Bacterial degradation of poly(*trans*-1,4-isoprene) (gutta percha)

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Gutta percha, the *trans*-isomer of polyisoprene, is being used for several technical applications due to its resistance to biological degradation. In the past, several attempts to isolate micro-organisms capable of degrading chemically pure poly(*trans*-1,4-isoprene) have failed. This is the first report on axenic cultures of bacteria capable of degrading gutta percha. From about 100 different habitats and enrichment cultures, six bacterial strains were isolated which utilize synthetic poly(*trans*-1,4-isoprene) as sole carbon and energy source for growth. All isolates were assigned to the genus *Nocardia* based on 16S rRNA gene sequences. Four isolates were identified as strains of *Nocardia nova* (L1b, SH22a, SEI2b and SEI5a), one isolate was identified as a strain of *Nocardia jiangxiensis* (SM1) and the other as a strain of *Nocardia takedensis* (WE30). In addition, the type strain of *N. takedensis* obtained from a culture collection (DSM 44801^T) was shown to degrade poly(*trans*-1,4-isoprene). Degradation of poly(*trans*-1,4-isoprene) by these seven strains was verified in mineralization experiments by determining the release of CO₂. All seven strains were also capable of mineralizing poly(*cis*-1,4-isoprene) and to use this polyisoprenoid as a carbon and energy source for growth. Mineralization of poly(*trans*-1,4-isoprene) after 80 days varied from 3 % (strain SM1) to 54 % (strain SEI2b) and from 34 % (strain L1b) to 43 % (strain SH22a) for the *cis*-isomer after 78 days. In contrast, *Gordonia polyisoprenivorans* strain VH2, which was previously isolated as a potent poly(*cis*-1,4-isoprene)-degrading bacterium, was unable to degrade poly(*trans*-1,4-isoprene). Scanning electron microscopy revealed cavities in solid materials prepared from poly(*trans*-1,4-isoprene) and also from poly(*cis*-1,4-isoprene) after incubation with *N. takedensis* strain WE30 or with *N. nova* strain L1b, whereas solid poly(*trans*-1,4-isoprene) material remained unaffected if incubated with *G. polyisoprenivorans* strain VH2 or under sterile conditions.

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INTRODUCTION

Two types of polyisoprenoides, which differ according to their isomerism, are almost exclusively synthesized by many plants: poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene). The *cis* isomer (natural rubber, NR) is produced by more than 2000 dicotyledonous plants (Backhaus, 1985) and is an industrially important polymer used for many technical applications such as fabrication of automobile tyres. The *trans* isomer (gutta percha, GP) is synthesized by much

fewer plants and occurs, for example, in South-east Asian trees *Palaquium gutta* and *Eucommia ulmoides*, the European shrub *Euonymus europaeus* and the South American tree *Couma macrocarpa*. GP is used for several applications due to its resistance to biological degradation. This polymer has been used since the last century as an insulation material for transatlantic telegraph cables, and it is still used for the production of conveyers, golf balls, decoration objects, chewing gum and in dentistry to fill root canals.

First investigations on the biodegradation of NR were carried out in the early 20th century (Söhngen & Fol, 1914; for a review, see Rose & Steinbüchel, 2005), but only recently have two genes encoding enzymes capable of poly(*cis*-1,4-isoprene) cleavage been isolated, independently from different bacteria. In the NR-degrading bacterium

Abbreviations: GP, gutta percha; NR, natural rubber.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences reported in this paper are DQ840025 (*N. takedensis* WE30), DQ840027 (*N. jiangxiensis* SM1), DQ840026 (*N. nova* L1b), DQ840028 (*N. nova* SH22a), DQ840029 (*N. nova* SEI2b) and DQ840030 (*N. nova* SEI5a).

Xanthomonas sp. 35Y, RoxA was identified (Braaz *et al.*, 2004) which acts as a dioxygenase on poly(*cis*-1,4-isoprene), resulting in the formation of short-chain isoprenoid intermediates. Lcp (Latex-clearing protein) was identified in *Streptomyces* sp. K30, and the *lcp* gene restored the wild-type phenotype in a chemically induced rubber-negative mutant of this bacterium lacking the ability to degrade NR as indicated by the absence of haloes if grown on NR latex-containing agar plates (Rose *et al.*, 2005). Intermediates resulting from cleavage of poly(*cis*-1,4-isoprene) by actinomycetes harbouring Lcp have been identified as isoprenoids with approximately 20 isoprene units (Ibrahim *et al.*, 2006). Apart from the number of isoprene moieties of the cleavage products, intermediates of poly(*cis*-1,4-isoprene) degradation produced by RoxA and Lcp exhibit a similar chemical structure, possessing one terminal ketone and one terminal aldehyde functional group. However, RoxA and Lcp share no sequence homologies, indicating two individually evolved enzyme systems for rubber cleavage. So far no proteins homologous to RoxA have been identified in bacteria other than *Xanthomonas* sp. 35Y, whereas the presence of genes encoding Lcp homologues has been shown in the genomes of several actinomycetes capable of rubber degradation. In *Streptomyces* sp. K30, two ORFs (*oxiA* and *oxiB*), putatively encoding a heterodimeric molybdenum-dependent aldehyde dehydrogenase, are located downstream of *lcp*. Recombinant strains of *Streptomyces lividans* TK23 expressing *lcp* and *oxiBA* do not accumulate aldehydes around the colonies if grown on NR latex as was observed for strains expressing only *lcp*. As revealed by transposon-induced rubber-negative mutants of *Gordonia polyisoprenivorans*, degradation intermediates of poly(*cis*-1,4-isoprene) enter the central metabolism via β -oxidation with the involvement of a specific α -methylacyl-coenzyme A race-mase (Banh *et al.*, 2005).

In contrast to NR degradation, no micro-organisms capable of degrading poly(*trans*-1,4-isoprene) have been described to date. In addition, only very few studies have been

published on microbial degradation of GP. Kupletskaya *et al.* (1960) tried to demonstrate biodegradability of GP by fermentation studies of *Eucommia* leaves; however, the micro-organisms isolated were incapable of degrading chemically pure poly(*trans*-1,4-isoprene). The weight losses of blends of poly(*trans*-1,4-isoprene) and starch observed by Arvanitoyannis *et al.* (1998) were solely due to degradation of the starch. So far, cleavage of either isomer of polyisoprene has only been demonstrated *in vitro* employing enzyme-mediator systems like lipooxygenase/linoleic acid or laccase/1-hydroxybenzotriazole/linoleic acid as radical producers (Enoki *et al.*, 2003). This study aimed to isolate and characterize axenic cultures of poly(*trans*-1,4-isoprene)-degrading bacteria and to compare microbial degradation of GP and NR.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Cells of *G. polyisoprenivorans* VH2, *Nocardia* sp. and *Rhodococcus opacus* PD630 were cultivated at 30 °C in Standard I nutrient broth (St-I; Merck), Caso medium (Merck) or mineral salts medium (MSM) as described by Schlegel *et al.* (1961). The following polyisoprenoids were added as carbon sources to liquid MSM: 0.6 % (w/v) cryomilled synthetic poly(*trans*-1,4-isoprene) with a mean molecular mass of approximately 410 kDa (Aldrich) or 0.6 % (w/v) milled synthetic poly(*cis*-1,4-isoprene) with a mean molecular mass of 800 kDa (Aldrich). Synthetic poly(*cis*-1,4-isoprene) was milled in the presence of inert talcum (3MgO.4SiO₂.H₂O) in the ratio 1:1 to prevent coagulation of the polymer particles and was added before autoclaving, whereas poly(*trans*-1,4-isoprene) was frozen in liquid nitrogen before cryomilling in a ZM200 rotation mill (Retsch), yielding particles with grain sizes between 62 µm and 1.4 mm. GP was sterilized with 70 % (v/v) ethanol, added after autoclaving and subsequently evaporated in a vacuum. For mineralization experiments, poly(*trans*-1,4-isoprene) and poly(*cis*-1,4-isoprene) with a defined range of grain size was obtained by sieving in an AS200 sieve shaker (Retsch).

Liquid cultures in Erlenmeyer flasks were incubated on a horizontal rotary shaker. Solid media were prepared by the addition of 1.5 % (w/v) agar. NR latex overlay agar plates, for characterizing the ability to

Table 1. Strains used in this study

Strain	Characteristics/origin	Reference
<i>Gordonia polyisoprenivorans</i> VH2	NR-degrading wild-type	DSM 44266
<i>Rhodococcus opacus</i> PD630	Degrades no polyisoprene	DSM 44193
<i>Nocardia takedensis</i>	NR- and GP-degrading wild-type isolated from activated sludge	DSM 44801 ^T
<i>Nocardia takedensis</i> WE30	NR- and GP-degrading wild-type isolated from soil of Amazon rainforest	This study
<i>Nocardia nova</i> L1b	NR- and GP-degrading wild-type isolated from soil of a <i>Euonymus europaeus</i> population in Lingen, Germany	This study
<i>Nocardia nova</i> SH22a	NR- and GP-degrading wild-type isolated from a root of <i>Couma macrocarpa</i> (Brazil)	This study
<i>Nocardia nova</i> SEI2b	NR- and GP-degrading wild-type isolated from soil below a <i>Couma macrocarpa</i> tree (Brazil)	This study
<i>Nocardia nova</i> SEII5a	NR- and GP-degrading wild-type isolated from soil below a <i>Couma macrocarpa</i> tree (Brazil)	This study
<i>Nocardia jiangxiensis</i> SM1	NR- and GP-degrading wild-type isolated from latex of <i>Couma macrocarpa</i> (Brazil)	This study

produce translucent haloes by degradation of the water-insoluble polymers, were prepared as described previously (Ibrahim *et al.*, 2006); GP latex from the South American tree *Couma utilis* was applied accordingly.

Enrichment and isolation of GP-degrading bacteria. Soil and plant samples were taken from different habitats of autochthonous *Euonymus europaeus* populations in Germany and also from different areas with *Couma macrocarpa* populations in the Amazon rainforest in Brazil. Samples were incubated in 50 ml MSM medium containing 0.6% (w/v) cryomilled poly(*trans*-1,4-isoprene). If growth was macroscopically detected by an increase in turbidity, several strains of the respective cultures were brought to axenic cultures on solid St-I medium. The strains obtained were then individually tested in liquid culture for their capability to degrade synthetic poly(*trans*-1,4-isoprene). Purity of cultures capable of GP degradation was verified both by smearing on St-I medium and by direct determination of 16S rRNA gene sequences (see below).

Taxonomic studies. Gram-staining was performed according to Gerhardt *et al.* (1994). Bactident oxidase test strips were applied for oxidase testing (Merck). Catalase activity was determined by adding 3% (v/v) H₂O₂ to a freshly grown colony. Cleavage of L-alanyl-nitroanilid was tested by applying Bactident LAAP test strips (Merck).

Isolation of total DNA. A sample (1.5 ml) of a well grown culture in MSM containing poly(*trans*-1,4-isoprene) was harvested by centrifugation and resuspended in 500 µl SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris/HCl, pH 7.5) containing 3 mg lysozyme ml⁻¹. After 2 h incubation at 37 °C, 50 µl of an SDS solution (100 g l⁻¹) and 20 µl of a proteinase K solution [20 g (l TE)⁻¹; TE, 1 mM EDTA and 10 mM Tris/HCl, pH 8.0] were added and mixed gently, followed by another incubation for 2 h at 55 °C. Subsequently, 0.33 vols of a 5 M NaCl solution and 1 vol. chloroform were added and mixed gently. After 30 min incubation at room temperature, the phases were separated by centrifugation and the aqueous phase was transferred to a fresh tube. DNA was then precipitated by the addition of 0.7 vols 2-propanol and the precipitate was washed twice with 70% (v/v) ethanol. After drying it was dissolved in an appropriate volume of TE buffer containing 0.01% (v/v) RNase A solution (10 mg ml⁻¹).

Determination of 16S rRNA gene sequence. Amplification of 16S rRNA genes was done by PCR using *Taq* DNA-polymerase (Invitrogen) and the primers 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3'). Purification of the PCR product was carried out as described previously (Rainey *et al.*, 1996). The purified 16S rRNA gene was then directly sequenced using the following primers: 27f (5'-GAGTTTGATCCTGGCTCAG-3'), 343r (5'-CTGCTGCCTCCGTA-3'), 357f (5'-TACGGGAGGCAGCAG-3'), 519r [5'-G(T/A)ATTACCGCGG(C/T/G)GCTG-3'], 536f [5'-CAGC(C/A)GCCGCGGTAAT(T/A)C-3'], 803f (5'-ATTAGATACCCTGGTAG-3'), 907r (5'-CCGTC-AATTCATTTGAGTTT-3'), 1114f (5'-GCAACGAGCGCAACCC-3'), 1385r [5'-CGGTGTGT(A/G)CAAGGCC-3'] and 1525r (5'-AGAAAGGAGGTGATCCAGCC-3') applying the Big Dye 3.1 kit and a 3730 DNA capillary sequence analyser (Applied Biosystems). Sequences were aligned manually with published sequences from representative actinomycetes obtained from EMBL. BLASTN was used to determine the percentage of identical nucleotides to 16S rRNA gene sequences in the GenBank database. Phylogenetic trees were constructed by applying CLUSTAL X software (www-igbmc.ustrasbg.fr/BioInfo/; Thompson *et al.*, 1997) using the neighbour-joining method (Saitou & Nei, 1987) and were visualized by NJPLOT (ftp://pbil.univ-lyon1.fr/pub/mol_phylogeny/njplot; Perrière & Gouy, 1996).

Determination of percentage mineralization. Evidence for biodegradation of the poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene) hydrocarbon chains to CO₂ was obtained by determination of respiratory CO₂ released during cultivation of cells in the presence of polyisoprene as sole carbon source. Determination was carried out in tightly closed Erlenmeyer flasks by using the property of Ba(OH)₂ to precipitate CO₂ as BaCO₃. The flasks, containing 100 ml MSM medium, 0.6% milled poly(*cis*-1,4-isoprene) or poly(*trans*-1,4-isoprene), and a test tube, containing 15 ml of a 0.2 M Ba(OH)₂ solution, were inoculated with 25 or 40 mg cells (fresh wt) of a culture grown for 48 h. At each measuring point, the flasks were aerated, and the test tubes were replaced by new tubes containing fresh Ba(OH)₂ solution. Consumption of HCl for precipitation of CO₃²⁻ as BaCO₃ was determined for each period by titration with 0.1 M HCl according to the following equation, yielding the percentage mineralization.

$$\text{Mineralization}(\% \text{ CO}_2) = \frac{\text{Required amount of HCl (ml)} \times 0.1}{\text{C content of amount of poly(1,4-isoprene) applied (mmol)} \times 2}$$

As indicator, phenolphthalein was added (20 µl of a 1%, w/v, solution in 2-propanol) and the end point of titration was determined by alteration of the colour from magenta to uncoloured. A non-inoculated Erlenmeyer flask was handled in the same way as control.

Scanning electron microscopy (SEM). Deterioration of samples of poly(*cis*-1,4-isoprene) and poly(*trans*-1,4-isoprene) by selected strains was verified by SEM. A sample (40 ml) of a 1% solution of poly(*trans*-1,4-isoprene) in chloroform was placed into a glass Petri dish to obtain a thin polymer film after evaporation of the solvent. The film was then cut into pieces. Poly(*cis*-1,4-isoprene) was applied as cubic pieces with a side length of about 1 cm. MSM cultures (200 ml) containing 1 g milled GP plus thin GP squares or 1 g milled poly(*cis*-1,4-isoprene) plus the cubes of poly(*cis*-1,4-isoprene) were inoculated with 70 mg cell fresh weight of the respective strain grown for 48 h in St-I medium. The polymer pieces were withdrawn from their culture vessel after the incubation periods indicated in the text and were then washed with distilled water or used directly for SEM. Samples were gold-sputtered with an Emitech vacuum sputter device K550x (Ashford) and examined with a Hitachi S-3000N scanning electron microscope at 20 kV under high vacuum conditions. Micrographs were recorded digitally.

RESULTS

Isolation of GP-degrading bacterial strains

At the beginning we undertook several unsuccessful attempts to enrich GP-degrading bacteria from various soil samples, streets, sewage sludge and other places. We then used soil samples taken from the neighbourhood of GP-producing plants, expecting that in decaying plant material GP-degrading bacteria would naturally be enriched. In total, more than 100 samples were taken from different habitats to start enrichment and isolation of GP-degrading bacteria.

Numerous enrichment cultures yielded only 13 poly(*trans*-1,4-isoprene)-degrading strains in total. Some strains, which were isolated from different enrichment cultures but inoculated with the same environmental sample, turned

out to be identical, as revealed by 16S rRNA gene sequencing. Thus, only six strains, differing in their 16S rRNA gene sequences, were obtained and subsequently characterized. Five of these different strains were isolated from cultures inoculated with samples received from *C. utilis* populations in the Amazon rainforest in Brazil, and only one strain was isolated from a culture inoculated with a soil sample from an autochthonous *E. europaeus* population in Germany (for details, see Table 1). No bacterial strains capable of GP degradation could be isolated from samples taken from autochthonous *E. europaeus* populations located near the villages Wustrow, Trochel, Freude and Bleckede (Germany), from a wastewater treatment plant (Münster, Germany), from a composting plant (Münster, Germany) and from more than 30 samples taken from unassigned locations in the Amazon rainforest in Brazil. All newly isolated bacterial strains colonized the surface of the GP grains in liquid cultures (data not shown). Later it was shown that the isolates grew adhesively on poly(*cis*-1,4-isoprene) particles as has been described for species of *Gordonia* and *Nocardia* (Arenskötter *et al.*, 2004; Ibrahim *et al.*, 2006). These strains exhibited no clear zone formation when cultivated on overlay agar plates prepared from GP or NR latex.

Taxonomic classification

In liquid MSM cultures containing GP as sole source of carbon and energy, all strains exhibited coccoid growth, and strains WE30, L1b and SH22a also formed mycelia on solid complex St-I or Caso medium. Growth was clearly reduced at temperatures above 30 °C and no growth was observed at temperatures higher than 37 °C. All strains were Gram-positive and non-motile; no spores could be detected. Oxidase and LAAP tests were negative for all strains, but the catalase test demonstrated enzyme activity.

The sequences of complete 16S rRNA genes were determined as described in Methods to characterize the taxonomic position of the novel isolates in more detail. The new sequences were aligned with sequences deposited in GenBank to determine closely related species. The 16S rRNA gene sequence of *N. takedensis* WE30 shared highest similarity (99.9 %) with that of *Nocardia takedensis* DSM 44801^T which was previously isolated from moat sediment and activated sludge (Yamamura *et al.*, 2005). Isolate WE30 is therefore referred to as *N. takedensis* WE30. The 16S rRNA gene sequences of the isolates L1b, SH22a, SEI2b and SEII5a exhibited highest similarities (99.8, 98.9, 98.7 and 99.5 %, respectively) to that of *Nocardia nova* DSM 44481^T (Wallace *et al.*, 1991). These strains are therefore referred to as *N. nova* strains L1b, SH22a, SEI2b and SEII5a, respectively. The 16S rRNA gene sequence of strain SM1 shared highest similarities (99.3 %) with that of *Nocardia jiangxiensis* DSM 17684^T (Cui *et al.*, 2005). This isolate is therefore referred to as *N. jiangxiensis* SM1. The phylogenetic tree shown in Fig. 1 illustrates the relationship between the novel isolates to all validly described type strains of the genus *Nocardia*. Since 16S rRNA analysis identified *N. takedensis* DSM 44801^T as the type strain with the closest relationship

to *N. takedensis* WE30, this strain was also investigated for its ability to degrade GP.

Quantification of poly(*trans*-1,4-isoprene) and poly(*cis*-1,4-isoprene) degradation

The capability of the newly isolated bacteria to utilize both isomers of poly(1,4-isoprene) as a carbon source had only been detected macroscopically up to this point. Thus, this property required verification by determining the respiratory evolution of CO₂ during growth on ground material of the respective polymer. Since GP is a solid and water-insoluble material, only the surface of poly(*trans*-1,4-isoprene) is expected to be exposed to microbial attack. Consequently, an increase in the available surface of the polymer should enhance the degradation rate, even if the total weight of the applied material is kept constant. The polymer was cryomilled to examine the effect of the surface area on the degradation rates of poly(*trans*-1,4-isoprene). The granules thus obtained were then sieved applying a Retsch AS 200 basic sieve shaker to obtain granule fractions with defined grain sizes of 125–250, 250–500, 500–710, 710–1000 and 1250–1400 µm, respectively. For each grain, a 100 ml MSM culture containing 0.6 g of the respective granule fraction was inoculated with 40 mg fresh cell weight of *N. takedensis* WE30, and mineralization was measured comparatively to observe the dependence of degradation on the available GP surface. Fig. 2(a) clearly illustrates a correlation between the size of the supplied granules and the extent of GP degradation by *N. takedensis* WE30. If smaller particles were provided, mineralization proceeded more rapidly when compared to cultures with larger granules. In cultures with GP grains of 125–250 µm, 40 % of the substrate was metabolized to CO₂ within 78 days, while only 25 % of the poly(*trans*-1,4-isoprene) granules of 1250–1400 µm was mineralized in the same period by *N. takedensis* WE30. This indicated that only the surface of the material is accessible to microbial attack, as is usually the case with solid biodegradable materials.

The capability of the newly isolated *Nocardia* strains and of *N. takedensis* DSM 44801^T to utilize both isomers of poly(1,4-isoprene) as carbon source was then compared in mineralization experiments. The highest mineralization values of poly(*trans*-1,4-isoprene) were obtained with *N. nova* strains L1b and SEI2b, and with *N. takedensis* WE30. After 80 days incubation, these strains had metabolized about 42–54 % of the supplied GP to CO₂ (Fig. 2b). The type strain of *N. takedensis* mineralized only about 20 % of the GP during the same period, and the slowest rate of degradation was observed with *N. jiangxiensis* SM1 which released only about 3 % of the poly(*trans*-1,4-isoprene) as CO₂ (Fig. 2b). Mineralization of poly(*trans*-1,4-isoprene) by cultures of *G. polyisoprenivorans* VH2 and *R. opacus* PD630 was also measured as controls. *G. polyisoprenivorans* VH2 has been characterized as a potent degrader of poly(*cis*-1,4-isoprene) by Linos & Steinbüchel (1998), but was incapable of degrading poly(*trans*-1,4-isoprene) (Fig. 2b). Also, *R. opacus* PD630 did not degrade poly(*trans*-1,

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences (1346 bp) of all type strains of validly described *Nocardia* species and of the novel bacterial isolates capable of degrading poly(*trans*-1,4-isoprene). Numbers at nodes indicate the level of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets; only values above 50% are given. Bar, 0.005 substitutions per nucleotide position.

4-isoprene) as indicated by mineralization studies (Fig. 2b). In addition, no growth on poly(*trans*-1,4-isoprene) could be detected macroscopically for these two bacteria.

In the past, numerous bacterial strains have been described that degrade both synthetic poly(*cis*-1,4-isoprene) and NR (Jendrossek *et al.*, 1997; for review, see Rose & Steinbüchel, 2005); however, these strains do not degrade the *trans* isomer GP as revealed from a laboratory screening of several of them (unpublished data). In this study we decided to

clarify if the novel GP-degrading strains and *N. takedensis* DSM 44801^T also are capable of degrading poly(*cis*-1,4-isoprene). Therefore, all six poly(*trans*-1,4-isoprene)-degrading strains and *N. takedensis* DSM 44801^T were tested for their ability to utilize poly(*cis*-1,4-isoprene) as a source for carbon and energy, and degradation was compared by mineralization experiments to that of *G. polyisoprenivorans* VH2 (Fig. 2c). All the novel *Nocardia* strains and *N. takedensis* DSM 44801^T were indeed able to utilize poly(*cis*-1,4-isoprene) very efficiently and metabolized

this polymer for the most part even more rapidly than *G. polyisoprenivorans* VH2. The highest degree of mineralization after 78 days was obtained with *N. nova* SH22a (43%). *N. nova* L1b metabolized only 34% of the poly(*cis*-1,4-isoprene) to CO₂, but was also more efficient than *G. polyisoprenivorans* VH2 (25%). However, the values for mineralization of poly(*cis*-1,4-isoprene) by the novel

Fig. 2. Percentage mineralization during growth of bacteria on polyisoprenoids. (a) Effect of GP granule size on mineralization. Cultures (100 ml) in mineral salts medium containing 0.6% (w/v) synthetic poly(*trans*-1,4-isoprene) with a defined granule size (●, 125–250; ○, 250–500; ▼, 500–710; △, 710–1000; ■, 1250–1400 µm) were inoculated with 40 mg cell fresh weight of isolate WE30. (b) Mineralization of GP by novel GP-degrading strains. Cultures (100 ml) in mineral salts medium containing 0.6% (w/v) synthetic poly(*trans*-1,4-isoprene) (granule size, 250–500 µm) were inoculated with 25 mg fresh cell weight of the respective strains (□, L1b; ◇, *N. takedensis*; ○, SEI2b; ▲, SH22a; ●, SEI5a; △, WE30; ◆, SM1), or of *G. polyisoprenivorans* VH2 (■) or *R. opacus* PD630 (▼) as negative controls. (c) Mineralization of poly(*cis*-1,4-isoprene) rubber by novel GP-degrading strains and by the NR-degrading strain *G. polyisoprenivorans* VH2. Cultures (100 ml) in mineral salts medium containing 0.6% (w/v) synthetic poly(*cis*-1,4-isoprene) rubber (granule size, 250–500 µm) were inoculated with 40 mg cell fresh weight of the respective strains (□, L1b; ◇, *N. takedensis*; ○, SEI2b; ◆, SM1; ▲, SH22a; ●, SEI5a; △, WE30; ■, *G. polyisoprenivorans*). All values represent the arithmetic mean of two individual cultures. The deviation from the arithmetic mean was 11.8% for all measurements with an SD of 18.7%.

GP-degrading strains diverged to a lesser extent than observed for their mineralization of the *trans* isomer.

Visualization of GP and rubber degradation by SEM

Films of GP and cubes of synthetic poly(*cis*-1,4-isoprene) were incubated for 1, 3 and 5 weeks with cells of *N. takedensis* WE30 and *N. nova* L1b, and were then examined by SEM to observe the degradation of poly(*trans*-1,4-isoprene) and poly(*cis*-1,4-isoprene) visually in a chronological sequence (Figs 3 and 4). GP films incubated with cells of *N. takedensis* WE30 exhibited a few cavities with diameters of approximately 10 µm after only 1 week (Fig. 3a). After 3 weeks incubation, approximately 20 cavities mm⁻² with diameters of about 30 µm were observed (Fig. 3b). After 5 weeks incubation, the surfaces of the GP films had almost totally deteriorated; cavities were confluent and separated cavities could hardly be distinguished (Fig. 3c); only a few holes with diameters of about 60 µm permeating the entire GP film were visible. This progression was also observed in cultures of *N. nova* L1b. In the samples withdrawn after 1 week incubation, signs of degradation were hardly visible by SEM (Fig. 3e), whereas after 3 weeks incubation, the material exhibited approximately 15 cavities mm⁻² with diameters of about 20 µm (Fig. 3f). The numbers and diameters of the cavities continued to increase, and after 5 weeks incubation with *N. nova* L1b approximately 50 holes mm⁻² with diameters of about 40 µm were observed (Fig. 3g). In contrast, GP films of a non-inoculated control exhibited no evidence of deterioration (Fig. 3d). Consequently, the appearance of cavities was clearly due to microbial attack of poly(*trans*-1,4-isoprene) by *N. takedensis* WE30 and *N. nova* L1b. In addition, the GP films exhibited

Fig. 3. Scanning electron micrographs of GP films after incubation with micro-organisms. GP films were incubated with cells of *N. takedensis* WE30 (a–c) and *N. nova* L1b (e–g). Films were incubated for 1 (a, e), 3 (b, f) and 5 weeks (c, g). (h) Surface of a GP film after 5 weeks incubation in the presence of *G. polyisoprenivorans*. (d) Surface of a GP film after 5 weeks incubation in a non-inoculated sterile control. The circle in (a) highlights a cavity which is difficult to recognize in this stage. Bars, 200 μm .

Fig. 4. Scanning electron micrographs of the surface of poly(*cis*-1,4-isoprene) particles after incubation with *G. polyisoprenivorans* VH2 (b), *N. takedensis* WE30 (c) and *N. nova* L1b (d) for 5 weeks. (a) Surface of poly(*cis*-1,4-isoprene) after 5 weeks incubation under the same conditions in the absence of bacterial cells. Bars, 200 µm.

no cavities or other signs of degradation when incubated with cells of *G. polyisoprenivorans* VH2 (Fig. 3h), thus confirming the inability of this poly(*cis*-1,4-isoprene)-degrading bacterium to degrade the *trans* isomer.

The surfaces of the poly(*cis*-1,4-isoprene) cubes, as imaged by SEM after an incubation period of 5 weeks, are presented in Fig. 4(a–d). In the absence of bacterial cells (uninoculated control), the surface was not modified (Fig. 4a). In contrast, cells of *G. polyisoprenivorans* VH2 caused remarkable deterioration as indicated by the appearance of large cavities in the rubber (Fig. 4b), as already shown for this strain (Linos *et al.*, 2000). The surfaces of the rubber particles were attacked to a similar extent by *N. takedensis* WE30 (Fig. 4c) and *N. nova* L1b (Fig. 4d), thereby confirming that these strains are also potent NR-degrading bacteria.

However, despite the adhesive growth of all the isolates on GP and poly(*cis*-1,4-isoprene), cells were never visible in the scanning electron micrographs. This is probably due to the procedure of sample preparation and due to only very weak adhesion of the cells to the polymer materials.

DISCUSSION

All previous efforts have failed to identify micro-organisms capable of degrading poly(*trans*-1,4-isoprene), also referred

to as GP. Vague hints for the biodegradability of poly(*trans*-1,4-isoprene) were obtained by fermentation of *Eucommia* leaves containing GP; however, it turned out that chemically pure poly(*trans*-1,4-isoprene) was not degraded (Kupletskaya *et al.*, 1960). Obviously, the growth of the micro-organisms in this case was only due to other compounds present in the leaves. Additionally, several bacterial strains well known as poly(*cis*-1,4-isoprene)-degraders cannot utilize the *trans* isomer of polyisoprene (unpublished data), and even purified RoxA, which is responsible for poly(*cis*-1,4-isoprene) cleavage by *Xanthomonas* sp. 35Y, was shown to be incapable of cleaving poly(*trans*-1,4-isoprene) *in vitro* (Braaz *et al.*, 2004). However, GP as a natural compound ought to be biodegradable (Steinbüchel, 2005). This assumption led us to investigate if poly(*trans*-1,4-isoprene)-degrading bacterial strains could be isolated from the habitats of plants synthesizing GP. This is the first report of micro-organisms which are unequivocally capable of degrading GP.

Although more than 100 environmental samples were investigated, bacterial growth could only be detected macroscopically in 6 enrichment cultures, each yielding one strain in axenic culture metabolizing poly(*trans*-1,4-isoprene). It is possible that cultivatable GP-degrading bacteria are endemic and occur only in habitats where

poly(*trans*-1,4-isoprene) is present due to decaying material from plants synthesizing GP. GP-utilizing bacteria are obviously not ubiquitously distributed in nature, otherwise previous investigations should have succeeded in isolating poly(*trans*-1,4-isoprene)-degrading strains.

Interestingly, all strains isolated in this study were taxonomically assigned to the genus *Nocardia* based on their 16S rRNA gene sequences. Based on these data the isolates L1b, SH22a, SEI2b and SEII5a were assigned as strains of *N. nova*, isolate WE30 as a strain of *N. takedensis* and isolate SM1 as a strain of *N. jiangxiensis*. These GP-degrading bacteria belong therefore to the mycolic-acid-containing *Actinobacteria* [*Corynebacterium*, *Mycobacterium*, *Nocardia* (CMN) group]. All GP-degrading bacteria were also able to degrade NR. In this regard they behaved like most rubber-degrading bacteria representing the so-called 'adhesively growing group' of rubber degraders (Linos *et al.*, 2000). In contrast to several NR-degrading streptomycetes, these GP-degrading bacteria do not produce translucent haloes on agar plates containing emulsified NR latex of *Hevea brasiliensis* or latex from the South American tree *Couma utilis* that contains both poly(*trans*-1,4-isoprene) and poly(*cis*-1,4-isoprene). Instead, they grew adhesively on both isomers if incubated in liquid cultures like all rubber-degrading species of the genus *Gordonia* do on NR (Arenskötter *et al.*, 2004).

Mineralization experiments clearly revealed that the GP-degrading isolates, as well as *N. takedensis* DSM 44801^T, metabolized synthetic poly(*trans*-1,4-isoprene), although they exhibited marked differences regarding the percentage of mineralization. Mineralization studies and SEM clearly revealed that the poly(*cis*-1,4-isoprene)-degrading bacterium *G. polyisoprenivorans* VH2 did not exhibit any detectable degradation of poly(*trans*-1,4-isoprene), and nor did the negative control *R. opacus* PD630. This confirmed previous observations that bacteria capable of degrading poly(*cis*-1,4-isoprene) do not degrade the *trans* isomer. In contrast, all poly(*trans*-1,4-isoprene)-degrading bacteria isolated described in this study and *N. takedensis* DSM 44801^T were also able to degrade poly(*cis*-1,4-isoprene). Therefore, the ability to degrade GP always seems to occur in combination with the capability to degrade NR. In general, biodegradation of both the *cis* and *trans* isomers is a similarly slow process. It will be interesting to determine if GP-degrading bacteria possess one unspecific enzyme system for degradation of both isomers of polyisoprenoides, or if two separate enzyme systems exist in these bacteria: one responsible for degradation of the *trans* isomer and the other for the *cis* isomer. In the latter case, it should be possible to isolate bacteria capable of utilizing only poly(*trans*-1,4-isoprene) but not poly(*cis*-1,4-isoprene). Previous observations that biodegradation of poly(*trans*-1,4-isoprene) is a rare process are supported by the fact that none of the many poly(*cis*-1,4-isoprene)-degrading bacteria isolated so far is able to degrade poly(*trans*-1,4-isoprene).

All newly isolated strains capable of degrading poly(*trans*-1,4-isoprene) colonized the surface of the water-insoluble polymer during growth in liquid cultures, as has been described for taxonomically related bacteria that degrade the *cis* isomer. Therefore, the surface area of GP particles or the surface to volume ratio affect the percentage of mineralization since only the exposed surface is supposed to be accessible to the bacteria and their enzymes. This was clearly demonstrated with granule fractions of GP varying in grain size; the highest percentage of mineralization was observed with particles of the smallest grain size (125–250 µm). Therefore, to obtain reliable and comparable quantitative data on the degradation of poly(*trans*-1,4-isoprene) or on degradation by different micro-organisms, the granule size should be kept within a defined and narrow range.

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