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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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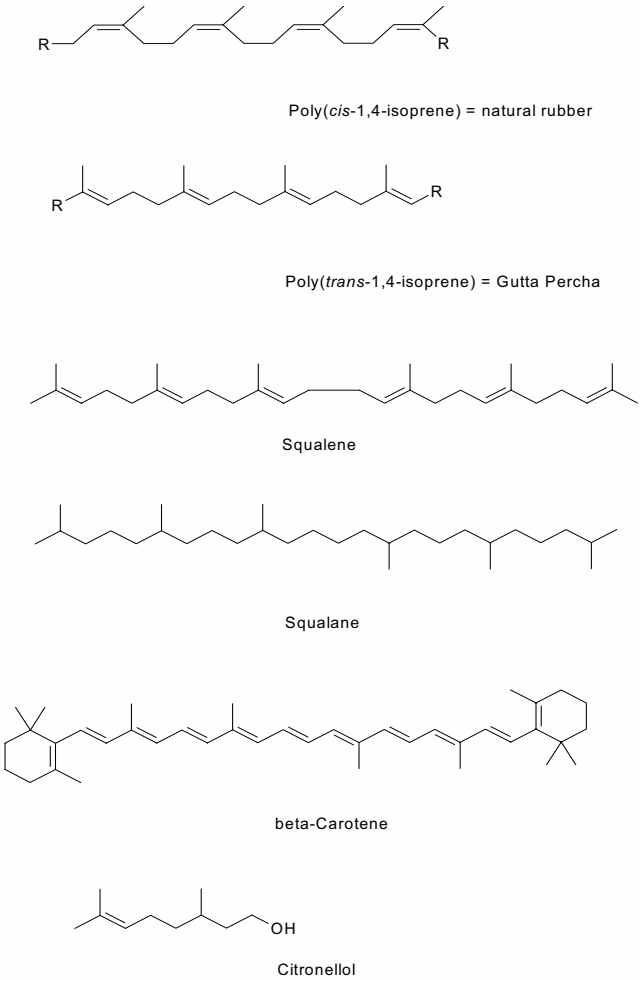

FIG. 1. Structural formulas of polyisoprenoids and putative low-molecular-weight model substances.

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

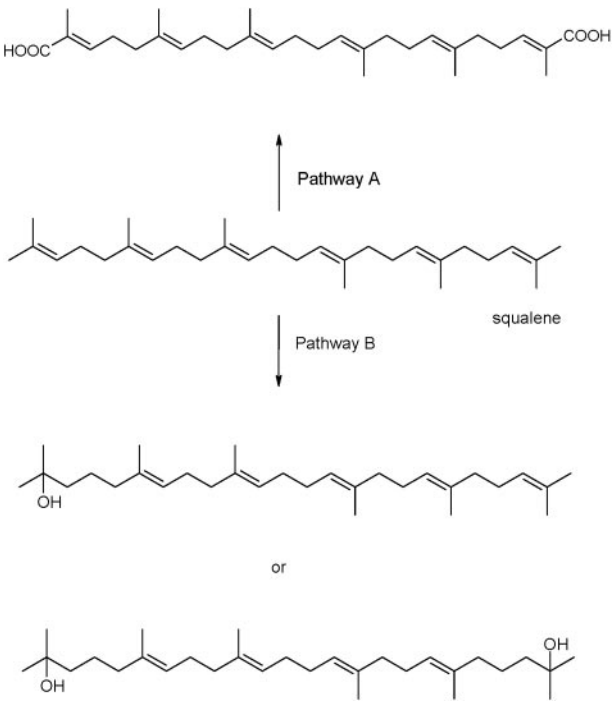

FIG. 2. Proposed oxidation of the terminal methyl groups of squalene to squalenedioic acid (pathway A) and hydration of squalene to mono- and dihydrated squalene (pathway B). Evidence for these pathways was obtained by using *Corynebacterium* sp. strain SY-79 (47) and *Corynebacterium* sp. strain S-401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

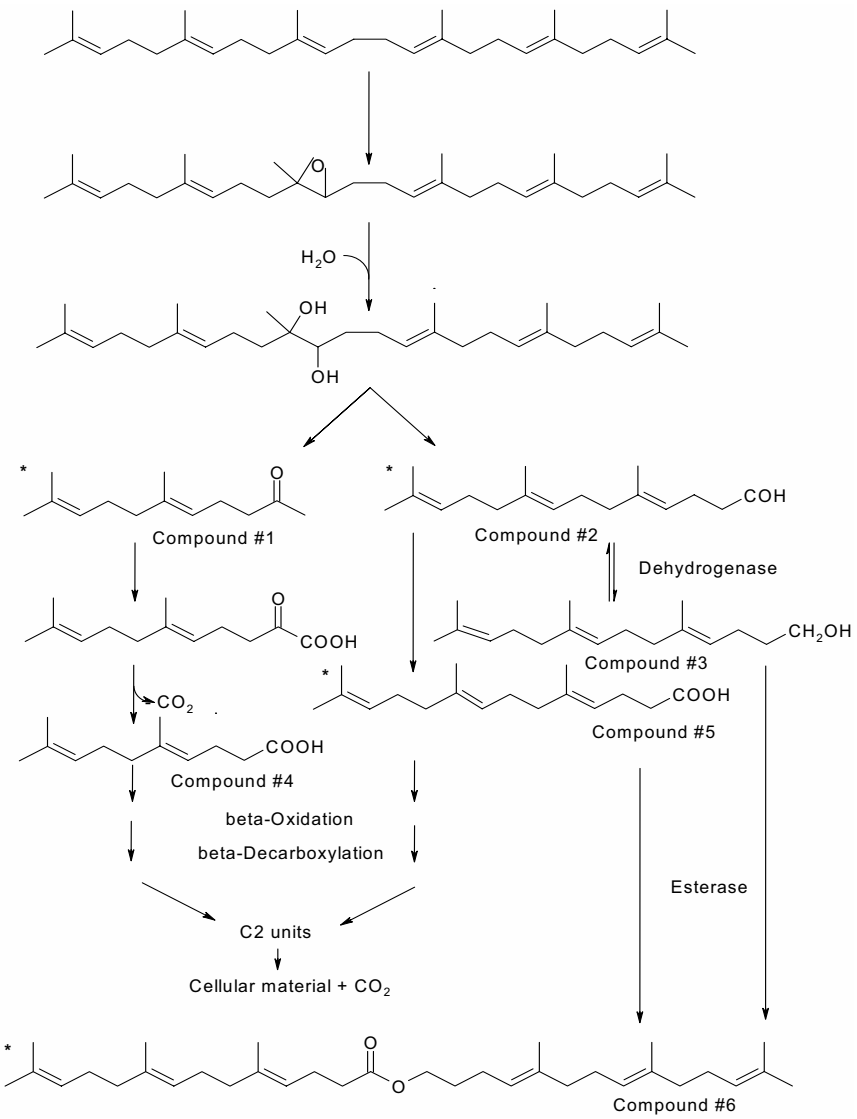

FIG. 3. Proposed oxygenase-catalyzed cleavage of squalene and pathways for aerobic metabolism. Evidence for this pathway was obtained by using *Marinobacter* strain 2Asq64 (36). Compound 1, geranylacetone; compound 2, 5,9,13-trimethyltetradeca-4*E*,8*E*,12-trienal; compound 3, 5,9,13-trimethyltetradeca-4*E*,8*E*,12-trien-1-ol; 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

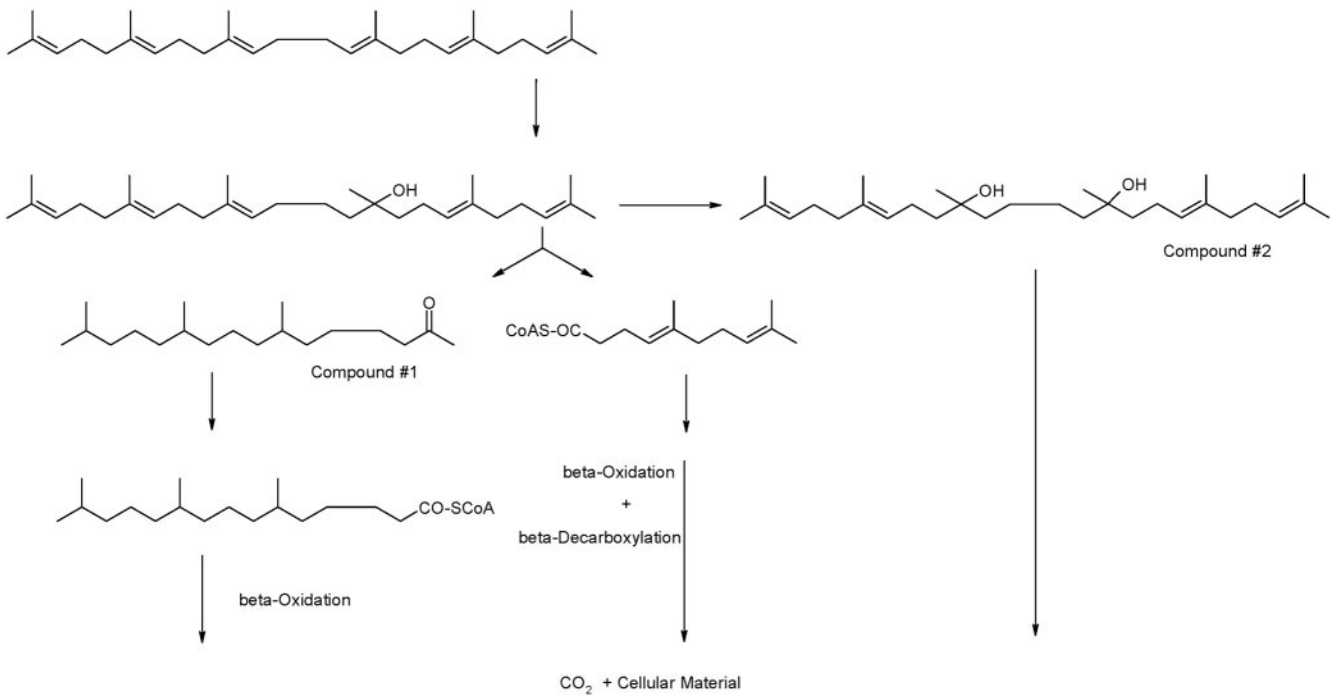

FIG. 4. Proposed pathway for the anaerobic degradation of squalene. Evidence for this pathway was obtained by using *Marinobacter* sp. strain 2sq31 (37). Compound 1 (2,6,10,15,19,23-hexamethyltetracosa-2,6*E*,18*E*,22*E*-tetraen-10,15-diol) and compound 2 (7,11,15-trimethylhexadeca-6*E*,10*E*,14-trien-2-one)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

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