Microbial Gutta-Percha Degradation Shares Common Steps with Rubber Degradation by *Nocardia nova* SH22a

Quan Luo,a Sebastian Hiessel,a Anja Poehelein,b Alexander Steinbüchel,a,c

Institut für Molekulare Mikrobiologie und Biotechnologie, Westfälische Wilhelms-Universität Münster, Münster, Germany; Department of Genomic and Applied Microbiology and Göttingen Genomics Laboratory, Institut für Mikrobiologie und Genetik, Georg-August-Universität Göttingen, Göttingen, Germany; King Abdulaziz University, Faculty of Biology, Jeddah, Saudi Arabia.a

*Nocardia nova* SH22a, a bacterium capable of degrading gutta-percha (GP) and natural rubber (NR), was used to investigate the GP degradation mechanism and the relations between the GP and NR degradation pathways. For this strain, a protocol of electroporation was systematically optimized, and an efficiency of up to 4.3 × 10⁷ CFU per µg of plasmid DNA was achieved. By applying this optimized protocol to *N. nova* SH22a, a Tn5096-based transposon mutagenesis library of this bacterium was constructed. Among about 12,000 apramycin-resistant transformants, we identified 76 stable mutants defective in GP or NR utilization. Whereas 10 mutants were specifically defective in GP utilization, the growth of the other 66 mutants was affected on both GP and NR. This indicated that the two degradation pathways are quite similar and share many common steps. The larger number of GP-degrading defective mutants could be explained in one of two ways: either (i) the GP pathway is more complex and harbors more specific steps or (ii) the steps for both pathways are almost identical, but in the case of GP degradation there are fewer enzymes involved in each step. The analysis of transposition loci and genetic studies on interesting genes confirmed the crucial role of an α-methylacyl-coenzyme A racemase in the degradation of both GP and NR. We also demonstrated the probable involvement of enzymes participating in oxidoreduction reactions, β-oxidation, and the synthesis of complex cell envelope lipids in the degradation of GP.

Gutta-percha (GP) and natural rubber (NR) are both natural polymers of isoprene but differ from each other in the stereostructure. Whereas GP consists of poly(trans-1,4-isoprene) and is synthesized only by a few plants mainly occurring in the tropical areas, NR consists of poly(cis-1,4-isoprene) and is synthesized by more than 2,500 plant species and also some fungi (1). Both the trans and the cis isomers are now important application materials and are extensively used in various fields of our daily life. For example, due to the good insulation properties, plastic-like solidity, and excellent resistance to microbial decomposition, GP is used for the production of undersea telegraph cables and golf balls and to fill transiently root canals in dentistry, whereas rubber (natural and synthetic) is applied more abundantly to produce mobile tires, conveyers, latex gloves, rubber hoses, condoms, etc., due to its superior physical properties. Although we enjoy the benefits from these products, the increasing production of poly(trans-1,4-isoprene) and poly(cis-1,4-isoprene) also leads to an accretive problem of waste management. Burning is the most widely applied method for the waste treatment, but this is not desirable since this causes a secondary pollution (CO, NOₓ, and SO₂) of ambient air (2).

As a potential waste treatment method that could be more natural and clean, biodegradation of polyisoprene products has been studied since the beginning of the last century (3), but nearly all of the studies were restricted to rubber degradation. Many efforts were undertaken to isolate suitable rubber-degrading bacteria and to unravel the molecular mechanism of biodegradation. With solid-state polymers (vulcanized and unvulcanized rubber) or NR latex as substrates, more than 100 bacteria were isolated (4–7). Two biodegradation strategies have been unraveled thus far (7): (i) the adhesive growing group of bacteria with cells colonizing the surfaces of rubber substances and (ii) the latex clearing group of bacteria that produce translucent halos on NR latex agar plates (8). Moreover, several novel genes, such as *roxA* (rubber oxygenase gene) (9), *lcp* (latex clearing protein gene) (10), and *mcr* (α-methylacyl-coenzyme A [CoA] racemase gene) (11), were identified in succession, and a relatively complex degradation pathway was unraveled by experimental studies and by *in silico* genome analysis (12). In contrast, studies investigating GP biodegradation are rare. Until now, only six bacterial isolates were identified as being capable of degrading GP (13). The rubber-degrading ability was also observed in these strains. They were all taxonomically assigned to the genus *Nocardia*, of which some species are reported to be capable of degrading hydrocarbons such as *n*-alkanes, phenylalkanes, aliphatic alcohols, fatty acids, polycyclic aromatic compounds, and even beeswax (14–19). Mineralization experiments showed that the degradation efficiency depended significantly on the size of polymer grains in the medium, indicating that an adhesive growth strategy was used by these bacteria. More interestingly, it was observed that the GP-degrading ability always occurs in combination with the ability to degrade rubber, but not vice versa. This raised two possible explanations about the degradation pathway: (i) GP-degrading bacteria possess one unspecific enzyme system responsible for the degradation of both, the *trans* and the *cis* polymers, or (ii) two separate enzyme systems exist in these bacteria acting independently on each polymer (13).

The present study sought to unravel the GP degradation mechanism and the potential relations between the GP and NR degra-
dation pathways, so the GP- and NR-degrading bacterium *N. nova* SH22a (13) was chosen as a model organism for this. Application of transposon mutagenesis using Tn5096 was established for strain SH22a, and a library was constructed for screening mutants defective in GP and/or NR degradation. However, a systematic optimization of the electroporation efficiency had to be performed before transposon mutagenesis could be successfully applied. As far as we know, this is the first genetic study on a GP-degrading bacterium. We provide here a solid basis for further studies on the GP and NR degradation mechanisms.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and cultivation conditions.** Bacterial strains and plasmids used in the present study are listed in Table 1. If not stated otherwise, *N. nova* was grown in CASO broth (Carl Roth, Germany) at 30°C, whereas *Gordonia polyisoprenivorans* and *Escherichia coli* were grown in lysogeny broth (LB) at 30 and 37°C, respectively. For optimization of electroporation efficiency, nutrient broth (NB), brain heart infusion (BHI), and GYM (DSMZ; no. 65 GYM) were used. In screening and growth experiments, *Gordonia polyisoprenivorans* \( \text{SH22a} \) was cryomilled to a defined grain size \( \text{(-1,4-isoprene)} \) or poly(\text{trans-1,4-isoprene}) liquid MSM, synthetic poly(\text{trans-1,4-isoprene}) (CAS no. 104389-32-4, 99%; Sigma-Aldrich), respectively, was used as the sole carbon and energy source. For growth experiments in MSM-NR sandwich agar plates were prepared according to a previously described method (21). For this, synthetic poly(\text{trans-1,4-isoprene}) and MSM-GP medium were also used. In screening and growth experiments, *N. nova* and *G. polyisoprenivorans* were grown in mineral salts medium (MSM) (20) at 30°C, and carbon sources were added as indicated in the text. MSM-GP site reference(s)

<table>
<thead>
<tr>
<th>Strain, plasmid, or oligonucleotide</th>
<th>Characteristics, description, or sequence (5′–3′)*</th>
<th>Restriction site</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>N. nova SH22a</em></td>
<td>GP- and NR-degrading wild type</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td><em>N. nova SH22a OC11-7-56</em></td>
<td>Transposon-induced mutant, GP- and NR-degrading</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><em>N. nova SH22a OC11-8-33</em></td>
<td>Transposon-induced mutant, GP-degrading defective</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><em>N. nova SH22a OC20-2-10</em></td>
<td>Transposon-induced mutant, GP-degrading defective</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><em>N. nova SH22a Δmcr</em></td>
<td>mcr(_{5096}), deletion mutant, GP- and NR-degrading defective</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><strong>G. polyisoprenivorans</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. polyisoprenivorans</em></td>
<td>NR-degrading wild type, DSM no. 44266</td>
<td></td>
<td>80</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli XL10-Gold</em></td>
<td>Tet(^r) (mcrA)(<em>{183}) Δ(mcrCB-hsdSMR-mrr)(</em>{173}) endA1 supE44 thi-1 recA1</td>
<td></td>
<td>Agilent Technologies, USA</td>
</tr>
<tr>
<td></td>
<td>gyrA96 relA lac Ht [F(^{−}) proAB lacPZΔM15 Tn10 (Tet(^r)) Amy Cam(^r)]</td>
<td></td>
<td>USA</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMAS096</td>
<td>Contains transposon Tn5096; Amp(^r) Apr(^r)</td>
<td></td>
<td>21</td>
</tr>
<tr>
<td>pNC9053</td>
<td><em>E. coli</em>/Rhodococcus, <em>Gordonia</em>, <em>Nocardia</em> shuttle vector, pNC903 ori; Km(^r)</td>
<td></td>
<td>28, 29</td>
</tr>
<tr>
<td></td>
<td><em>Thi(^{r})</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pJET1.2/blunt</td>
<td>Blunt-end cloning vector; Amp(^r)</td>
<td></td>
<td>Thermo Fisher Scientific, USA</td>
</tr>
<tr>
<td>pJET:MCRKN</td>
<td>For mcr(_{5096}), deletion expt; Amp(^r) MCR(^{r})</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pJET:MCRKN::tsr</td>
<td>For mcr(_{5096}), deletion expt; Amp(^r) <em>Thi(^{r})</em></td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pNV18.1</td>
<td><em>E. coli</em>/Nocardia shuttle vector; Km(^r) <em>Neo(^{r})</em></td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>pNV18.1::mcr(_{5096})</td>
<td>For complementation expt</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pNV18.1::mcr(_{5096})</td>
<td>For complementation expt</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>pDBMC5-S</td>
<td><em>E. coli</em>/Gordonia shuttle vector; Gm(^r)</td>
<td></td>
<td>30</td>
</tr>
<tr>
<td>pDBMC5-S::mcr(_{5096})</td>
<td>For complementation expt</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td><strong>Oligonucleotides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCR-kn1</td>
<td>AAAAATGATCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>MCR-kn2</td>
<td>CTCAGTTGGGCCCCACAGCTCGAATCCCCTACCTCGGTTCGTCG</td>
<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>MCR-kn3</td>
<td>CGACGGAGACCGGTTAGGAGGAACGCAAGCGGTTAGGGCACTAG</td>
<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>MCR-kn4</td>
<td>AAAAATGATCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>FACL-kn1</td>
<td>AACTAGTCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>FACL-kn2</td>
<td>GTAGTTGGGCCCCACAGCTCGAATCCCCTACCTCGGTTCGTCG</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>FACL-kn3</td>
<td>GATGTGACAGCTCGTTGTTGACGCTGGTTCGTCG</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>FACL-kn4</td>
<td>AACTAGTCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>NMO-kn1</td>
<td>AACTAGTCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>NMO-kn2</td>
<td>CTCAGTTGGGCCCCACAGCTCGAATCCCCTACCTCGGTTCG</td>
<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>NMO-kn3</td>
<td>CAGCGGAGACCGGTTAGGAGGAACGCAAGCGGTTAGGGCACTAG</td>
<td>EcoRI</td>
<td></td>
</tr>
<tr>
<td>NMO-kn4</td>
<td>AACTAGTCAGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>MCR-exp1</td>
<td>CTCAGTTGGGCCCCACAGCTCGAATCCCCTACCTCGGTTCG</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>MCR-exp2</td>
<td>TCGGCGAGGCGGTAGGAGGCAACCAACCCTACTCGGTTCG</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>VH2-MCR-1</td>
<td>ACTCGTACGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
<tr>
<td>VH2-MCR-2</td>
<td>ACTCGTACGTTGCAAGCTGGTGCACCGAC</td>
<td>SpeI</td>
<td></td>
</tr>
</tbody>
</table>

*Neor*, neomycin resistance; *Thir*, thioestrepton resistance; *Tetr*, tetracycline resistance; *Ampr*, ampicillin resistance; *Camr*, chloramphenicol resistance; *Aprr*, apramycin resistance; *Kmr*, kanamycin resistance; *Gmr*, gentamicin resistance. Underlining in the sequences indicates the restriction endonuclease sites for DNA cloning.
were performed in Erlenmeyer flasks that were incubated on a horizontal rotary shaker at 140 rpm. Solid media were prepared by adding 1.6% (wt/vol) agar-agar. Antibiotics such as 100 μg of ampicillin/ml, 50 μg of apramycin/ml, 50 μg of kanamycin/ml, 50 μg of neomycin/ml, or 25 μg of thiostrporen/ml were applied when required.

**Optimized electroporation protocol.** Precultures of *N. nova* were cultivated in 50 ml of CASO medium at 30°C for 5 days. Subsequently, 100 ml of BHI medium supplemented with 0.5% (wt/vol) glycine, 6.0 mg of nisin, and 0.02% (vol/vol) Tween 80 in a ml of BHI medium were inoculated with 2.0% (vol/vol) of a preculture and cultivated at 30°C for 24 h. The cells were harvested at 4°C, washed twice with cold sterile water (Milli-Q, Millipore, USA), and then resuspended in water to an optical density of 6.0 at 600 nm. Freshly prepared competent cells were stored at −80°C. Before the electroporation pulse, 400 μl of competent cells was mixed with foreign DNA (final concentration, 0.015 μg/ml) and preincubated at 10°C for 5 min. The mixture was electroporated at 5 ms in electrocuvettes (Peqlab, Germany) with gaps of 2 mm by an Equibio Easyject electroporator at the following settings: 12.5 kV/cm, 201 Ω, and 25 μF. Pulsed cells were immediately supplemented with 600 μl of CASO medium and regenerared at 30°C for 4 h before they were plated on CASO agar plates containing the appropriate antibiotics. Restorantin competent were selected after 2 to 7 days of incubation at 30°C. More details about the optimization procedures are described in Results.

**Tn5096-based mutagenesis.** The Tn5096-containing suicide plasmid pMA5096 (21) was transferred into *N. nova* competent cells according to the electroporation protocol described above. Apramycin-resistant transformants obtained on CASO agar plates were characterized for their abilities to utilize GP and NR.

**Screening for mutants defective in GP or NR degradation.** Transposon-induced transformants were transferred onto MSM-GP and MSM-NR sandwich agar plates. Cells were inoculated by toothpicks through the top MSM agar layer into the GP or NR film to provide cells with direct contact to the polysoprenes. Mutants showing defective phenotypes in GP or NR utilization were then incubated on MSM agar medium containing 0.5% (wt/vol) glucose as the sole carbon source to identify auxotrophic mutants. This screening procedure was performed twice, thereby ensuring to obtain only mutants with stable defective phenotypes in the degradation of polysoprenes. These mutants were then cultivated in 70 ml of liquid MSM containing either type of polymer grain. The optical densities of the cultures were measured at 600 nm.

**DNA extraction and manipulation.** Plasmid DNA was isolated from crude cell lysates by the alkaline extraction method (22). The cells were broken in lysis buffer with additional lysozyme (1 mg/ml) at 37°C for 1 h when plasmids were prepared from *N. nova* and *G. polyisoprenivorans*. Total DNA of *N. nova* was isolated according to a previously described method (23). Other genetic procedures and molecular manipulations were performed according to standard protocols (24). The primers used for DNA cloning and sequencing are listed in Table 1.

**Sequence analysis.** Nucleotide sequences adjacent to the transposition loci were obtained by sequencing and comparison to the draft genome of *N. nova* SH22a (unpublished data). Open reading frames (ORFs) adjacent to the transposition loci were predicted using the software package ARTEMIS (25), and the corresponding amino acid sequences were analyzed by using BLAST (26) against protein databases (NR, TrEMBL, and SWISS-Prot). Corresponding nucleotide sequences are available in GenBank under accession numbers KC181133, KC181134, and KC181135 (see Fig. 2).

**Construction of deletion mutants.** In order to construct an mcr_{SH22a} deletion mutant, the flanking regions upstream (flankA, 1.0 kb) and downstream (flankB, 0.93 kb) of mcr_{SH22a} were amplified by using PfX polymerase (Invitrogen, USA) employing the primer pairs MCR-kn1/MCR-kn2 and MCR-kn3/MCR-kn4 and the total DNA of *N. nova* SH22a, respectively. The resulting fragments (flankA and flankB) were combined together to yield a fused fragment (flankA-B, 1.93 kb) via fusion PCR (27).

The fragment flankA-B was subsequently cloned into the cloning vector pJET1.2/blunt (Thermo Fisher Scientific, USA), yielding pJET::MCRKN. A 1.1-kb thiorostrporen-resistant cassette (tsr), which was excised from pNC9503 (28, 29) by using the endonuclease EcoRI, was cloned into the single EcoRI site between flankA and flankB, yielding pJET::MCRKN::tsr. The 3.03-kb linear fragment (flankA::tsr::flankB) was excised from pJET::MCRKN::tsr by SpeI and transferred into *N. nova* SH22a competent cells by electroporation for homologous recombination on thiostrporen plates. Genotypes of recombimant mutants were confirmed by PCR using internal and external primers. The fadD (long-chain fatty acid-CoA ligase gene) and nmo (nitromonooxygenase gene) deletion mutants were constructed by a similar procedure employing the primer pairs FACL-kn1/FACL-kn2, FACL-kn3/FACL-kn4, NMO-kn1/NMO-kn2, and NMO-kn3/NMO-kn4.

**Cloning of mcr_{SH22a} and mcr_{VH2}.** A 2.0-kb fragment of mcr_{SH22a} and a 1.6-kb fragment of mcr_{VH2} containing native promoters were amplified from total DNA of *N. nova* SH22a (primers MCR-exp1/MCR-exp2) and *G. polyisoprenivorans* VH2 (primers VH2-MCR-1 and VH2-MCR-2), respectively, by PfX polymerase. The fragments were cloned into the HindII site of pNV18.1, yielding pNV18.1::mcr_{SH22a} and pNV18.1::mcr_{VH2}. Either plasmid was independently transformed into *N. nova* SH22a Δmcr for complementation. In the case of *G. polyisoprenivorans*, the mcr_{SH22a} gene was excised from pNV18.1::mcr_{SH22a} with HindIII/XbaI and cloned into a HindIII/XbaI-digested shuttle vector pDBMCS-5 (30), yielding pDBMCS-5::mcr_{SH22a}. The plasmid was transferred into competent cells of the mcr disruption mutant *G. polyisoprenivorans* VH2 2-22 (11) by electroporation (29).

**RESULTS**

**Optimization of electroporation efficiency of *N. nova*.** Our first attempts to yield transposon-induced mutants by electrotcransformation of pMA5096 (21) into *N. nova* SH22a competent cells gave only very few apramycin-resistant transformants. With such a low efficiency, we could not generate a transposon mutagenesis library with a sufficient number of transformants to screen for GP or NR degradation-defective mutants. Therefore, we performed a systematic optimization of the electroporation efficiency for *N. nova* by altering the cultivation conditions and the electroporation procedure. The optimization was started by applying the following starting settings: (i) cultivation of cells in CASO medium containing 0.5% (wt/vol) glucose, 1.0% (wt/vol) sucrose, and 0.02% (vol/vol) Tween 80; (ii) 1.5 μg of foreign DNA/ml; (iii) a field strength of 12.5 kV/cm; and (iv) electroporation that was performed at 0°C. Each condition was altered independently, whereas all other parameters were kept constant. In order to make it easy to count the efficiency, we used the *E. coli/Rhodococcus, Gordonia* shuttle vector pN9503 (28, 29), which also showed the ability to shuttle between *E. coli* and *N. nova*.

Previous studies described that growth medium and cultivation time to prepare competent cells greatly affected electroporation efficiency (31, 32). Four media—CASO, NB, BHI, and GYM—were investigated in the present study. Cells grew much slower in NB medium than in the others; therefore, CASO, BHI, and GYM were used for the next step. We observed that cells from the early exponential phase (24 h) gave much higher efficiencies than from the middle (40 h) or the late (54 h) exponential phase in either medium. The highest efficiency (2.4 × 10^8 CFU/μg of DNA) was achieved with the cells from BHI medium.

The relationship between the electroporation efficiency and the used plasmid DNA concentration was also determined (Fig. 1A). Different concentrations of plasmid DNA at 0, 0.015, 0.15, 0.5, 1.5, and 2.5 μg/ml were examined. We observed that low DNA con-
centrations (**\(0.15 \mu g/ml\)**) resulted in a much higher efficiency than relatively high DNA concentrations. The highest efficiency (**1.1 \(10^7\) CFU/g DNA**) was achieved at a DNA concentration of **0.015 g/ml**. When the concentration was higher than **1.5 g/ml**, the efficiency remained constant.

The presence of the additives glycine and INH, which appeared to affect the assembly of the cell walls (33,34), and of sucrose enhanced transformation efficiencies in some Gram-positive bacteria such as *Corynebacterium glutamicum* (32), *Bacillus anthracis* (35), *Rhodococcus rhodochrous* CW25 (36), and *R. opacus* PD630 (28). In the present study (Fig. 1B to D), *N. nova* cells cultivated in the presence of **0.5% (wt/vol)** glycine showed the highest efficiency (**1.2 \(10^7\) CFU/g DNA**). The addition of **6.0 \(\mu g/ml\)** INH resulted in an **2.5-fold increase** in efficiency (as high as **4.3 \(10^7\) CFU/g DNA**) in comparison to the absence of INH. In contrast to our expectation, the addition of sucrose did not exert any positive effect on the electroporation efficiency. While the concentration of sucrose increased from **0** to **2.0% (wt/vol)**, the efficiency exhibited a tendency to decrease with only a little rise at a concentration of **2.0% (wt/vol)** (Fig. 1D).

The effects of short temperature treatments were also examined. When the competent cell-DNA mixture was applied with a treatment at **10°C** for **5 min**, the electroporation efficiency increased **~4.5-fold** compared to experiments without treatment (Fig. 1E and F). Finally, an efficiency could be stably achieved at the level of **10^7\) CFU/\mu g of DNA** for *N. nova* SH22a by using the optimized protocol (see Materials and Methods).

**pMA5096-mediated transposon mutagenesis of N. nova.** The suicide plasmid pMA5096 (21) containing Tn5096 (37) was used in the present study for generating transposon-induced mutants of *N. nova* SH22a. Totally, we obtained about **12,000 apramycin** resistant transformants. Banh et al. reported that Tn5096 mediated the integration of the entire plasmid pMA5096 into the genome of *G. polyisoprenivorans* VH2 and not only of Tn5096 (21). Therefore, before mapping the transposition loci, we randomly chose and examined five mutants to determine whether this also happens in *N. nova* SH22a. The total DNA from the five mutants was isolated, digested by ApaI (since no ApaI site exists in pMA5096), and subsequently self-ligated. The self-ligation mixtures were transformed into *E. coli* XL10-Gold cells. All transformants showed not only apramycin resistance encoded on the Tn5096 part but also ampicillin resistance encoded on the pBluescript SK(−) vector part, which supported the assumption that also in *N. nova* SH22a the entire pMA5096 plasmid integrated.
FIG 2 Locations of pMA5096 insertions in the genomes of N. nova SH22a transposon-induced mutants and identification of genes adjacent to the transposition loci. (A) Mutant OC11-7-56 (GenBank accession no. KC181133; orf1, putative ferredoxin reductase; orf3, putative transcriptional regulator); (B) mutant OC11-8-33 (GenBank accession no. KC181134; orf1, putative enoyl-CoA hydratase; orf2, putative oxidoreductase; orf3, AKR superfAMILY oxidoreductase; orf3, hypothetical protein); (C) OC20-2-10 (GenBank accession no. KC181135; orf1, hypothetical protein; fadD, putative long-chain fatty-acid CoA ligase; nmo, putative nitrate monoxygenase; orf2, hypothetical protein; orf3, putative polypeptide synthase; orf4, putative long-chain fatty acyl-AMP ligase). The lengths and directions of arrows showing the genes indicate the proportional lengths and directions of transcription, respectively, of the corresponding genes.

in the genome. In line with our expectations, replicable plasmids could be isolated from these E. coli transformants. For each transposon-induced mutant, plasmids were isolated from five randomly chosen E. coli transformants and restricted with ApaI. One main band occurred in all five plasmids digested, and up to two small bands were additionally observed in some cases. Moreover, the main bands derived from different mutants differed from each other in size. These results indicated that the transposition had occurred only once in one mutant and that pMA5096 had integrated into different sites of the genome. This assumption was also verified by subsequent sequence analysis of the self-ligation plasmids derived from each mutant employing primers hybridizing adjacent to pMA5096.

Screening for mutants defective in GP or NR degradation. All transposon-induced transformants were screened on both MSM-GP and MSM-NR sandwich plates, which contained poly-(trans-1,4-isoprene) or poly(cis-1,4-isoprene) as the sole carbon and energy sources, respectively. Because it was sometimes difficult to differentiate the degree of deficiency on solid plates, growth experiments were also performed in liquid medium with all candidate mutants after two rounds selection on sandwich plates. After the detection of 116 auxotrophic mutants, which did not show growth on glucose as the sole carbon and energy sources, we finally obtained 76 mutants stably displaying a deficiency in polyisoprene utilization. Among all 76 mutants, 10 mutants showed impaired growth on GP but not on NR, whereas the other 66 mutants were defective in the degradation of both GP and NR.

Identification of genes involved in GP or NR degradation. In order to identify genes that were disrupted in the mutants defective in GP or NR degradation, the transposition loci of pMA5096 were analyzed as described above. The data from the first three mutants, which were characterized in more detail, indicated that the integration of pMA5096 had occurred in three chromosomal regions of N. nova SH22a; the related genes will be described below.

In the genome of mutant OC11-7-56 (Table 1, Fig. 2A), which was defective in both GP and NR degradation, pMA5096 was mapped in a 1.101-kb ORF (mcr). This ORF is predicted to encode an α-methylacyl-CoA racemase (MCR; EC 5.1.99.4) catalyzing the racemization of (R)- and (S)-isomers of a variety of α-methyl-branched CoA thioesters (38, 39). The corresponding translation product showed high sequence identities to function-verified MCRs derived from various species (11, 38, 40) (64% identity to the MCR G. polyisoprenivorans VH2, GenBank accession no. AFA74657.1; 58% identity to the MCR of Mycobacterium tuberculosis, GenBank accession no. AFN49044.1; 47% identity to the AMACR of rat, GenBank accession no. EDL82982.1). The homologues could also be detected in the genomes of other known NR-degrading bacteria, regardless of whether they grew adhesively (Nocardia farcinica IFM10152 [41, 42]) or exhibited clear zone-forming abilities [Streptomyces coelicolor A3(2) (10, 43), Streptomyces flavogriseus ATCC 33331 (12), and Actinosynnema mirum DSM43827 (12, 44)]. In the adjacent region of mcr, the genes encoding a putative ferredoxin reductase (orf1), a putative amidohydrolase (orf2), and a putative transcriptional regulator (orf3) were detected.

In order to exclude polar effect exerted by the transposon insertion, deletion and complementation experiments were performed for mcr. A deletion mutant, N. nova SH22a Δmcr, in which mcr was replaced by the 1.1-kb thiostrepton-resistant cassette tsr, was constructed via homologous recombination. Like the corresponding transposon-induced mutant, the deletion mutant displayed a significant deficiency in the utilization of either polyisoprene in comparison to the wild-type strain (Fig. 3). When plasmid pNV18.1::mcr was harboring one copy of the intact mcr gene of N. nova SH22a, was transferred into N. nova SH22a Δmcr, the deficiency was completely restored in the resulting recombinant mutant (Fig. 3). Plasmids were isolated from the complemented mutant after 35 days cultivation and transformed into E. coli cells for proliferation. The same restriction patterns demonstrated that the plasmids were derived from the original parents and could replicate in N. nova stably even under the condition without selective pressure.

Since MCRs accept a wide range of α-methyl-branched CoA thioesters as substrates (38, 39) and play a crucial role in the β-oxidation of the degradation products of certain methyl-branched compounds (11, 45, 46), the growth of N. nova SH22a on 0.5% (vol/vol) squalane, squalene, phytol, farnesol, and geranylacetone was examined. On solid MSM, wild-type SH22a showed good growth on phytol (Fig. 4A) and squalene (Fig. 4B) as the sole carbon sources, whereas no growth occurred on squalane, farnesol, and geranylacetone. As on polyisoprene, the mcr deletion mutant was also defective in utilization of phytol and squalene, whereas growth on water-soluble substrates such as glucose, acetate, and pyruvate was not affected. The deficiencies regarding
phytol and squalene utilization were also immediately restored when the mutant was complemented with mcr.

In a previous study, Arenskötter et al. identified an MCR homologue in *G. polyisoprenivorans* VH2 and verified its essential role in the degradation of methyl-branched isoprenoids including poly(cis-1,4-isoprene) (11, 21). This MCRVH2 homologue exhibited a high sequence identity (≥65%) to MCRSH22a. In order to evaluate whether these two homologues could functionally complement each other, mcr and the adjacent regions were cloned in and introduced into *N. nova* SH22a Δmcr and *G. polyisoprenivorans* VH2 2-22 (an mcr disruption strain), respectively. The impaired growth of strain SH22a Δmcr on trans- and cis-polyisoprenes was completely abolished upon heterologous expression of *mcr*VH2 (Fig. 4C and D). Furthermore, the expression of *mcr*VH2 also successfully restored the NR-degrading capability in strain VH2 2-22 (Fig. 4F). However, a capability of GP degradation was not additionally conferred to *G. polyisoprenivorans* VH2 by expression of *mcr*VH2 (Fig. 4E).

In mutant OC11-8-33 (Table 1, Fig. 2B), which exhibited no growth on GP, pMA5096 was mapped in a gene (orf1) encoding for a putative oxidoreductase belonging to the aldo-keto reductase (AKR) superfamily (PF00248). When the adjacent regions were analyzed, two ORFs (orf1 and orf2) were identified in the downstream region. One, orf1, exhibited the same transcriptional direction as orf1 and was predicted to code for an enoyl-CoA hydratase. The other, orf2, was predicted to encode another oxidoreductase but organized in the opposite direction toward orf1. A 0.6-kb orf3 was detected upstream of orf1, and the deduced amino acid sequence was predicted as a conserved protein, which is also found in many actinobacteria (e.g., *Streptomyces*, *Thermomonospora*, *Nocardiopsis*, and *Kitasatospora*). However, its function is still unknown.

In another mutant, OC20-2-10 (Table 1, Fig. 2C), which exhibited impaired growth on GP, pMA5096 was mapped in the chromosomal region between two genes coding for a putative long-chain fatty-acid CoA ligase (fadD) and a probable nitronate monooxygenase (nmo). The insertion of pMA5096 might interrupt the promoter of either gene. In order to identify which gene is really related to the GP degradation, fadD and nmo were deleted separately. Neither the fadD deletion mutant nor the nmo deletion mutant exhibited a defective phenotype in GP degradation. Furthermore, in the downstream region of nmo, three function-related ORFs were organized in a possible operon. The deduced amino acid sequences of orf2 and orf3 showed similarities to phthiocerol/phenolphthiocerol synthesis polyketide synthases (PPSs) of mycobacteria (47, 48), and the deduced amino acid sequence of orf4 showed high similarities to many mycobacterial FdD proteins. All of these FdD belong to the group of long-chain fatty acyl-AMP ligases (FAALs) (49) and are thought to play a role in the synthesis of complex lipids of mycobacterial cell envelopes (50–54). On the other side, a 2.37-kb ORF (orf1) was detected in the downstream region of fadD. It was predicted to encode a function unknown protein which is conserved in many *Corynebacterineae* species. However, it was organized in

### Figure 3
Growth profiles of the *N. nova* SH22a wild-type strain and of mutants in liquid MSM with poly(trans-1,4-isoprene) (A) and poly(cis-1,4-isoprene) (B) as the sole carbon and energy sources. Symbols: ○, wild type; ▲, *N. nova* SH22a Δmcr; ■, *N. nova* SH22a Δmcr (pNV18.1::*mcr*SH22a). The data are presented as means from three independent replicates with the standard deviations.

### Figure 4
Growth of *N. nova* SH22a or *G. polyisoprenivorans* VH2 on solid MSM media with phytol (A), squalene (B), poly(trans-1,4-isoprene) (C and E), and poly(cis-1,4-isoprene) (D and F) as the sole carbon and energy sources. A and B: I, *N. nova* SH22a wild-type strain; II, *N. nova* SH22a Δmcr; III, *N. nova* SH22a Δmcr (pNV18.1::*mcr*SH22a); C and D: I, *N. nova* SH22a wild-type strain; II, *N. nova* SH22a Δmcr; III, *N. nova* SH22a Δmcr (pNV18.1::*mcr*SH22a). E and F: I, *G. polyisoprenivorans* VH2 wild-type strain; II, *G. polyisoprenivorans* VH2 2-22; III, *G. polyisoprenivorans* VH2 2-22 (pDBMCS-5::*mcr*SH22a); IV, *N. nova* SH22a wild-type strain.
the opposite direction toward fadD and thought not likely to be involved in GP degradation.

DISCUSSION

For many actinobacteria exhibiting a variety of special properties in cell structures and biochemical functions, genetic studies are often restricted due to the lack of molecular tools, including efficient electroporation methods. In the present study, we optimized the electroporation efficiency for N. nova SH22a systematically by altering the cultivation conditions and the electroporation procedure in order to meet the needs for transposon mutagenesis, achieving a stable efficiency of $10^7$ CFU/μg of DNA. This efficiency will allow most genetic engineering processes in N. nova, and it will also provide benefits for studies on other species of the genus Nocardia.

Applying the optimized protocol, a transposon mutagenesis library comprising about 12,000 mutants was constructed by electrottransformation of pMA5096 into the GP- and NR-degrading bacterium N. nova SH22a. Analysis of the transposition mutants indicated that pMA5096 introduced the integration of the entire plasmid randomly into the genome of N. nova, which therefore provides us with an abundant mutational source to identify genes involved in GP and/or NR degradation. This type of integration of pMA5096 also provided a benefit to analyze the transposon loci, since replicable plasmids containing parts of the genomic DNA could be readily isolated and sequenced employing appropriate primers. In addition to the capabilities of hydrocarbon degradation (14–19), many members of the genus Nocardia are pathogens causing occasional infection diseases affecting the lungs, the central nervous system, cutaneous tissues, or whole bodies of either humans or animals. In addition, these pathogens are capable of producing a number of bioactive metabolites (55–60). The pMA5096-based transposon mutagenesis described here could therefore also be an appropriate method for unraveling the anabolism pathway of bioactive metabolites and for studying the mechanism of pathogenesis.

In contrast to the degradation of NR, the degradation of GP, differing from NR only in the stereo-structure, has been rarely studied. In 2007, six Nocardia strains (four N. nova strains, one N. jiangxensis strain, and one N. takedensis strain) capable of degrading GP were isolated and identified (13). That study provided the first description of axenic cultures capable of degrading GP. Interestingly, all isolates showed both GP- and NR-degrading abilities and the same adhesiveness and growth behaviors on polymer compounds as other NR-degrading bacteria, such as G. polyisoprenivorans (7), M. fortuitum (7), and N. farcinica (61). In the present study, we identified a total of 76 mutants defective in GP and/or NR degradation. Whereas 10 mutants were only impaired in GP degradation but not in NR degradation, the other 66 mutants showed impaired growth on both GP and NR. This makes it conceivable that the degradation of GP and NR may undergo a similar pathway sharing many common steps. The larger number of GP-degrading defective mutants could be explained as follows. (i) The GP pathway is more complex and harbors more specific steps, or (ii) the steps for both pathways are almost identical, but in the case of GP degradation there are fewer enzymes involved in each step, and they are more specific toward the substrates. Therefore, the probability to obtain GP-defective mutants will be higher than to obtain NR-defective mutants. However, it cannot be excluded that specific steps exist in the degradation of NR, although such NR-specific mutants were not identified. This could be due to the fact that the mutants detected here may not comprise all genes involved in the degradation of GP and/or NR or that homologues of the particular gene exist that encode enzymes also capable of catalyzing the respective step, as has been shown for the lacto-clearing protein in G. polyisoprenivorans (12). Since compounds which are structurally related to GP, such as squalene, squalane, phytane, phytol, and rubber, are degraded via β-oxidation (12, 62–65) and because the degradation of squalene and phytol is also affected in the MCR deletion mutant, it is conceivable that β-oxidation is also involved in GP degradation. Enzymes catalyzing the steps of β-oxidation and closely related steps may account for most of the common steps in the degradation of either polyisoprene.

This inference was supported by the results of the subsequent transposition mapping analysis. A gene coding for α-methylacyl-CoA racemase (MCR) that was inactivated by insertion was identified in the GP and NR mutant OC11-7-56. MCRs are involved in β-oxidation and catalyze the interconversion of the “R” and “S” configurations of a variety of (R)- and (S)-2-methyl-branched-chain fatty acyl-CoAs (38, 39). Because the first step of β-oxidation is a stereospecific reaction, the involved enzymes, either the acyl-CoA oxidases or dehydrogenases act only on substrates possessing the “S” configuration. This critical position of MCRs accords with the fact that they are involved in the metabolism of many methyl-branched compounds, including GP and NR. In mammals, they play an important physiological role in the metabolism of the bile acid intermediates di- and trihydroxycoprostanic acids (3α,7α-dihydroxy- and 3α,7α,12α-trihydroxy-5β-cholestanoic acids) (38, 66), as well as methyl-branched fatty acids such as phytanic acids (3,7,11,15-tetramethylhexadecanoic acids) (67).

In bacteria, MCRs are also required for growth on pristine, phytane, squalane, or isoprenoids (11, 46). In the present study, the deletion of mcr in N. nova SH22a caused a significantly impaired growth not only on GP and NR but also on phytol and squalene. We detected a difference in the growth behavior of the deletion mutant growing on solid media containing the trans or the cis configuration of polyisoprene. A reason for this could be the difference in stability. It is known that GP is much more stable than rubber. Therefore, the mutant might grow on intermediates such as levulinic aldehyde and limonene (68), resulting from the decomposition of the rubber (e.g., from auto-oxidation) during cultivation. More interestingly, the growth deficiencies caused by the inactivation of MCRs in N. nova SH22a and G. polyisoprenivorans VH2 were completely abolished by the other MCR, indicating that the function of MCRs from either strain could be replaced by the other homologue. In coincidence with this, the expression of MCRsh22, did not confer GP-degrading ability to G. polyisoprenivorans VH2, thereby demonstrating that MCRs are not specifically involved in only GP or NR degradation.

Insertion of pMA5096 in mutants OC11-8-33 and OC20-2-10 led to impaired growth on GP but did not at all affect the growth on NR. The phenotypes of these mutants most likely pointed to GP-specific degradation steps. In mutant OC11-8-33, pMA5096 disrupted an aldo-keto reductase gene (akr). AKRs are a large superfamily showing low level of sequence similarity among members and comprise many NADPH-dependent oxidoreductases (69). More than 150 known and putative members are distributed into 15 families (70), catalyzing the reduction of carbonyl groups from a wide range of substrates, such as aldehydes/ketones.
aldoses, steroids, and prostaglandins (71–73). Some AKRs showed activities in the detoxification, signals processing and oxidative stress defense (72, 74). In the present study, the AKR was allocated to the AKR subfamily based on the sequence similarity analysis toward all classified AKR members from the AKR superfamily database (70), and it showed highest similarity to a Trypanosoma brucei prostaglandin F2α (PGF2α) synthase, which catalyzes the reduction of the 9,11-endoperoxide group of PGH2 to two hydroxy groups of PGF2α (75). Previous studies have shown that ketones and aldehydes arose in the degradation of rubber (76, 77). This can also be the case in GP degradation. The AKR identified in the present study may act on these ketone and aldehyde intermediates by catalyzing their reduction. However, a particular function of this AKR cannot be allocated due to the lack of further information on reaction substrates and degradation intermediates generated in GP degradation.

In mutant OC20-2-10, the insertion of pMA5096 was mapped in the region between a putative long-chain fatty-acid CoA ligase gene and a probable nitrate monooxygenase gene, leading to the postulation that the promoter of either gene was inactivated. However, subsequent deletion experiments of either gene demonstrated that none of these genes is directly involved in GP degradation. The deficiency might be caused by the polar effect of the insertion on genes located downstream of the insertion. Downstream of nmo, three ORFs (orf2, orf3, and orf4) were detected that probably constitute an operon. The corresponding protein products showed high similarities to the mycobacterial enzymes PPSs and FadDs that are involved in the synthesis of phthiocerol dimycocerolates (48), which are extremely hydrophobic constituents (78) that are primarily found in the cell envelope of pathogenic mycobacteria (79). Therefore, it could be assumed that these genes are responsible for the synthesis of hydrophobic cell envelope compounds and that these compounds may play a role in the adhesive growth behavior of N. nova via hydrophobic interaction with hydrophobic substances such as polyisoprenes. The fact that mutant OC20-2-10 exhibited a defective phenotype only on GP but not at all on NR suggests that this hydrophobic interaction is more important for GP than for NR degradation. The role of these hydrophobic compounds needs to be further studied.

The results of this study are a first and important step to unraveling the unknown mechanism of GP degradation and also showed relations between GP and NR degradation. For this, an efficient electroporation protocol for N. nova SH22a was systematically optimized. It provides a powerful tool not only for the analysis of the degradation of abundant natural resources but probably also for studies on other clinically and industrially important Nocardia strains with respect to the anabolism of bioactive metabolites and the mechanism of pathogenesis.

ACKNOWLEDGMENT

We acknowledge financial support from the Deutsche Forschungsgemeinschaft (STE-386/10-1) for this study.

REFERENCES

10. Rose K, Tenberge KB, Steinbüchel A. 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.
38. Schmitz W, Fingerhut R, Conzelmann E. 1994. Purification and prop-


43. Schmitz W, Fingerhut R, Conzelmann E. 1994. Purification and prop-

52. Gokhale RS, Suxena P, Chopra T, Mohanty D. 2007. Versatile polyketide enzymatic machinery for the biosynthesis of complex mycobacterial lip-
63. Silva RA, Grossi V, Alvarez HM. 2007. Biodegradation of phytane (2,6,10,14-tetramethylhexadecane) and accumulation of related iso-

Downloaded from http://aem.asm.org on October 24, 2017 by guest


