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MINIREVIEWS

Biodegradation of Natural Rubber and Related Compounds: Recent Insights into a Hardly Understood Catabolic Capability of Microorganisms

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Natural rubber latex is produced by over 2,000 plant species, and its main constituent is poly(*cis*-1,4-isoprene), a highly unsaturated hydrocarbon. Since 1914 there have been efforts to investigate microbial rubber degradation; however, only recently have the first proteins involved in this process been identified and characterized and have the corresponding genes been cloned. Analyses of the degradation products of natural and synthetic rubbers isolated from various bacterial cultures indicated without exception that there was oxidative cleavage of the double bond in the polymer backbone. A similar degradation mechanism was postulated for the cleavage of squalene, which is a triterpene intermediate and precursor of steroids and triterpenoids. Aldehyde and/or carbonyl groups were detected in most of the analyzed degradation products isolated from cultures of various rubber-degrading strains. The transient formation of intermediate degradation products with molecular masses of about 10^4 Da from poly(*cis*-1,4 isoprene) having a molecular mass of about 10^6 Da by nearly all rubber-degrading bacteria investigated without detection of other intermediates requires an explanation. Knowledge of rubber degradation at the protein and gene levels and detailed analyses of detectable degradation products should result in a detailed understanding of these obviously new enzymatic reactions.

OCCURRENCE AND CHEMICAL STRUCTURE OF NATURAL RUBBER

The term natural rubber or caoutchouc (from Indian: caa = tears; ochu = tree; cahuchu = weeping tree) refers to a coagulated or precipitated product obtained from latex of rubber plants (*Hevea brasiliensis*), which forms nonlinked but partially vulcanizable polymer chains having molecular masses of about 10^6 Da with elastic properties; at higher temperatures natural rubber is plastically ductile and useful for production of elastomers. Latex serves as a clogging material during healing of wounds caused by mechanical injury of plants.

Natural rubber consists of C_5H_8 units (isoprene), each containing one double bond in the *cis* configuration (Fig. 1). However, polyisoprene of *H. brasiliensis* contains in addition two *trans*-isoprene units in the terminal region (52). Although approximately 2,000 plants synthesize poly(*cis*-1,4-isoprene), only natural rubber of *H. brasiliensis* (99% of the world market) and guayule rubber of *Parthenium argentatum* (1% of the world market) are produced commercially (52). Latex of *Hevea* plants contains about 30% poly(*cis*-1,4-isoprene) and is harvested by a "tapping" procedure after the bark of the plants is notched diagonally, which yields 100 to 200 ml latex resin within 3 h. Such "tapping" is usually carried out every 2 to 3 days, yielding up to 2,500 kg of natural rubber per year per ha. In 1998, the world production of natural rubber was about 6.6 million tons; more than 70% of this rubber was produced in only three countries (Thailand, Indonesia, and Malaysia), and about 40% was purchased by only three countries (United States, China, and Japan). Most of the natural rubber (75%) is used for production of automobile tires (33).

Dehydrated natural rubber of *H. brasiliensis* contains approximately 6% nonpolyisoprene constituents. Depending on the clone, seasonal effects, and the state of the soil, the average composition of latex is as follows: 25 to 35% (wt/wt) polyisoprene; 1 to 1.8% (wt/wt) protein; 1 to 2% (wt/wt) carbohydrates; 0.4 to 1.1% (wt/wt) neutral lipids; 0.5 to 0.6% (wt/wt) polar lipids; 0.4 to 0.6% (wt/wt) inorganic components; 0.4% (wt/wt) amino acids, amides, etc.; and 50 to 70% (wt/wt) water (51). The polymer is present in 3- to 5-μm so-called rubber particles, which are covered by a layer of proteins and lipids (20), which separate the hydrophobic rubber molecules from the hydrophilic environment. Because some *Hevea* proteins have allergenic potential, methods were developed to remove these proteins. An efficient method involves cleaning the latex by centrifugation and employing enzymatic digestion with alkaline proteases or papain or treatment with sodium or potassium hydroxide. This allows production of condoms and latex gloves with low protein contents (less than 20 μg/g of natural rubber).

Only a few plant species synthesize polyisoprenes in the *trans* configuration (Fig. 1). Chicle (*Manikara zapota*), gutta-percha (*Pallaquium gutta*), and balata (*Manikara bidentata*) are typical representatives of *trans*-polyisoprene-synthesizing plants. Gutta-percha and balata produce *trans*-polyisoprenes with high molecular weights (1.4×10^5 to 1.7×10^5). The chicle tree is unique, because it produces latex with about equal amounts of *cis*- and *trans*-polyisoprenes.

The discovery of the classical vulcanization process by Goodyear in 1839 allowed production of materials with im-

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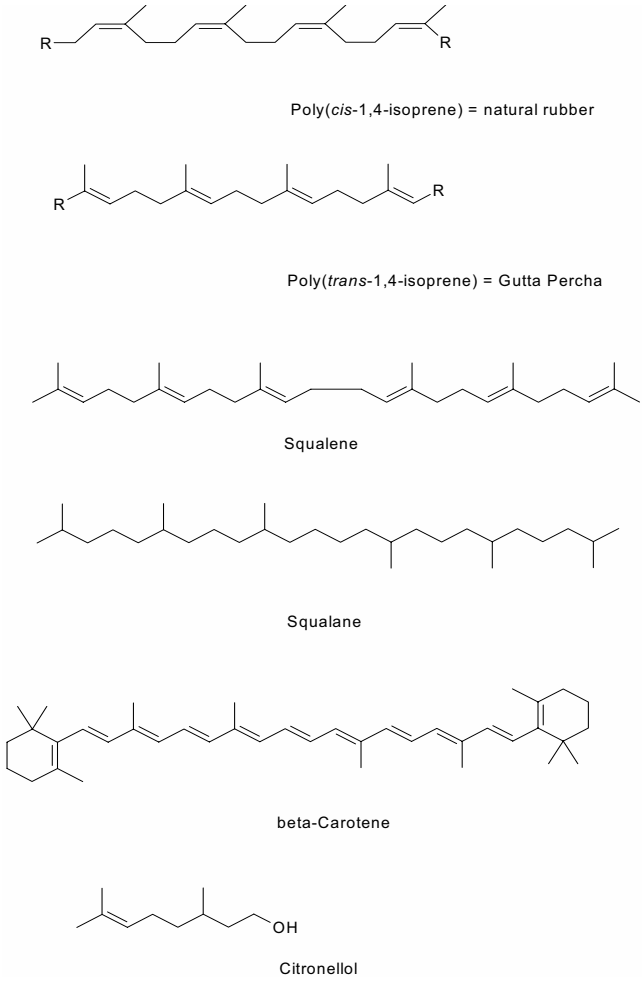

FIG. 1. Structural formulas of polyisoprenoids and putative low-molecular-weight model substances.

proved properties from natural rubber. The polyisoprene molecules are covalently linked by bridges of elemental sulfur at the double bonds (13). Alternatively, vulcanization is also achieved by employing organic peroxides (32) or radiation (51); such vulcanized materials have lower long-term stability since the polymer chains are cross-linked solely by carbon bonds. Although the first synthetic rubbers were produced at the beginning of the last century, only after 1950, after the development of stereospecific catalysts, could polyisoprene be synthesized in the *cis* and *trans* configurations (52). Today it is possible to produce synthetic polyisoprene that has physical properties similar to those of natural rubber with a purity of 98 to 99%. However, the stress stability, processability, and other parameters of synthetic polyisoprene are still less satisfying than those of natural rubber (52).

OCCURRENCE AND CHEMICAL STRUCTURE OF SQUALENE

Squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6*E*,10*E*,14*E*,18*E*,22*E*-hexaene) (Fig. 1) was discovered first in the liver of "dogfish" (*Squalus acanthias*), an organism belonging to the class Squalidae that was the origin of the name squalene (55). Squalene is a natural triterpene which plays an important role as a precursor in the biosynthesis of steroids and triterpenoids. Biosynthesis of squalene results from a "tail-to-tail" condensation of two molecules of the sequiterpene farnesylpyrophosphate (16). It occurs, for example, in human sebum and in olive oil. In the latter, the squalene content decreases significantly only after 6 to 8 months, indicating that the molecule has considerable stability (35). Squalene was also identified as an essential molecule in anal gland secretions of beavers that keep their pelts water repellent (41). Squalene also occurs in many microorganisms; e.g., 0.4% (wt/wt) of the cell dry mass of *Nannocystis exedens* is composed of squalene (25).

PROBLEMS AND DIFFICULTIES HAMPERING STUDIES OF THE MICROBIAL DEGRADATION OF RUBBER

Several serious difficulties hamper investigation of microbial rubber degradation. Rubber biodegradation is a slow process, and the growth of bacteria utilizing rubber as a sole carbon source is also slow. Therefore, incubation periods extending over weeks or even months are required to obtain enough cell mass or degradation products of the polymers for further analysis. This is particularly true for members of the clear-zone-forming group (see below). Periods of 10 to 12 weeks have to be considered for *Streptomyces coelicolor* 1A (8), *Thermomonospora curvata* E5 (22), or *Streptomyces* sp. strain K30 (40); the only exception is *Xanthomonas* sp. strain 35Y (54). Although members of the non-clear-zone-forming group exhibit slightly faster growth, cultivation periods of at least 6 weeks are also required for *Gordonia westfalica* (11), e.g., to determine whether a putative mutant is able to grow on the polymer.

Frequently, newly isolated strains must be used to study rubber biodegradation. These isolates are often members of poorly characterized taxa, and established genetic tools are not applicable. Therefore, for a newly isolated strain of the clear-zone-forming bacterium *Micromonospora aurantiaca* W2b and for some representatives of the genus *Gordonia*, efficient transformation systems based on conjugation and electroporation were established (3, 39). For example, it was shown that the origin of replication (*oriV*) of the native *Rhodococcus rhodochrous* plasmid pNC903 permitted replication of this plasmid in some *Gordonia* species. In addition, *oriV* of the megaplasmid pKB1 from the rubber-degrading bacterium *G. westfalica* Kb1 was used for construction of *Escherichia-Gordonia* shuttle vectors, which were also applicable to other *Gordonia* species and other bacteria (11). In addition, the genome sequence of no rubber-degrading bacterium has been determined.

Additional problems arise from the presence of other natural biodegradable compounds in natural rubber and latex (see above) or from additives which are required for vulcanization or to influence the material properties. To avoid allocation of growth or CO_2 release to degradation of, e.g., proteins and lipids present in the material, growth and mineralization experiments must be performed carefully. Additives can promote (e.g., fillers and stoppers) or inhibit (accelerators, antioxidants, and preservation material) biodegradation of rubber material (20, 31). The inhibitory effect of antioxidants extracted from synthetic polyisoprene, which was prepared for tire production, on the growth of *G. westfalica* was demonstrated by Berekaa et

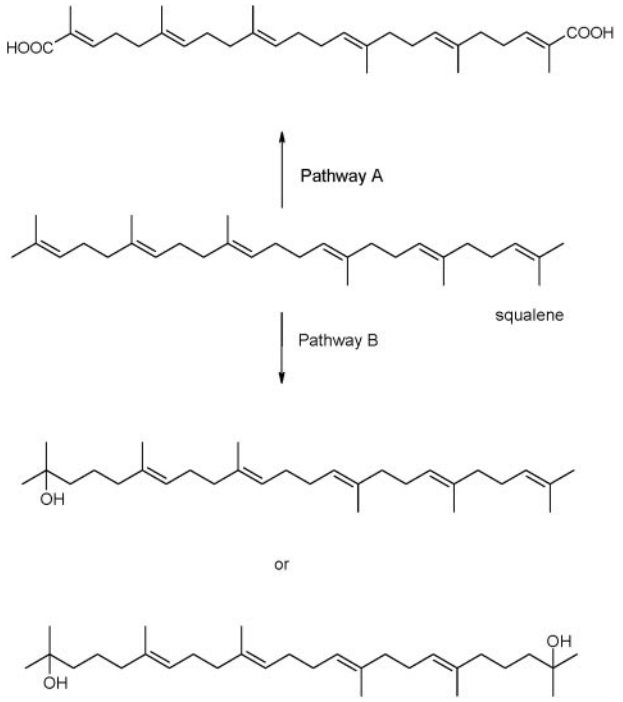

FIG. 2. Proposed oxidation of the terminal methyl groups of squalene to squalenedioic acid (pathway A) and hydration of squalene to mono- and dihydrated squalene (pathway B). Evidence for these pathways was obtained by using *Corynebacterium* sp. strain SY-79 (47) and *Corynebacterium* sp. strain S-401 (46).

al. (6). It was also shown that extraction of latex gloves with organic solvents before incubation enhanced the growth of some rubber-degrading strains (6).

Various difficulties in the study of microbial rubber degradation could be overcome by the use of low-molecular-weight model substances. Molecules like squalene, squalane, β-carotene, or citronellol may be suitable for this purpose (Fig. 1), although the chemical structures of all these compounds differ from that of natural rubber with regard to the configuration of the methyl groups or the existence of double bonds. Oligomers exactly matching the chemical structure of natural rubber are not available.

MICROBIAL DEGRADATION OF SQUALENE AND SQUALANE

Squalene can be regarded with some restrictions as a low-molecular-weight model substance to study microbial polyisoprene degradation, although the configuration of the methyl groups is *trans*. Interestingly, all rubber-degrading bacteria which do not form clear zones on latex agar (see below) are able to metabolize squalene, whereas all clear-zone-forming rubber-degrading strains (see below) are unable to use squalene as a sole carbon source (unpublished data).

Examination of the aerobic degradation of squalene revealed three different metabolic pathways, including (i) oxidation of the terminal methyl groups that leads to squalenedioic acid (Fig. 2) (47) and (ii) hydratation of the double bond that leads to tertiary alcohols (Fig. 2) (46). These pathways occurred in species of the genus *Corynebacterium* if the cells were cultivated in squalene medium supplemented with yeast extract, and the metabolites resisted further degradation and were excreted into the culture broth.

The third pathway involves oxygenase-catalyzed cleavage of the internal double bonds and leads to geranylacetone and 5,9,13-trimethyltetradec-4*E*,8*E*,12-trienic acid (Fig. 3) (58). This pathway is of particular interest with regard to microbial rubber cleavage, because all internal double bonds in squalene involve carbon atoms that carry a methyl group like that in polyisoprene. The hypothetical degradation pathway shown in Fig. 3 was postulated for *Arthrobacter* sp. and for *Marinobacter squalenivorans* (36). Investigations of the latter organism led to detection of several metabolites that occur during growth on squalene. With regard to these metabolites, oxygenase-catalyzed cleavage of internal double bonds, oxidation of keto-terminal methyl groups, decarboxylation of the resulting keto acid, and esterase activity were proposed for squalene degradation by *M. squalenivorans*, although no enzymes or genes were identified. Microbial epoxidation of alkenes, proposed for squalene cleavage by *M. squalenivorans*, was first demonstrated for cells of *Pseudomonas aeruginosa* when the formation of 1,2-epoxyoctane from 1-octene was observed (56).

In contrast to aerobic degradation of squalene, information about the anaerobic catabolism of squalene is scarce. Incomplete conversion of squalene by a methanogenic enrichment culture was studied by Sawada et al. (44). Several denitrifying and squalene-degrading bacteria were recently isolated and characterized (9). In a denitrifying *Marinobacter* species hydration of double bonds to tertiary alcohols occurred as the first step (Fig. 4), and methyl ketones were formed as products of carbon chain cleavage (37). The methyl ketones may be carboxylated, yielding acids, which are then probably metabolized via β-oxidation and β-decarboxylation reactions; asymmetric diols have not been detected.

So far, no enzymes or genes involved in microbial squalene degradation have been identified. Only squalene epoxidase has been characterized in detail. However, this epoxidase is an anabolic enzyme that catalyzes the conversion of squalene to (*3S*)-2,3-oxidosqualene. Together with the cyclization of (*3S*)-2,3-oxidosqualene to sterols, it catalyzes a key step in the conversion of acyclic lipids into sterols in plants, fungi, and vertebrates (1, 27, 59). Inhibition of squalene epoxidase is an important target in the design of therapeutically important antifungal agents like terbinafin (1, 12, 43).

For squalane degradation by *Mycobacterium* spp., a pathway based on carboxylation and deacetylation was proposed (5), as such a pathway was also found for the degradation of citronellol (17). However, for both molecules cleavage at the double-bond positions did not occur. In contrast, β-carotene cleavage of the double bond by a β-carotene 15,15′-monooxygenase occurred at the C-15 position (57); however, this double bond does not involve a carbon atom carrying a methyl group like all double bonds in polyisoprene and squalene.

MICROBIAL DEGRADATION OF NATURAL AND SYNTHETIC RUBBER

Microbial degradation of natural rubber has been investigated for 100 years (48) (Table 1). It became obvious that

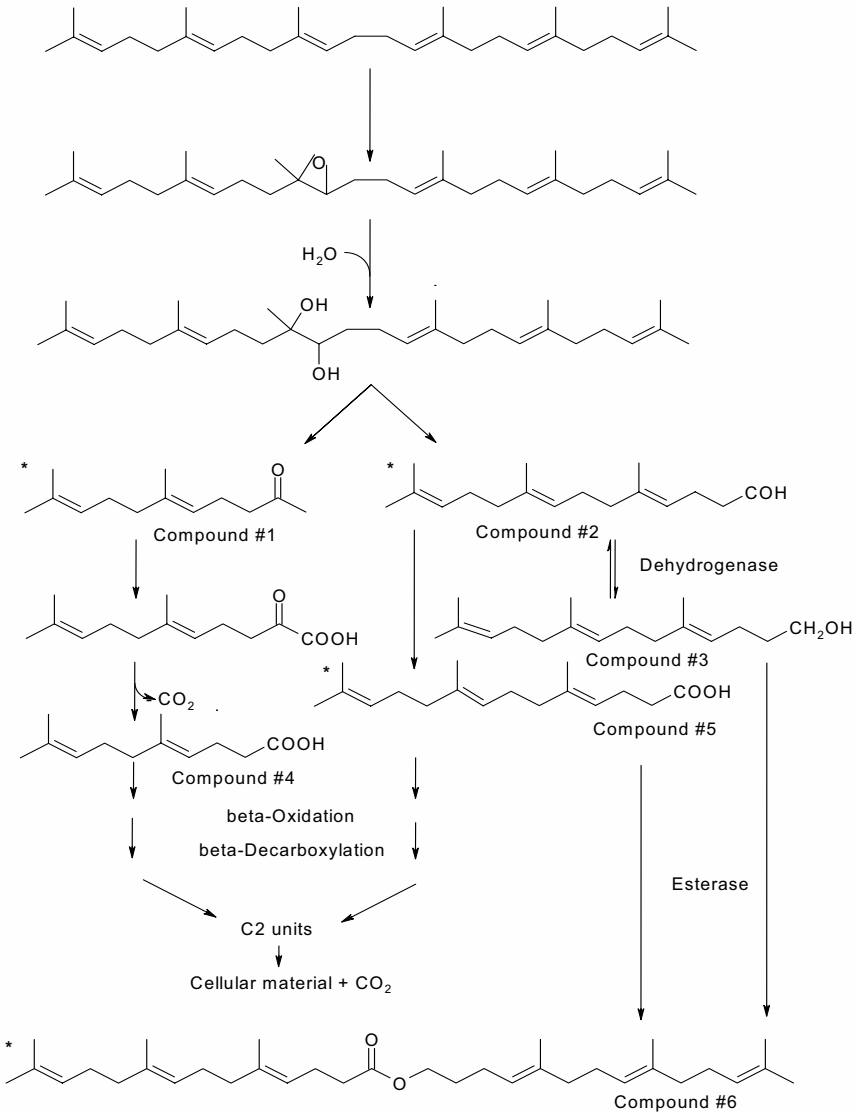

FIG. 3. Proposed oxygenase-catalyzed cleavage of squalene and pathways for aerobic metabolism. Evidence for this pathway was obtained by using *Marinobacter* strain 2Asq64 (36). Compound 1, geranylacetone; compound 2, 5,9,13-trimethyltetradeca-4*E*,8*E*,12-trienal; compound 3, 5,9,13-trimethyltetradeca-4*E*,8*E*,12-trien-1-ol; compound 4, 5,9-dimethyldeca-4*E*,8-dienoic acid; compound 5, 5,9,12-trimethyltetradeca-4*E*,8*E*,12-trienoic acid; compound 6, 5,9,13-trimethyltetradecyl-5,9,13-trimethyltetradecanoate. Detected metabolites are indicated by asterisks.

bacteria, as well as fungi, are capable of degrading rubber and that rubber biodegradation is a slow process (14, 19, 21, 23, 34, 50). The introduction of latex overlay agar plates, which consisted of a bottom agar layer of mineral salt medium and a layer of latex or latex agar on top, for isolation and cultivation of rubber-degrading microorganisms was an important achievement (50). Microorganisms growing on such plates formed clear zones around their colonies. When 1,220 different bacteria were investigated for the ability to degrade rubber employing the latex overlay agar plate technique, 50 clear-zone-forming, rubber-degrading strains all belonging to the mycelium-forming actinomycetes (Table 1) were identified (23). Formation of clear zones was inhibited by addition of glucose, indicating that there was regulation of the expression of rubber-degrading enzymes. Growth of some of the strains on natural rubber led to significant weight loss (10 to 30%, wt/wt) of the material used and to a decrease in the average molecular weight of the polymer from 640,000 to about 25,000.

One disadvantage of latex overlay agar plates is that not all rubber-degrading bacteria can be cultivated in this way, because many do not form halos on such plates and because too little polyisoprene is locally available to allow formation of visible colonies by these organisms. Rubber-degrading bacteria were therefore divided into two groups according to the growth type and other characteristics (29). With one exception, representatives of the first group belong to the clear-zone-forming actinomycetes mentioned above and metabolize the polyisoprene by secretion of one or several enzymes. Most representatives of this group show relatively weak growth on natural or synthetic rubber. Members of the second group do not form halos and do not grow on latex plates; they require direct contact with the polymer, and growth on rubber is adhesive in

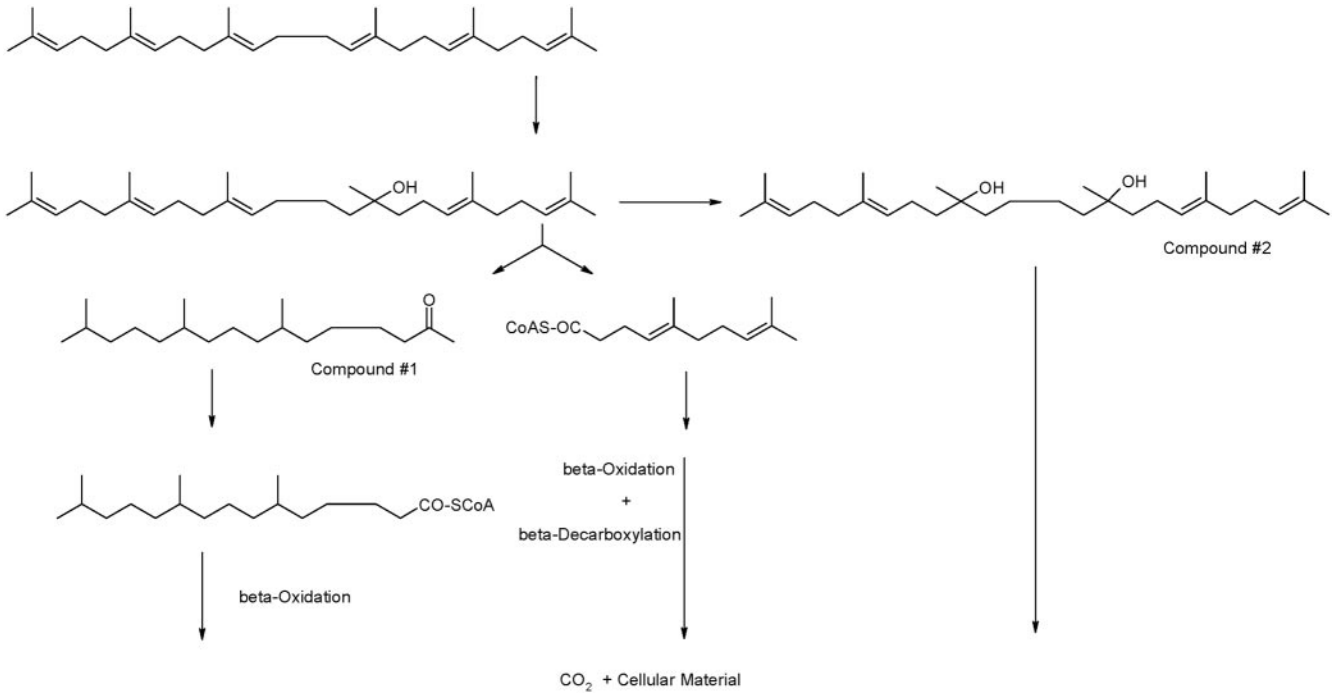

FIG. 4. Proposed pathway for the anaerobic degradation of squalene. Evidence for this pathway was obtained by using *Marinobacter* sp. strain 2sq31 (37). Compound 1 (2,6,10,15,19,23-hexamethyltetracosa-2,6*E*,18*E*,22*E*-tetraen-10,15-diol) and compound 2 (7,11,15-trimethylhexadeca-6*E*,10*E*,14-trien-2-one) were detected in the cultivation broth.

an obligatory sense. Members of this group show relatively strong growth on polyisoprene and belong to the *Corynebacterium-Nocardia-Mycobacterium* group. Some new rubber-degrading strains belonging to the *Corynebacterium-Nocardia-Mycobacterium* group, such as *Gordonia polyisoprenivorans* strains VH2 and Y2K, *G. westfalica* strain Kb1, and *Mycobacterium fortuitum* strain NF4, were isolated recently (2, 30) (Table 1). Species of the genus *Gordonia* very frequently are rubber degraders (4).

TABLE 1. Rubber-degrading bacteria mentioned

Bacterium	Type of rubber degradation[a]	Reference
Actinomadura sp.	B	23
Actinomyces candidus	?	34
Actinomyces elastica	?	48
Actinomyces elasticus	?	34
Actinomyces fuscus	?	48
Actinoplanes (three species)	B	23
Dactylosporangium sp.	B	23
Gordonia polyisoprenivorans VH2	A	29
Gordonia polyisoprenivorans Y2K	A	2
Gordonia westfalica Kb1	A	30
Micromonospora aurantiaca W2b	B	29
Micromonospora (five strains)	B	23
Mycobacterium fortuitum NF4	A	29
Nocardia sp.	B	23
Nocardia sp. strain 835A	?	53
Nocardia farcinica S3	A	22
Proactinomyces ruber	?	34
Streptomyces (31 strains)	B	23
Streptomyces sp.	B	28
Streptomyces sp.	B	38
Streptomyces sp. strain La7	B	19
Streptomyces sp. strain K30	B	40
Thermomonospora sp. strain E5	B	22
Xanthomonas sp. strain 35Y	B	54

[a] A, rubber-degrading bacteria which are unable to grow or form clear zones on latex overlay plates B, rubber-degrading bacteria which form clear zones on latex overlay agar plates. For the type of rubber degradation see reference 29.

Biodegradation of vulcanized rubber material is also possible, although it is even more difficult due to the interlinkages of the poly(*cis*-1,4-isoprene) chains, which result in reduced water absorption and gas permeability of the material (45). Two *Streptomyces* strains were isolated from vulcanized gaskets of cement water tubes, which were the cause of 1.5-mm-diameter holes in the material after 12 months of incubation (38). Continuation of these studies led to development of the so-called Leeflang test bath, in which rubber material is examined in a steady aquatic stream with regard to its stability against microbial degradation (28).

So far, there have been no reports which have definitely demonstrated biodegradation of poly(*trans*-1,4-isoprene), the main constituent of gutta-percha and balata. Although isolation of several microorganisms capable of destroying cast films of gutta extracted from *Eucommia* was reported by Kupletskaya et al. (26), no further details were determined. Intensive attempts in our laboratory to enrich and isolate poly(*trans*-1,4-isoprene)-degrading bacteria or to demonstrate poly(*trans*-1,4-isoprene) degradation by known rubber degraders failed.

BIOCHEMICAL ANALYSIS OF RUBBER BIODEGRADATION

Enzymes involved in rubber biodegradation, particularly enzymes catalyzing cleavage of the rubber backbone, were one of

TABLE 2. Degradation products obtained from natural rubber or synthetic polyisoprene after incubation with different bacteria

Strain	Poly(*cis*-1,4-isoprene)[a]	Mol wt of degradation products	No. of isoprene units	Method of identification[b]	Functional groups[c]	Reference(s)
Nocardia sp. strain 835A	NR	~7,800	114	NMR, GPC	A, K	53
		~1,300	19	NMR, GPC	A, K	
Xanthomonas sp. strain 35Y	NR	~7,700	113	NMR, GPC	A, K	54
		236	2	NMR, GPC	A, K	10, 54
S. coelicolor 1A	NR	226	2	NMR, EI	K, Ac	7
		196	2	NMR, EI	K	
		264	3	NMR, EI	K	
S. lividans TK23 pIJ702::*lcp*	IR	~12,000	~180	GPC	A	40
Nocardia farcinica S3	IR	~13,000	~190	GPC	A, C	22
Thermomonospora sp. strain E5	IR	~13,000	~190	GPC	A	22
		~570	~8	GPC	K?	

[a] NR, natural rubber; IR, poly(*cis*-1,4-isoprene).
[b] EI, electron ionization mass spectrometry.
[c] Functional groups were detected by NMR, infrared spectrometry, or staining with Schiff's reagent. A, aldehyde group; K, keto group; Ac, acid group; C, carbonyl group.

the last obstacles to biopolymer degradation and were unknown until recently. Chemical analysis of degradation products which were transiently formed due to incomplete biodegradation, analysis of mutants not capable of using natural rubber as a carbon source for growth, and finally identification of the first genes coding for enzymes catalyzing cleavage of polyisoprene revealed some information about the biochemistry of rubber biodegradation.

Rubber biodegradation by *Gordonia* sp. The occurrence of isoprene oligomers containing aldehyde and ketone groups after incubation of latex gloves with *G. polyisoprenivorans* and other bacteria and a decrease in the number of double bonds in the polyisoprene chain were demonstrated by staining with Schiff's reagent and using Fourier transform infrared spectroscopy with attenuated total reflectance (29). This was consistent with oxidative cleavage of the polyisoprene molecules.

Analyses of plasmid-free mutants of *G. westfalica* strain Kb1, which had lost the ability to grow on natural rubber as a sole carbon source, suggested that genes located on a 101-kbp megaplasmid comprising 105 open reading frames play an essential role in rubber degradation (11). In addition, transposon mutagenesis of *Gordonia* species using a transposon based on the IS*493* element (49) from *Streptomyces lividans* TK66 revealed mutants defective in pigmentation, anabolic pathways, and also mutants with defects in rubber utilization that are currently being investigated in our laboratory (4).

Rubber biodegradation by *Nocardia* sp. strain 835A. *Nocardia* sp. strain 835A, which exhibited reasonable growth on natural and synthetic rubber, was one of the first strains that was investigated in detail with regard to rubber biodegradation, and it was postulated that there was oxidative cleavage of poly(*cis*-1,4-isoprene) at the double-bond position (53). Weight losses of the rubber material used of 75 and 100% (wt/wt) after 2 and 8 weeks of incubation, respectively, and of the latex glove material used of 90% (wt/wt) after 8 weeks were obtained. Gel permeation chromatography (GPC) of the chloroform-soluble fraction of degraded glove material revealed two fractions of fragments with molecular masses of 1×10^4 and 1.6×10^3 Da, comprising 114 and 19 isoprene molecules, respectively. Both fractions exhibited infrared spectra identical to those of aldehyde derivatives of dolichol, and based on these results together with the results of ^{1}H nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR studies molecules with aldehyde and keto groups having the following formula were postulated: OHC-CH_2-[CH_2-C(-CH_3)═CH-CH_2]$_n$-CH_2-C(═O)-CH_3 (Table 2). Unfortunately, investigations of rubber degradation by this strain were not continued at a biochemical or molecular level.

Rubber biodegradation by *Streptomyces* sp. Species of the genus *Streptomyces* have frequently been investigated with regard to rubber biodegradation. The protein content of cultures of the clear-zone-forming organism *S. coelicolor* strain 1A increased from 240 μg/ml to 620 μg/ml during incubation of the cells with natural rubber latex after 10 weeks of incubation (7). GPC analysis of the rubber material remaining after cultivation of this strain for 6 weeks with synthetic poly(*cis*-1,4-isoprene) showed a shift in the molecular mass distribution from about 800 kDa to about 2×10^4 Da. Analysis of the degradation products of disintegrated latex gloves revealed several compounds, which could be separated by high-performance thin-layer chromatography. Three of the compounds isolated were identified by one- and two-dimensional ^{1}H-NMR spectroscopy as 2,6-dimethyl-10-oxo-undec-6-enoic acid, 5,6-methyl-undec-5-ene-2,9-dione, and 5,9-6,10-dimethyl-pentadec-5,9-diene-2,13-dione. From this analysis and the occurrence of acetonyldiprenylacetoaldehyde (Ap_2A), which was first identified as a rubber degradation product in a *Nocardia* sp. strain 835A culture (see above), the hypothetical pathway for degradation of poly(*cis*-1,4-isoprene) shown in Fig. 5 was suggested. However, the authors pointed out that the compounds identified (compounds 2 to 4) were not necessarily intermediates of rubber degradation, so that these metabolites may have been dead end products. Unfortunately, UV-induced mutants of *Streptomyces griseus* 1D and *S. coelicolor* 1A which were not able to form clear zones on latex overlay agar plates, which showed no increase in protein content in liquid culture containing latex as a sole carbon source, and which did not produce a weight loss in glove material or changes in the molecular weight of the polymer were not analyzed further (8).

Streptomyces sp. strain K30 is another strain from which UV-induced mutants defective in rubber degradation were obtained (40). About 1% of the mutants analyzed exhibited a

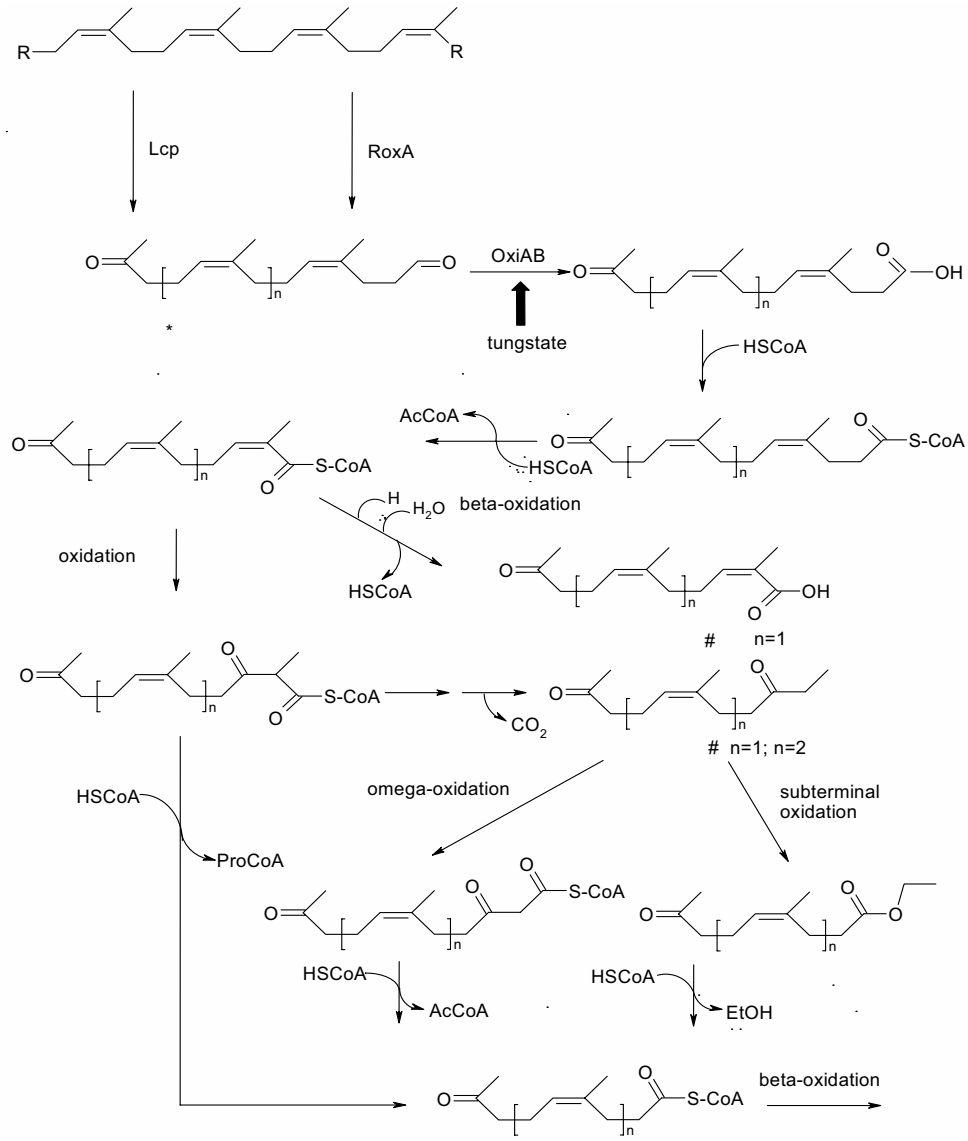

FIG. 5. Hypothetical pathway for rubber degradation. Evidence for this pathway was obtained by using *S. coelicolor* 1A (7) and *Streptomyces* sp. strain K30 (40). Metabolites detected by Tsuchii and Takeda (54) are indicated by asterisks ($n = 1$), and metabolites detected by Bode et al. (7) are indicated by number signs. The position of inhibition of this pathway by tungstate is indicated (40). Lcp, latex clearing protein from *Streptomyces* sp. strain K30; OxiAB, oxidoreductase from *Streptomyces* sp. strain K30; RoxA, rubber oxygenase from *Xanthomonas* sp. (10); CoA, coenzyme A.

clear-zone-negative phenotype on latex overlay plates. However, only a few of these latex-negative mutants retained the ability to form clear zones on xylan like the wild type, thus indicating a correlation between rubber and xylan degradation possibly due to defects in protein secretion. One of these rubber-negative mutants was used to identify three genes encoding the rubber-degrading capability of *Streptomyces* sp. strain K30 by phenotypic complementation (40). The cloned *lcp* (latex clearing protein) gene restored clear zone formation in the rubber-negative mutants described above and also enabled a recombinant strain of *S. lividans* TK23 to grow and to form clear zones on latex overlay agar plates. Furthermore, genes for a heterodimeric molybdenum hydroxylase homologue (*oxiAB*) were located downstream of *lcp* in *Streptomyces* sp. strain K30 (40). Whereas heterologous expression of *lcp* in *S. lividans* TK23 resulted in the accumulation of 12-kDa degradation products containing aldehyde groups, heterologous expression of *lcp* plus *oxiAB* yielded aldehydes only if 10 mM tungstate was present. Since tungstate is known to be a specific inhibitor of molybdenum hydroxylases, OxiAB probably oxidized the aldehydes formed by Lcp to the corresponding acids, which could then be further metabolized via the β-oxidation pathway (Fig. 5). This is consistent with the observation that the presence of 0.1% acrylic acid in the medium prevented growth of *Streptomyces* species on latex (8; Rose, unpublished data).

Rubber biodegradation by *Xanthomonas* sp. strain 35Y. Incubation with the gram-negative, clear-zone-forming organism

Xanthomonas sp. strain 35Y resulted in a weight loss of 60% in natural rubber after only 7 days (54). GPC analysis of the degradation products obtained after incubation of natural rubber with a crude enzyme extract revealed compounds with apparent molecular weights of less than 10^4 and 10^3 (Table 2), comprising about 113 and only 2 isoprene units, respectively. ^{1}H-NMR and ^{13}C-NMR analyses revealed the same molecular structure for the degradation products as that obtained with *Nocardia* sp. strain 835A (see above). Gas chromatography (GC)-mass spectrometry (MS) analysis identified the compound in the low-molecular-weight fraction as acetonyldiprenylacetoaldehyde. A crude enzyme extract prepared from the supernatant of a culture of this *Xanthomonas* strain incubated with natural rubber latex for 5 days revealed activity with natural rubber, poly(*cis*-1,4-isoprene), dolichol, and ficaprenol but not with the *trans* oligoisoprenoid squalene. Degradation studies with crude enzyme and latex in the presence of ^{18}O revealed incorporation of ^{18}O into AP_2A. After incubation for 1 h, the incorporation of one ^{18}O atom into AP_2A was 77% and the incorporation of two atoms of ^{18}O was 4%. Under a nitrogen atmosphere, no detectable AP_2A was produced. Therefore, it was concluded that molecular oxygen is necessary for rubber cleavage at the double-bond position of the polymer.

This *Xanthomonas* strain secretes a protein having an apparent molecular mass of 65 kDa during growth on latex (10, 24), which was referred to as rubber oxygenase (RoxA). Analysis of the sequence of the cloned gene resulted in identification of a signal peptide sequence in the nonmature protein and two heme-binding motifs and a 20-amino-acid region conserved in diheme cytrochrome *c* peroxidases. RoxA of *Xanthomonas* sp. strain 35Y did not exhibit any homology with Lcp of *Streptomyces* sp. strain K30. The purified RoxA protein contained about 2 mol heme per mol RoxA protein and had strong absorption at 406 nm (10), which is characteristic of heme-containing proteins; the absorption shifted to 409 nm upon incubation with synthetic rubber, indicating that the substrate binds to the heme site(s). Absorption bands at 418 nm, 522 nm, 549 nm, and 553 nm appeared after reduction with dithionite. Incubation of the purified RoxA protein with latex and oligo(*cis*-1,4-isoprene) resulted in accumulation of a major 236-Da degradation product (12-oxo-4,8-dimethyltrideca-4,8-diene-1-al) (10), which was also isolated and identified previously (see above) (54), and some homologous degradation products with one less or one to three more isoprene units than the major metabolite. Cyanide and carbon monoxide inhibited this reaction (10). GC-MS analysis of 12-oxo-4,8-dimethyltrideca-4,8-diene-1-al formed in the presence of $^{18}O_2$ and of derivatives indicated that both oxygen atoms were incorporated into the molecule (D. Jendrossek, personal communication). These data indicate that RoxA is a hemoprotein belonging to the cytochrome *c* group representing a novel type of dioxygenase.

IN VITRO DEGRADATION OF POLYISOPRENE BY OXIDATIVE ENZYMES

Recently, three different enzyme mediator systems consisting of radical-generating enzymes and their substrates acting as radical precursors were investigated with regard to biodegradation of polyisoprene and rubber material. Although these enzymes certainly have a different physiological function, these studies demonstrated that biochemically generated radicals are capable of degrading polyisoprenoids.

If *cis*-polyisoprene and *trans*-polyisoprene were incubated with lipoxygenase from *Glycine max* (soybean) or peroxidase from *Amoracia rusticana* (horseradish) in the presence of the radical mediators linoleic acid and 1-hydroxybenzotriazole, respectively, for 24 h at 37°C, GPC analysis revealed a decrease in the molecular weight of the polymers (15). Biodegradation of rubber was completely inhibited by 10 mM butylated hydroxytoluene, indicating that there was consumption of free radicals. These findings matched well the postulated reaction mechanism for lipoxygenase–linoleic acid or peroxidase–1-hydroxybenzotriazole with polyisoprene, resulting in generation of linoleic acid or 1-hydroxybenzotriazole radicals and subsequent chain cleavage of alkoxyl radicals derived from 1,4-polyisoprene by β-scission (15). The lipoxygenase could be replaced by Fenton's reagent; in this case, the radicals were chemically generated. Both systems yielded compounds with aldehyde or keto groups, which were detected by a 2,4-dinitrophenylhydrazine assay (15). Furthermore, examination of latex gloves treated with these enzyme mediator systems for 48 h revealed hole formation in the material, as detected by scanning electron microscopy.

Similarly, manganese peroxidase (MnP) isolated from *Ceriporiopsis subvermispora* strain FP-90031 was incubated with nonvulcanized synthetic poly(*cis*-1,4-isoprene) for 48 to 96 h at 35°C (42). Only if unsaturated fatty acids like linoleic acid or arachidonic acid were used as radical mediators was a decrease in the molecular weight of the polyisoprene observed. Laccase from *Coriolus* sp., horseradish peroxidase, or Fenton's reagent gave similar molecular weight reductions for incubated rubber sheets and GPC profiles of the degradation products. Double shot (DS)-pyrolysis-GC-MS of degraded vulcanized rubber sheets by lipid peroxidation yielded isoprene, 1,4-dimethyl-4-vinylcyclohexene, 1-methyl-5-(1-methylethenyl)cyclohexene, and limonene. Isoprene and the dimers were formed by intramolecular cyclization and subsequent chain scission of the CH_2-CH_2 bonds in polyisoprene.

PUTATIVE MECHANISMS OF POLYISOPRENE-CLEAVING ENZYMES

All the studies with various microorganisms indicated that during rubber degradation oxidative cleavage of the double bond in the poly(*cis*-1,4-isoprene) backbone must occur as the first step. Furthermore, most of the degradation products detected (22, 40, 54) contained aldehyde and keto groups (Table 2). This can be explained by oxygenases like RoxA. Even enzyme mediator systems yielded in vitro degradation products containing aldehyde or keto groups (15, 42). Enzyme systems like the lipoxygenase, peroxidase, or laccase system depend on mediators, which are radicalized by these enzymes and which subsequently generate polyisoprene radicals that are cleaved by β-scission. If a radical mechanism also applies to the in vivo cleavage of polyisoprene by bacteria, radicals must be generated. Fetzner (18) postulated a reaction mechanism for cofactor-independent dioxygenases in which an amino acid residue (probably histidine) functions as a proton acceptor. A combi-

nation of both enzyme mechanisms may allow a new mechanism that does not require a fatty acid mediator.

Interestingly, the degradation products, which were initially generated by most cultures of rubber-degrading bacteria and transiently accumulated in the medium before they were further metabolized, had molecular masses close to 10^4 Da. This observation indicates that there is an endocleavage rather than an exocleavage mechanism of rubber degradation. However, this observation is not consistent with random endocleavage of the polyisoprenoid chain. Random endocleavage may be prevented due to structural constraints related to either the rubber-degrading enzyme itself, which requires binding of the polymer in a particular manner to the surface of the enzyme, or the microstructure of the solid polymer, which is exposed to the surface accessible to the enzyme at only defined distances. The situation might be different if excess purified enzyme is employed and if the reaction continues to completion, as shown for RoxA (10). Understanding the reasons for the nonrandom endocleavage may also help explain why these microorganisms degrade only poly(*cis*-1,4-isoprene) and generally not the *trans* isomer. For example, only the *cis* polymer might be flexible enough to bind to the enzyme protein and to the catalytic domains.

CONCLUSIONS

Natural rubber and other natural polyisoprenoids are the only biopolymers whose cleavage by enzymes is still mostly unknown. However, recently there has been considerable progress in our understanding of microbial rubber degradation. Analyses of the degradation products have indicated a possible biodegradation pathway for this abundant and technically important polymer. Two nonhomologous enzymes involved in this process and the respective genes were recently identified in species of the genera *Streptomyces* and *Xanthomonas*. This should allow detailed molecular and biochemical studies to determine the mechanisms by which natural and synthetic polyisoprenoids are degraded.

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