substantial amounts of polyisoprene. The portion of natural rubber that is produced by non-*Hevea* species is difficult to estimate, but it is reasonable to assume that polyisoprene compounds are ubiquitous.

Most of the artificially produced polyisoprene is released to the environment after use and, together with polyisoprene-containing biomass, constitutes an abundantly available carbon source for microorganisms. Streptomycetes and related actinobacteria are well-known for their potential to degrade biopolymers, such as proteins, polysaccharides, or polyesters. Previous analysis of the distribution of rubber-degrading bacteria showed that, indeed, members of the actinobacteria are frequently able to utilize polyisoprene as a source of carbon and energy (1, 6, 22–24). As far as it has been investigated, most—if not all—of the rubber-degrading actinobacteria employ latex-clearing protein (Lcp) as the catalyst for the primary attack of polyisoprene (6, 7). *Xanthomonas* sp. 35Y is so far the only known rubber-degrading Gram-negative bacterium, and this strain does not have Lcp but produces rubber oxygenase (RoxA). Reports on the isolation of other Gram-negative bacteria that can utilize natural rubber as a sole source of carbon and energy have not been published since the description of latex degradation by *Xanthomonas* sp. 35Y, and even a directed search among related strains was not successful (1). One could assume that *Xanthomonas* sp. 35Y represents an exception and that most Gram-negative bacteria are not able to utilize polyisoprene as a carbon source. Few gene products with high similarity to RoxA that are described in this study have been deposited in databases, and none were deposited before 2009, suggesting that RoxA, at least, is not widespread in nature. We verified the function of RoxA as a polyisoprene-cleaving enzyme in at least 3 species (*H. ochraceum*, *M. fulvus*, and *C. coralloides*) by functional expression. On the basis of the high amino acid sequence similarities (76 to 92%; Table 2), it is very likely that *C. apiculatus* has a functional RoxA ortholog as well.

Myxobacteria are known to prefer a variety of insoluble biopolymers, such as cellulose, chitin, and starch, for growth. They are also able to grow bacteriotrophically by lysing intact bacterial cells and proteins (25). However, their ability to degrade poly(*cis*-1,4-isoprene) has not been described prior to this study but seems plausible for myxobacteria, which are decomposers of recalcitrant substrates. The finding of RoxA orthologues only in myxobacteria, besides *Xanthomonas* sp. 35Y, is indeed conspicuous and suggests that RoxA is overrepresented in this group (compared to other Gram-negative bacteria). The rubber degradation ability may generally be restricted to certain groups of bacteria, like the Gram-positive actinomycetes or the Gram-negative myxobacteria, which both share the capacity to decompose hardly accessible molecules. Although the rubber degradation ability among Gram-negative bacteria was not discovered first in myxobacteria, this group may represent the counterpart to the actinomycetes, which combine the majority of Gram-positive bacteria that are rubber degraders. Our results suggest that rubber-degrading Gram-negative bacteria use RoxA instead of Lcp as the rubber-cleaving enzyme. Inspection of the genome sequences of *Xanthomonas* sp. 35Y and that of the myxobacteria indicated that none of them harbor any Lcp orthologs. In conclusion, nature has evolved polyisoprene-cleaving enzymes at least two times: the Lcp system is widespread in and apparently specific for Gram-positive bacteria (*Streptomyces*, *Gordonia*, and other actinomycetes), and the RoxA system is present only in Gram-negative bacteria. It will be interesting to see whether exceptions to this rule can be found in future.

Nevertheless, a commonality of all rubber degraders seems to be the metabolization of polyisoprene cleavage products like ODTD. β-Oxidation has been proposed to be a further metabolic route for rubber latex cleavage products by Lcp in Gram-positive bacteria that are rubber degraders (26, 27). Moreover, genes necessary for β-oxidation are also found in all the myxobacteria investigated in the genomic context surrounding the respective *roxA* genes. Only a small region of 7.6 kb around *roxA* of *Xanthomonas* sp. 35Y has been sequenced. However, genes coding for a putative aminoglycoside-phosphotransferase, enoyl coenzyme A (enoyl-CoA) hydratase, and acetyl-CoA acetyltransferase are located within this region, suggesting that a β-oxidation cluster is also present around *roxA*$_{Xsp}$ and that there is a metabolic connection between the function of RoxA and β-oxidation. One could speculate that the localization of *roxA* in this metabolic gene cluster is indicative of the potential of the rubber oxygenase to use other hydrocarbons, in addition to poly(*cis*-1,4-isoprene), as substrates which are metabolized by β-oxidation.

Expression of active RoxA in *E. coli* or other heterologous hosts was not successful, regardless of the absence or presence of cytochrome *c* maturation genes (*ccm* genes). We assume that the maturation machinery for the covalent attachment of two hemes and/or for the formation of two disulfide bridges proceeds via different mechanisms in *Xanthomonas* sp. 35Y than in *E. coli* or *B. subtilis*. Since expression of RoxA from three species of myxobacteria was possible in recombinant *Xanthomonas* cells, we assume that the RoxA maturation systems for *H. ochraceum*, *C. coralloides*, and *M. fulvus* are related and are interchangeable. We postulate

the existence of a yet unknown (or at least modified) cytochrome *c* maturation system in *Xanthomonas* sp. 35Y and RoxA-expressing myxobacteria, and we assume that RoxA of *C. apiculatus* and other not yet described RoxA orthologs from other Gram-negative bacteria can be functionally expressed in recombinant *Xanthomonas* sp. 35Y as well.

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