Identification of Poly(*cis*-1,4-Isoprene) Degradation Intermediates during Growth of Moderately Thermophilic Actinomycetes on Rubber and Cloning of a Functional *lcp* Homologue from *Nocardia farcinica* Strain E1

Ebaid M. A. Ibrahim,¹ Matthias Arenskötter,¹ Heinrich Luftmann,² and Alexander Steinbüchel^{1*}

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, D-48149 Münster, Germany,¹ and Organisch-Chemisches Institut, Westfälische Wilhelms-Universität Münster, Corrensstrasse 40, D-48149 Münster, Germany²

Received 13 December 2005/Accepted 2 March 2006

The enrichment and isolation of thermophilic bacteria capable of rubber [poly(*cis*-1,4-isoprene)] degradation revealed eight different strains exhibiting both currently known strategies used by rubber-degrading mesophilic bacteria. Taxonomic characterization of these isolates by 16S rRNA gene sequence analysis demonstrated closest relationships to *Actinomadura nitritigenes*, *Nocardia farcinica*, and *Thermomonospora curvata*. While strains related to *N. farcinica* exhibited adhesive growth as described for mycolic acid-containing actinomycetes belonging to the genus *Gordonia*, strains related to *A. nitritigenes* and *T. curvata* formed translucent halos on natural rubber latex agar as described for several mycelium-forming actinomycetes. For all strains, optimum growth rates were observed at 50°C. The capability of rubber degradation was confirmed by mineralization experiments and by gel permeation chromatography (GPC). Intermediates resulting from early degradation steps were purified by preparative GPC, and their analysis by infrared spectroscopy revealed the occurrence of carbonyl carbon atoms. Staining with Schiff's reagent also revealed the presence of aldehyde groups in the intermediates. Bifunctional isoprenoid species terminated with a keto and aldehyde function were found by matrix-assisted laser desorption ionization–time-of-flight and electrospray ionization mass spectrometry analyses. Evidence was obtained that biodegradation of poly(*cis*-1,4-isoprene) is initiated by endocleavage, rather than by exocleavage. A gene (*lcp*) coding for a protein with high homology to Lcp (latex-clearing protein) from *Streptomyces* sp. strain K30 was identified in *Nocardia farcinica* E1. *Streptomyces lividans* TK23 expressing this Lcp homologue was able to cleave synthetic poly(*cis*-1,4-isoprene), confirming its involvement in initial polymer cleavage.

Many bacterial isolates that are able to utilize poly(*cis*-1,4-isoprene) (rubber) as the sole source of carbon and energy have been described during the last hundred years (for details, see references 14 and 18). These bacteria can be divided into two groups, which follow different strategies to degrade rubber (12). Members of the first group form translucent halos if they are cultivated on solid media containing dispersed latex particles, indicating the excretion of rubber-cleaving enzymes. Mycelium-forming actinomycetes such as *Actinoplanes*, *Micromonospora*, and *Streptomyces* species belong to this group. The second group comprises mycolic acid-containing *Actinobacteria* belonging to the genera *Gordonia*, *Mycobacterium*, and *Nocardia*. These bacteria are not able to form translucent halos, but they grow adhesively at the surface of rubber particles in liquid culture, and they represent the most potent rubber-degrading bacterial strains (2). All rubber-degrading species described so far are mesophilic species, with only one exception, identified as a *Streptomyces* sp. closely related to *Streptomyces albobrissolus* and *Streptomyces viridodiateticus*, which was able to grow at temperatures of up to 50°C (8). It belongs to the so-called

clear-zone-forming group of rubber-degrading bacteria. Recently, genes encoding rubber-cleaving enzymes of mesophilic clear-zone-forming bacteria have been reported (7, 15). Thermophilic bacteria and enzymes produced by them are of great interest for biotechnological processes, because they would allow conversion of rubber into valuable chemicals and performance of such processes at a technical scale with fewer problems caused by contaminating bacteria (20). This study aimed at the isolation and characterization of thermophilic bacteria capable of rubber degradation.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cells of *Actinomadura*, *Nocardia*, and *Thermomonospora* spp. were cultivated at 50°C in Standard I nutrient broth (St-I) (Merck, Darmstadt, Germany) or mineral salts medium (MSM) as described by Schlegel et al. (17). The following carbon sources were added to liquid MSM: 0.2% (wt/vol) sodium acetate, 0.5% (vol/vol) natural latex concentrate (Neotex Latz; Weber & Schaer, Hamburg, Germany), or 0.3% (wt/vol) synthetic poly(*cis*-1,4-isoprene) with an average molecular mass of 800 kDa (no. 182141; Aldrich, Steinheim, Germany). Liquid cultures in Erlenmeyer flasks were incubated on a horizontal rotary shaker. Solid media were prepared by addition of 1.5% (wt/vol) agar-agar. Latex overlay agar plates were used for growth of clear-zone-forming strains. For this, MSM agar plates were covered with an overlay of MSM agar containing 0.2% (vol/vol) latex concentrate. Cells of *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani broth (16). Antibiotics were applied as described by Sambrook et al. (16). Protoplasts of *Streptomyces lividans* TK23 were prepared from cultures grown in modified YEME (3%

* Corresponding author. Mailing address: Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Corrensstrasse 3, D-48149 Münster, Germany. Phone: 49-251-8339821. Fax: 49-251-8338388. E-mail: steinbu@uni-muenster.de.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics and origin	Source and/or reference
Strains		
<i>Escherichia coli</i> Top10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>deoR recA1 araD139</i> Δ (<i>araA-lei</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen GmbH, Karlsruhe, Germany
<i>Actinomyces</i> sp. strain E6	Rubber-degrading wild type, isolated from food residues of animal husbandry in Egypt (clear zone forming)	This study
<i>Gordonia polyisoprenivorans</i> VH2	Rubber-degrading wild type (adhesive)	DSM 44266 (1)
<i>Gordonia westfalica</i> Kb1	Rubber-degrading wild type (adhesive)	DSM 44215 ^T (11)
<i>Nocardia farcinica</i> E1	Rubber-degrading wild type, isolated from top soil from Egypt (adhesive)	This study
<i>Nocardia farcinica</i> E2	Rubber-degrading wild type, isolated from sandy soil from Egypt (adhesive)	This study
<i>Nocardia farcinica</i> E3	Rubber-degrading wild type, isolated from soil of a gas station in Egypt (adhesive)	This study
<i>Streptomyces lividans</i> TK23		9
<i>Thermomonospora curvata</i> E4	Rubber-degrading wild type, isolated from food residues of animal husbandry in Egypt (clear zone forming)	This study
<i>Thermomonospora curvata</i> E5	Rubber-degrading wild type, isolated from compost in Germany (clear zone forming)	This study
<i>Nocardia farcinica</i> S3	Rubber-degrading wild type, isolated from sandy soil from Egypt (adhesive)	This study
<i>Nocardia farcinica</i> I7	Rubber-degrading wild type, isolated from sandy soil from Cambodia (adhesive)	This study
Plasmids		
pBluescript SK(-)	Ap ^r <i>lacPOZ'</i> , 2.95 kb	Stratagene, San Diego, Calif. 9
pIJ702		
pIJ702::lcpNFE1	Contains an <i>lcp</i> homologue from <i>N. farcinica</i> E1	This study

[wt/vol] yeast extract, 5% [wt/vol] Bacto peptone, 3% [wt/vol] malt extract, 34% [wt/vol] sucrose) medium (9). R5 agar plates were used for protoplast regeneration (9).

Enrichment and isolation of thermophilic rubber-degrading bacteria. For enrichment and isolation of moderately thermophilic bacteria able to degrade poly(*cis*-1,4-isoprene), several soil samples collected at various places in Egypt, Germany, and Cambodia were incubated separately at 50°C in Erlenmeyer flasks containing 50 ml MSM and 0.5% (vol/vol) latex concentrate or 0.3% (wt/vol) synthetic poly(*cis*-1,4-isoprene) as the sole source of carbon and energy. If growth was macroscopically detected within the first 3 weeks, 1 ml of the respective enrichment culture was transferred into 50 ml of fresh MSM containing the corresponding carbon source, and the cells were incubated again under the same conditions until an increase of the optical density was obtained. This procedure was repeated one more time. Subsequently, numerous strains were brought to axenic cultures on solid St-I medium and on latex overlay agar plates. The strains obtained were individually tested in liquid cultures for their ability to degrade synthetic poly(*cis*-1,4-isoprene).

Isolation, analysis, and manipulation of DNA. Plasmid DNA was prepared from crude lysates of cells by the alkaline extraction method (4). Total DNA of *Actinomyces*, *Nocardia*, and *Thermomonospora* spp. was prepared as described by Ausubel et al. (3), with modifications as follows. Cells from 50-ml cultures were harvested by centrifugation and suspended in 8.5 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and 1 ml lysozyme solution (10 mg/ml TE) was added. After incubation at 37°C for 2 h, 500 μ l of a sodium dodecyl sulfate solution (100 g/liter) and 50 μ l of a proteinase K solution (20 g/liter TE) were added and mixed gently. After additional incubation at 37°C for 1 h, 5 ml 5 M NaCl and 1.5 ml of a solution of 100 g hexadecyltrimethyl ammonium bromide per liter of 0.7 M NaCl were added, and the solution was incubated at 65°C for 20 min. Recombinant DNA techniques with *Streptomyces lividans* TK23 were performed as described by Kieser et al. (9). All other genetic procedures and manipulations were conducted as described by Sambrook et al. (16).

Determination of 16S rRNA gene sequence. PCR-mediated amplification of 16S rRNA genes, using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA) and the primers 27f (5'-GAGTTTGATCTGGCTCAG-3') and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3'), and purification of the PCR product were carried out as described previously (13). The purified 16S rRNA gene fragment was cloned into SmaI-digested pBluescript SK(-) DNA and sequenced. DNA sequences were determined with the primers 27f (5'-GAGTTT

GATCCTGGCTCAG-3'), 343r (5'-CTGCTGCCTCCCGTA-3'), 357f (5'-TACGGGAGGCAGCAG-3'), 519r (5'-G[T/A]ATTACCGCGGC[T/G]GCTG-3'), 536f (5'-CAGC(C/A)GCCGCGGTAAT[T/A]C-3'), 803f (5'-ATTAGATACCC TGGTAG-3'), 907r (5'-CCGTCAATTCATTTGAGTTT-3'), 1114f (5'-GCAA CGAGCGCAACCC-3'), 1385r (5'-CGGTGTGT[A/G]CAAGGCC-3'), and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3'), using a SequiTherm EXCEL II Long-Read L-C kit (Epicenter, Madison, WI) and a Li-COR model 4200 sequencer (LI-COR Biosciences, Lincoln, NE). Sequences were aligned manually with published sequences from representative actinomyces obtained from EMBL. BlastN was used to determine the percentages of nucleotides identical to 16S rRNA gene sequences in the GenBank databases.

GPC. Cleavage of poly(*cis*-1,4-isoprene) by the newly isolated bacterial strains was verified by gel permeation chromatography (GPC) and compared to the cleavages catalyzed by *Gordonia polyisoprenivorans* VH2 (1) and *Gordonia westfalica* DSM 44215^T (11). Five milliliters of synthetic poly(*cis*-1,4-isoprene) diluted in pentane (2.5%, wt/vol) was poured into sterile 300-ml Erlenmeyer flasks and the solvent was evaporated, resulting in a thin poly(*cis*-1,4-isoprene) layer adhering to the bottoms of the flasks. The flasks were then filled with 100 ml MSM and inoculated with 500 μ l of a well-grown culture washed twice with sterile saline. The cultures were cultivated for different periods to observe the cleavage chronologically. At each measurement point, the culture broth of the respective culture was discarded, and the remaining poly(*cis*-1,4-isoprene) layer was dried and subsequently dissolved in pentane. Particles were removed by centrifugation, and the supernatants were filtered through a Minisart SRP 4 syringe filter (PTFE membrane, 0.45- μ m pore size; Sartorius, Göttingen, Germany). The solvent was evaporated again, and the residue was dissolved in an appropriate volume of chloroform. The resulting samples were then analyzed by gel permeation chromatography with a Waters GPC system (Waters, Milford, CT) consisting of a 515 high-pressure liquid chromatography pump, a 410 differential refractometer, a 717plus autosampler, and four in-series-connected Styragel columns (HR3, HR4, HR5, and HR6, with pore sizes of 10³, 10⁴, 10⁵, and 10⁶ Å, respectively). The molecular weights of poly(*cis*-1,4-isoprene) and products resulting from cleavage were calculated from the retention times of defined poly(*cis*-1,4-isoprene) standards (PSS Polymer Standards Service GmbH, Mainz, Germany).

Isolation of degradation products. The degradation products were isolated by preparative GPC. For this, GPC was performed as described above. The relevant fractions containing the degradation products were collected, and the respective

FIG. 1. Mineralization of synthetic poly(*cis*-1,4-isoprene) and latex. (A) Adhesively growing strains and (B) clear-zone-forming strains were cultivated at 50°C in MSM containing 0.3% (wt/vol) synthetic poly(*cis*-1,4-isoprene) (IR) or 0.5% (vol/vol) latex concentrate. Mineralization was determined as described in Materials and Methods.

fractions from 15 to 20 GPC runs were combined. Afterwards, purification of degradation products was verified by GPC analysis.

Aldehyde staining of poly(*cis*-1,4-isoprene) and degradation products. Poly(*cis*-1,4-isoprene) and intermediates resulting from the cleavage were dissolved in chloroform (5 mg/ml), and 30- μ l portions of the solutions were spread on glass slides. After evaporation of the solvent, aldehyde groups were stained for 20 min with Schiff's reagent. Afterwards, the staining reagent was removed, and the slides were washed with sulfite solution. The composition of the staining solution was as follows: 2 g of fuchsin dissolved in 50 ml of glacial acetic acid, 10 g $\text{Na}_2\text{S}_2\text{O}_5$, 100 ml of 0.1 N HCl, and 50 ml H_2O . The composition of the sulfite solution was 5 g of $\text{Na}_2\text{S}_2\text{O}_5$ plus 5 ml of concentrated HCl (37 to 38%) in a 100-ml aqueous solution.

Infrared spectroscopy. Spectra were taken with a Varian (formerly Digilab) (Darmstadt, Germany) FTS4000 apparatus equipped with an ATR MKII sample stage.

MALDI-TOF. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF) was carried out using a LAZARUS III DE time-of-flight mass spectrometer (constructed by H. Luftmann, Organisch-Chemisches Institut, WWU-Münster, Germany) and an N_2 Laser (337 nm, 3 ns) operated at 19 kV with delayed extraction (600 ns). The matrix consisted of 50 mg *trans*-2-[3-(4-*tert*-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) and 1 mg $\text{Cu}(\text{NO}_3)_2$ in 1 ml tetrahydrofuran, which were mixed at a 1:1 ratio with 1 mg of the GPC-purified intermediates diluted in 1 ml tetrahydrofuran. The mixture was applied to the stainless steel target; when dry and crystallized, it was introduced into the mass spectrometer ion source.

Electrospray ionization mass spectrometry (ESI-MS). Measurements were made with a Quattro LCZ system (Waters-Micromass, Manchester, United Kingdom) equipped with a nanospray inlet. One milligram of the GPC-purified degradation product and 2 mg Girard-T-reagent were suspended by a short ultrasonic treatment in 0.5 ml ethanol and 50 μ l formic acid. The reaction mixture was then incubated at 60°C for 30 min and diluted 20-fold with methanol before measurement. Additionally, the mixture was measured with a MicroTof (Bruker, Daltonik, Bremen, Germany) to obtain exact mass information. The sample was introduced through a sample loop; the mass scale was calibrated with sodium formate immediately before the sample was measured.

Determination of mineralization rates. Evidence for biodegradation of the poly(*cis*-1,4-isoprene) hydrocarbon chain to CO_2 was obtained by determination of respiratory CO_2 evolution during cultivation of cells in the presence of poly(*cis*-1,4-isoprene) as the sole carbon source. Determination was carried out in tightly closed Erlenmeyer flasks by using the property of $\text{Ba}(\text{OH})_2$ to precipitate CO_2 as BaCO_3 . The flasks, containing 50 ml MSM culture, the rubber substrate [latex concentrate or poly(*cis*-1,4-isoprene)], and a test tube containing 15 ml of a 0.2 M $\text{Ba}(\text{OH})_2$ solution, were inoculated with 0.3% (vol/vol) of a well-grown culture. At each measurement point, the flasks were aerated and the test tubes were replaced by new tubes containing fresh $\text{Ba}(\text{OH})_2$ solution. Consumption of carbonate by precipitation of CO_3^{2-} as BaCO_3 was determined for each period

by titration with HCl and compared to that of a noninoculated control. The mineralization rate was calculated as follows: mineralization (% CO_2) = (required amount HCl [ml] $\times$ 0.252 M)/(C content of applied amount of *cis*-1,4-polyisoprene [mmol]) $\times$ 2.

Nucleotide sequence accession numbers. The nucleotide sequences of the 16S rRNA genes were deposited in GenBank and are available under the following GenBank accession numbers: isolate E1, AY524857; E2, AY524858; E3, AY524859; E4, AY525765; E5, AY525766; E6, AY766302; S3, AY524860; and I7, AY524861. The sequence of *lcp* from *N. farcinica* E1 is available under GenBank accession number DQ323117.

RESULTS

Isolation and characterization of thermophilic rubber-degrading bacteria. In total, eight strains from different habitats (Table 1) could be isolated as moderately thermophilic rubber-degrading bacteria. Three of these isolates (strains E4, E5, and E6) were able to produce halos on latex overlay plates as was described for various mycelium-forming actinomycetes, whereas five isolates (strains E1, E2, E3, S3, and I7) exhibited an adhesive growth behavior as was described for rubber-degrading bacteria belonging to the genera *Gordonia*, *Mycobacterium*, and *Nocardia*. For all strains, the optimal cultivation temperatures were determined. For this, cultures of strains E1, E2, E3, S3, and I7 were incubated in MSM containing 0.2% (wt/vol) sodium acetate at 30, 37, 45, 50, and 55°C, and the optical densities at 600 nm were determined after 24 h. Since measurement of the optical densities of cultures of isolates E4, E5, and E6 was not possible due to the formation of mycelia, the optimal growth temperature was determined by measuring the cellular dry matter after 72 h of incubation in St-I nutrient broth. All isolated strains exhibited the best growth at 50°C (data not shown).

To characterize the novel isolates taxonomically in more detail, 16S rRNA gene sequences were determined as described in Materials and Methods and were aligned with sequences deposited in GenBank to determine closely related species. The 16S rRNA gene sequences of isolates E1, E2, E3, S3, and I7 showed the highest similarities to the 16S rRNA

FIG. 2. Degradation products occurring during incubation of *N. farcinica* strain S3 and *T. curvata* strain E5 on poly(*cis*-1,4-isoprene). GPC profiles of the chloroform-soluble fraction recovered from cultures of strains S3 (A) and E5 (B) containing poly(*cis*-1,4-isoprene) as the sole source of carbon and energy were obtained after incubation for different periods. (C) Elution of the chloroform-soluble fraction of a control incubated for 70 days in the absence of cells. (D) Profile of intermediates purified from cultures of *N. farcinica* S3 after incubation for 7 days. This fraction was used for MALDI-TOF and ESI-MS analyses.

gene sequence of *Nocardia farcinica* DSM 43665^T (= ATCC 3318^T) (99.9%, 99.8%, 99.5%, 99.7%, and 99.9%, respectively), which is a moderately thermophilic actinomycete that is frequently isolated from patients exhibiting predisposing conditions. Below, isolates E1, E2, E3, S3, and I7 will therefore be referred to as *N. farcinica* strains E1, E2, E3, S3, and I7, respectively. Only 96.5% of the nucleotides of isolate E6's 16S rRNA gene sequence were identical to nucleotides of the 16S rRNA gene sequence of *Actinomadura nitritigenes* DSM 44137^T; therefore, this isolate will be referred to as *Actinomadura* sp. strain E6. The 16S rRNA gene sequences of isolates E4 and E5 exhibited the highest similarities to the 16S rRNA gene sequence of *Thermomonospora curvata* DSM 43183^T (= ATCC 19995^T) (99.2% and 98.8%, respectively), which is the type species of this genus. Members of the genus *Thermomonospora* are characterized by the formation of single spores on aerial mycelium; however, the taxonomic classification has been frequently revised in recent years (19). Apart from *T. curvata*, only one other species, *Thermomonospora chromogena*, still belongs to this genus. Other species were reclassified into the genera *Actinomadura*, *Microbispora*, and *Thermobifida*

(22). Isolates E4 and E5 will therefore be referred to as *T. curvata* strains E4 and E5, respectively.

Quantification of rubber degradation. The capacity of all newly isolated strains for rubber degradation was quantified as described in Materials and Methods by measuring the carbon dioxide released during growth on synthetic poly(*cis*-1,4-isoprene) and latex concentrate. The mineralization rates were then compared to those previously obtained for mesophilic rubber-degrading bacteria of the adhesively growing and the clear-zone-forming groups. Figure 1 presents the progression of CO₂ release during the time course of cultivation in the presence of synthetic poly(*cis*-1,4-isoprene) or latex concentrate. The poly(*cis*-1,4-isoprene) contained in latex concentrate was more readily utilized as a carbon source than synthetic poly(*cis*-1,4-isoprene). The adhesively growing bacteria (*N. farcinica* strains E1, E2, E3, S3, and I7) metabolized the polymeric carbon source more rapidly to CO₂ than the halo-forming bacteria (*T. curvata* strains E4 and E5 and *Actinomadura* sp. strain E6). Up to 10% (wt/wt) of the applied synthetic poly(*cis*-1,4-isoprene) and 15% (wt/wt) of the poly(*cis*-1,4-isoprene) contained in latex were catabolized to CO₂ by *N. far-*

FIG. 3. MALDI-TOF spectrum of the intermediates resulting from poly(*cis*-1,4-isoprene) cleavage by *N. farcinica* S3. The GPC-purified intermediate was analyzed. A distribution of peaks from 900 to 4,500 Da was observed. The intervals of 68 *m/z* correspond to the mass of one isoprene unit.

cinica strains E1, E2, E3, S3, and I7 after 50 days of incubation at 50°C. These values reached approximately one-third of the mineralization rates described previously for the adhesively growing strain *G. polyisoprenivorans* VH2. However, the investigations with that strain were carried out with a poly(*cis*-1,4-isoprene) exhibiting a significantly lower average molecular weight (10). Since the molecular weight of the substrate has an effect on the mineralization rate, these values cannot be directly compared (data not shown). *T. curvata* strains E4 and E5 converted not more than 5% (wt/wt) of synthetic poly(*cis*-1,4-isoprene) and about 9% (wt/wt) of the poly(*cis*-1,4-isoprene) of the latex concentrate to CO₂ within 60 days. These values were in the same range as the mineralization rates obtained with cultures of clear-zone-forming bacterial strains (K. Rose, personal communication).

Cleavage of the poly(*cis*-1,4-isoprene) backbone. Cleavage of the polymer backbone was visualized by GPC analysis, employing synthetic poly(*cis*-1,4-isoprene) with a molecular mass of approximately 800 kDa (approximately 10,000 isoprene units). All isolated strains were able to cleave the rubber substrate as demonstrated by the occurrence of an additional peak representing a product of one of the initial degradation steps. Intermediates of the same size were also observed in cultures of the mesophilic rubber-degrading bacteria *G. polyisoprenivorans* VH2 and *G. westfalica* DSM 44215^T (data not shown). Degradation of poly(*cis*-1,4-isoprene) by adhesively growing and clear-zone-forming strains was studied in more detail for one representative of each group (isolates S3 and E5, respectively). The time-dependent courses of polymer cleavage catalyzed by each representative diverged significantly (Fig. 2), as follows. (i) While isolate S3 produced the intermediates already after 3 days, degradation products with about the same molecular weight distribution were produced by strain E5 only after 28 days. (ii) In cultures of *N. farcinica* strain S3, the applied *cis*-1,4-polyisoprene had almost completely disappeared after 15 days, whereas this extent of polymer degradation was reached only after 70 days of incubation with *T. curvata* E5.

(iii) In GPC profiles of cultures of both strains, the peak resulting from noncleaved poly(*cis*-1,4-isoprene) decreased continuously as the peak indicating cleavage increased. Prolonged cultivation times also led to a decrease of the latter peak. In cultures of *N. farcinica* strain S3, these intermediates began to disappear to a significant extent already after 7 days of incubation. In contrast, in cultures of *T. curvata* strain E5, disappearance of the intermediates became apparent only after 49 days.

Purification and characterization of intermediates. Approximately 50 mg of the intermediate resulting from polymer cleavage by *N. farcinica* S3 was purified as described in Materials and Methods. Analysis by Fourier transform infrared spectroscopy clearly indicated the presence of carbonyl groups, most likely as a keto functional group (1,715 cm⁻¹). The poly(*cis*-1,4-isoprene) which was recovered from incubated cultures, with a molecular mass of 800 kDa (approximately 10,000 isoprene units), also contained detectable carbonyl functional groups. Staining with Schiff's reagent was clearly positive for the intermediate, indicating the introduction of aldehyde functional groups during cleavage of poly(*cis*-1,4-isoprene) in these degradation products. The presence of aldehyde functional groups was confirmed by H nuclear magnetic resonance (H-NMR) (data not shown). The average molecular mass of the degradation intermediate calculated by GPC analysis with poly(*cis*-1,4-isoprene) standards (Polymer Standard Service, Mainz, Germany) was approximately 13 kDa. In contrast, MALDI-TOF revealed that the degradation intermediate was a polymeric substance with a mass distribution of 900 to 4,500 Da as indicated by the spectrum of compounds representing molecules differing by 68 Da in molecular mass (Fig. 3). The sum of the end groups could be calculated as $(31.1 + n \times 68) \pm 1$; the relatively low precision is due to the noisy signal. ESI-MS analysis confirmed these results (Fig. 4). An end group sum of 100 Da is expected for an intermediate with keto (57-Da) and aldehyde (43-Da) terminal groups (for details, see Fig. 5).

FIG. 4. ESI-MS (Quattro LCZ) measurement of the intermediate derivatives obtained with Girard-T reagent. The GPC-purified intermediate was analyzed. As indicated by the intervals of 0.5 of the isotope peaks, the molecules contain two charges due to a twofold derivatization. Peak intervals of 34 m/z show the 68-Da isoprene repeating unit. The molecular mass of a bifunctional isoprenoid intermediate with 21 isoprene units can be calculated from the detailed isotope peak series shown on the right.

Derivatization of carbonyl compounds with Girard-T reagent, yielding compounds with charged functional groups, is a proven tool to greatly enhance their sensitivity for ESI-MS analysis. The resulting derivatives revealed doubly charged peaks with intervals of 34 m/z (Fig. 4). Thus, the degradation products were identified as polyisoprene polymers in which each molecule contained two carbonyl groups. Applying the mixture of the resulting derivatives to a MicroTof, the precise mass (± 6.4 mDa) was determined at selected peaks and agreed with the calculated values for the expected sum formulas (data not shown).

Cloning of an *lcp* homologue gene from *N. farcinica* strain E1. Recently, *lcp* from *Streptomyces* sp. strain K30 was cloned, and it was demonstrated that its translational product is responsible for the initial cleavage of poly(*cis*-1,4-isoprene) (15). A homologous gene (locus nfa21740) was identified in the genome sequence of *N. farcinica* IFM10152 by BLAST search (GenBank Accession number AP006618). With *lcp* from *Streptomyces* sp. strain K30 as a digoxigenin-labeled probe for Southern hybridization, DNA sequences homologous to *lcp* were detected in total genomic BamHI-restricted DNAs from *N. farcinica* strains E1, E2, E3, and S3. Employing the primers P1 (5'-AAAAGATCTATAGTCCGCTCGTCCCATAC-3') and P2 (5'-AAAGATCTACCATCTCTACGGCGGCTTC-3') (restriction recognition sites for BglII are underlined), which were deduced from the *N. farcinica* IFM10152 genome sequence, a 1.6-kbp DNA fragment from *N. farcinica* strain E1 comprising 191 bp upstream of the putative start codon and 135 bp downstream of the proposed stop codon was amplified by PCR. This DNA fragment was then digested with BglII and ligated into plasmid pIJ702 that was previously linearized with BglII. The resulting plasmid, pIJ702:*lcp*NFE1, and plasmid pIJ702 as a vector control were transferred to *S. lividans* TK23 by protoplast transformation. The recombinant strains of *S. lividans* TK23 were then cultivated in MSM cultures with poly(*cis*-1,4-isoprene) as the sole source of carbon and energy. After 6 and 8 weeks, the polymer was characterized by GPC

analysis (data not shown). Interestingly, *S. lividans* TK23 harboring pIJ702:*lcp*NFE1 was able to cleave the rubber material, as indicated by the occurrence of the degradation intermediate typically produced by the rubber-degrading bacterial strains characterized in this study. This intermediate was also positively stained with Schiff's reagent. In contrast, *S. lividans* TK23 harboring only pIJ702 did not produce this degradation product.

DISCUSSION

In total, eight moderately thermophilic bacterial strains capable of degrading poly(*cis*-1,4-isoprene) at 50°C were isolated from different geographic regions and habitats. Three strains, E4, E5, and E6, belonged to the clear-zone-forming group of rubber-degrading bacteria; they produced translucent halos if cultivated on solid mineral salts media containing poly(*cis*-1,4-isoprene) in the form of latex concentrate and exhibited mycelial growth. This feature is common among mesophilic rubber-degrading strains belonging to the genus *Streptomyces* and among other, closely related taxa such as *Actinoplanes* and *Micromonospora* (14). The 16S rRNA gene sequences of strains E4 and E5 showed the highest similarities to the 16S rRNA gene sequence of the type strain of *Thermomonospora curvata* (DSM 43183^T [= ATCC 19995^T]), of 99.2% and 98.8%, respectively. The phylogenetic position of *Actinomyces* sp. strain E6 remains fairly unknown, since identities to 16S rRNA gene sequences of validly described species were below 96%. The other five strains exhibited growth characteristics during rubber degradation as previously described for mesophilic species of the genera *Gordonia*, *Mycobacterium*, and *Nocardia* (2, 14). The 16S rRNA gene sequences of these five strains shared 99.5 to 99.9% identical nucleotides with the 16S rRNA gene sequence of the type strain of *Nocardia farcinica* (DSM 43665^T [= ATCC 3318^T]) and comprise with this species a distinct phylogenetic branch within the genus *Nocardia*. Therefore, all hitherto-isolated bacteria exhibiting adhe-

sive growth during rubber degradation belong to the suborder *Corynebacterineae*, the mycolic acid-containing actinomycetes. Presumably, the adhesive growth behavior is associated with the occurrence of mycolic acids in the cell walls of these bacteria.

Consequently, this study demonstrates that thermophilic rubber-degrading bacteria also can be divided into two groups regarding their degradation strategies, as was previously shown for mesophilic strains (12). The previous finding that adhesively growing bacteria comprise the more potent rubber degraders applies also to the thermophilic strains described in this study, as demonstrated by mineralization experiments and GPC analysis. On average, the adhesively growing strains of *N. farcinica* mineralized about 8.5% (wt/wt) of the synthetic poly(*cis*-1,4-isoprene) and approximately 13% (wt/wt) of the natural poly(*cis*-1,4-isoprene) applied as latex concentrate after 50 days. In contrast, the halo-forming *T. curvata* strains E4 and E5 converted only about 4% (wt/wt) of synthetic poly(*cis*-1,4-isoprene) and 7.5% (wt/wt) of the latex to CO₂ during the same period.

The thermophilic isolates of both groups converted poly(*cis*-1,4-isoprene) transiently to intermediates as revealed by GPC analysis. The molecular mass distributions of these intermediates were calculated by both GPC (~13 kDa) and MALDI-TOF (0.9 to 4.5 kDa). The significant deviation might be due to a change of the elution behavior during GPC analysis caused by the introduction of carbonyl groups affecting the interaction of the degradation intermediates with the column material. Fourier transform infrared spectroscopy analysis clearly indicated the occurrence of C=O bonds in the intermediates, but the signals resulting from carbonyl functional groups of ketones and aldehydes were not clearly resolved. A signal typical for a ketone was measured at 1,715 cm⁻¹. However, the presence of aldehyde groups was verified with Schiff's reagent staining and by H-NMR. Theoretically, the intermediates should contain one of both functional groups. ESI-MS clearly identified the degradation products as dicarbonyl isoprenoids. These data are in agreement with a previously proposed scheme for poly(*cis*-1,4-isoprene) cleavage by oxygen attack at the double bonds of the polymer (21) resulting in the formation of one aldehyde and one ketone per O₂, as was determined for the intermediates in this study and as illustrated in Fig. 5.

Because the retention time of the original peak representing the polymeric material employed did not change during degradation, endocleavage must occur as the initial step of rubber degradation by these strains. In the case of exocleavage, the retention time of the partially degraded polymer would continuously increase due to successive truncation of the supplied polymeric substrate, and intermediates with a low range of dispersity should not be generated. Since *N. farcinica* strain S3 and *T. curvata* strain E5 obviously exhibit different strategies for rubber degradation as indicated by their growth behavior, the similarity of the GPC profiles recorded during cultivation with poly(*cis*-1,4-isoprene) as the sole source of carbon and energy was surprising. However, nonrandom endocleavage cannot be excluded. The occurrence of distinct intermediates may be due to a particular molecular conformation of the poly(*cis*-1,4-isoprene) molecules in the solid material preventing random endocleavage of the polymer. In this case, the

FIG. 5. Proposed cleavage of poly(*cis*-1,4-isoprene) by strains expressing *lcp*. Two individual cleaving reactions presumably catalyzed by Lcp result in the formation of a bifunctional isoprenoid species terminated with a keto function (57 Da) and an aldehyde function (43 Da). The molecular mass of an intermediate with *n* 68-Da isoprene units can be calculated as $(43 + 57) + n \times 68$.

polydispersity of the remaining poly(*cis*-1,4-isoprene) should increase, and this should result in a significant broadening of the peak. The GPC profiles recorded from the chloroform-soluble rubber fractions of cultures of isolates S3 and E5 significantly differed from those previously described by Bode et al. (5, 6). They reported that the molecular weight of the rubber material decreased continuously, and they did not observe the appearance of intermediates of a distinct size. Despite this, the data on the chemical groups occurring in the cleavage products reported in this study are in full agreement with previous reports from other laboratories (5, 6, 7, 21) and our laboratory (12, 15).

Previously, *lcp* from *Streptomyces* sp. strain K30 was identified to be responsible for poly(*cis*-1,4-isoprene) degradation. Since species of *Streptomyces* and related genera differ from mycolic acid-containing *Actinobacteria* regarding their strategy for degradation of rubber, the occurrence of genes homologous to *lcp* in *N. farcinica* strains was surprising. However, *lcp* from *N. farcinica* strain E1 also conferred cleavage of poly(*cis*-1,4-isoprene) on *S. lividans* TK23. This indicates that Lcp homologues are responsible for the initial rubber degradation by both clear-zone-forming and adhesively growing *Actinobacteria*. Therefore, differences in growth behavior on polyisoprene substrates are influenced by additional, still-unknown factors. The lack of similarities between Lcp of *Actinobacteria* (reference 14 and this study) and RoxA of *Xanthomonas* sp. strain 35Y (7) also indicates that gram-positive and gram-negative bacteria have evolved different types of enzymes for rubber biodegradation. However, it has to be considered that *N. farcinica* is an opportunistic pathogen, which will restrict its use for biotechnological applications.

ACKNOWLEDGMENTS

We are grateful for financial support provided by the Deutsche Bundesstiftung Umwelt (Osnabrück, Germany) in the context of an ICBIO project (AZ. 13072). This work was also supported in part by a grant from the Egyptian Government Fellowship Program.

We are indebted to Klaus Bergander (Organisch-Chemisches Institut, WWU-Münster, Germany) for performing H-NMR analysis.

REFERENCES

1. Arenskötter, M., D. Baumeister, M. M. Berekaa, G. Pötter, R. M. Kroppenstedt, A. Linos, and A. Steinbüchel. 2001. Taxonomic characterization of two rubber degrading bacteria belonging to the species *Gordonia polyisoprenivorans* and analysis of hyper variable regions of 16S rDNA sequences. *FEMS Microbiol. Lett.* **205**:277–282.
2. Arenskötter, M., D. Bröker, and A. Steinbüchel. 2004. Biology of the metabolically diverse genus *Gordonia*. *Appl. Environ. Microbiol.* **70**:3195–3204.
3. Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struni. 1987. Current protocols in molecular biology, vol. 1. John Wiley & Sons, New York, N.Y.
4. Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**:1513–1523.
5. Bode, H. B., A. Zeeck, K. Plückhahn, and D. Jendrossek. 2000. Physiological and chemical investigations into microbial degradation of synthetic poly(*cis*-1,4-isoprene). *Appl. Environ. Microbiol.* **66**:3680–3685.
6. Bode, H. B., K. Kerkhoff, and D. Jendrossek. 2001. Bacterial degradation of natural and synthetic rubber. *Biomacromolecules* **2**:295–303.
7. Braaz, R., P. Fischer, and D. Jendrossek. 2004. Novel type of heme-dependent oxygenase catalyzes oxidative cleavage of rubber (poly-*cis*-1,4-isoprene). *Appl. Environ. Microbiol.* **70**:7388–7395.
8. Gallert, C. 2000. Degradation of latex and of natural rubber by *Streptomyces* strain La 7. *Syst. Appl. Microbiol.* **23**:433–441.
9. Kieser, T., M. J. Bipp, M. J. Buttner, K. F. Chater, and D. A. Hopwood. 2000. Practical streptomyces genetics. John Innes Foundation, Norwich, United Kingdom.
10. Linos, A., and A. Steinbüchel. 1998. Microbial degradation of natural and synthetic rubber by novel bacteria belonging to the genus *Gordonia*. *Kautsch. Gummi Kunstst.* **51**:496–499.
11. Linos, A., M. M. Berekaa, A. Steinbüchel, K. K. Kim, C. Spröer, and R. M. Kroppenstedt. 2002. *Gordonia westfalica* sp. nov., a novel rubber-degrading actinomycete. *Int. J. Syst. Evol. Microbiol.* **52**:1133–1139.
12. Linos, A., M. M. Berekaa, R. Reichelt, U. Keller, J. Schmitt, H. C. Flemming, R. M. Kroppenstedt, and A. Steinbüchel. 2000. Biodegradation of poly(*cis*-1,4-isoprene) rubbers by distinct actinomycetes: microbial strategies and detailed surface analysis. *Appl. Environ. Microbiol.* **66**:1639–1645.
13. Rainey, F. A., N. Ward-Rainey, R. M. Kroppenstedt, and E. Stackebrandt. 1996. The genus *Nocardiopsis* represents a phylogenetically coherent taxon and a distinct actinomycete lineage: proposal of *Nocardiopsaceae* fam. nov. *Int. J. Syst. Bacteriol.* **46**:1088–1092.
14. Rose, K., and A. Steinbüchel. 2005. Biodegradation of natural rubber and related compounds: recent insights into a hardly understood catabolic capability of microorganisms. *Appl. Environ. Microbiol.* **71**:2803–2812.
15. Rose, K., K. B. Tenberge, and A. Steinbüchel. 2005. Identification and characterization of genes from *Streptomyces* sp. strain K30 responsible for clear zone formation on natural rubber latex and poly(*cis*-1,4-isoprene) rubber degradation. *Biomacromolecules* **6**:180–188.
16. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. Schlegel, H. G., H. Kaltwasser, and G. Gottschalk. 1961. Ein Submersverfahren zur Kultur wasserstoffoxidierender Bakterien: Wachstumsphysiologische Untersuchungen. *Arch. Mikrobiol.* **38**:209–222.
18. Söhngen, N. L., and J. G. Fol. 1914. Die Zersetzung des Kautschuks durch Mikroorganismen. *Zentbl. Bakteriol. Abt. II* **40**:87–98.
19. Stackebrandt, E., F. A. Rainey, and N. L. Ward-Rainey. 1997. Proposal for a new hierarchic classification system, Actinobacteria classis nov. *Int. J. Syst. Bacteriol.* **47**:479–491.
20. Taylor, I. N., R. C. Brown, M. Bycroft, G. King, J. A. Littlechild, M. C. Lloyd, C. Praquin, H. S. Toogood, and S. J. C. Taylor. 2004. Application of thermophilic enzymes in commercial biotransformation processes. *Biochem. Soc. Trans.* **32**:290–292.
21. Tsuchii, A., T. Suzuki, and K. Takeda. 1985. Microbial degradation of natural rubber vulcanizates. *Appl. Environ. Microbiol.* **50**:965–970.
22. Zhang, Z., Y. Wang, and J. Ruan. 1998. Reclassification of *Thermomonospora* and *Microtetraspora*. *Int. J. Syst. Bacteriol.* **48**:411–422.