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MINI-REVIEW

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Recent advances in understanding resin acid biodegradation: microbial diversity and metabolism

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Abstract Resin acids are tricyclic diterpenoids that are found in the oleoresin of coniferous trees. Resin-acid-degrading microorganisms are ubiquitous in the environment. The bacterial isolates that grow on resin acids as sole organic substrates are physiologically and phylogenetically diverse, and include psychrotolerant, mesophilic, and thermophilic bacteria. Recent studies of the biodegradation of resin acids by these organisms have demonstrated that in gram-negative bacteria, distinct biochemical pathways exist for the degradation of abietane- and pimerane-type resin acids. One of these organisms, *Pseudomonas abietaniphila* BKME-9, harbors a convergent pathway that channels the nonaromatic abietanes and dehydroabietic acid into 7-oxodehydroabietic acid. This dioxygenolytic pathway is encoded by the recently cloned and sequenced *dit* gene cluster. The *dit* cluster encodes the ferredoxin and the α- and β-subunits of a new class of ring-hydroxylating dioxygenases as well as an extradiol ring-cleavage dioxygenase. Although it was previously thought that resin acids are very recalcitrant under anoxic conditions, recent investigations have demonstrated that they are partially metabolized under anoxic conditions by undefined microorganisms. The anaerobic degradation of resin acids principally generates aromatized and decarboxylated products (such as retene) that are thought to persist in the environment.

Key words Resin acid · Abietane · Pimerane · Diversity · Biodegradation · Dioxygenase · Diterpene

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Introduction

Investigations have elucidated pathways for the degradation of many plant compounds. These studies have focused principally on polymers such as cellulose and lignin, or on low-molecular-weight compounds such as phenolics and flavonoids. Because of their potential commercial and pharmaceutical applications, the microbial degradation products of mono- and triterpenoids have also been researched extensively (Barz and Weltring 1985; Van der Werf et al. 1997). This mini-review focuses on the microbial degradation of resin acids, a group of diterpenoids found mainly in the oleoresin of coniferous trees.

Resin acids can be classified into abietanes and pimeranes. Abietanes have an isopropyl side chain at the C-13 carbon atom, whereas pimeranes have vinyl and methyl substituents at this position (Fig. 1). Although the biodegradation of resin acids is a significant process in the global carbon cycle, it is research on the microbiology of pulp and paper mill effluent detoxification that has been the driving factor behind the biochemical studies of resin acid degradation. Resin acids are extracted from wood during the pulping process and are discharged in waste-

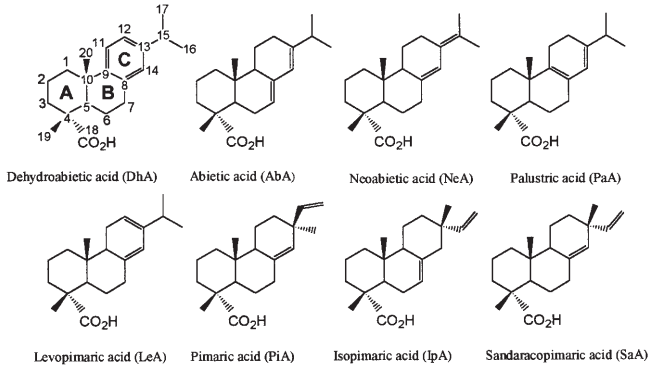

Fig. 1 Chemical structure of resin acids discussed in this paper. Carbon atom numbering and ring designation are shown for dehydroabietic acid

Table 1 Bacterial and fungal isolates capable of the biotransformation of resin acids (*SBR* sequencing batch reactor, *BKME* bleached kraft mill effluent, *ETASR* elevated-temperature activated sludge reactor, *DhA* dehydroabietic acid, *IpA* isopimaric acid, *AbA* abietic acid, *G* use as sole organic substrate, *SR* substrate removed from medium, and *CM* cometabolism of substrate)

Organism	Source	Substrate	Metabolism	Reference
Bacteria				
Sphingomonas sp. strain DhA-33[a]	SBR	DhA	G	Mohn (1995)
Zoogloea resiniphila DhA-35[a]	SBR	DhA	G	Mohn (1995)
Ralstonia sp. strain BKME-6[a]	BKME	DhA	G	Bicho et al. (1995)
Burkholderia sp. strain IpA-51[a]	Forest soil	IpA	G	Mohn et al. (1999a)
Burkholderia sp. strain DhA-54[a]	Forest soil	DhA	G	Mohn et al. (1999a)
Pseudomonas resinovorans[a]	Wheat field soil	DhA	G	Biellmann and Wennig (1971)
Pseudomonas vancouverensis DhA-51[a]	Forest soil	DhA	G	Mohn et al. (1999a)
Pseudomonas multiresinivorans IpA-1[a]	SBR	IpA	G	Wilson et al. (1996)
Pseudomonas sp. strain IpA-2[a]	SBR	IpA	G	Wilson et al. (1996)
Pseudomonas abietaniphila BKME-9[a]	BKME	DhA	G	Bicho et al. (1995)
Pseudomonas sp.	Unknown	DhA	G	Biellmann et al. (1973b)
Flavobacterium resinovorum	Pine forest soil	DhA	G	Biellmann et al. (1973a) Biellmann and Wennig (1968)
Mycobacterium sp. strain DhA-55[a]	Forest soil	DhA	G	Mohn et al. (1999a)
Mycobacterium sp. strain IpA-13[a]	SBR	IpA	G	Wilson et al. (1996)
Arthrobacter sp.	Lodgepole pine	Methyl DhA	G	Levinson and Carter (1968)
Pseudomonas sp. strain A19-6a[a,b]	BKME	AbA	G	Morgan and Wyndham (1996)
Alcaligenes sp. strain D11-13	BKME	DhA	SR	Morgan and Wyndham (1996)
Alcaligenes sp.	Soil	AbA	G	Cross and Myers (1968)
Alcaligenes eutrophus	Unknown	DhA	G	Biellmann et al. (1973b)
Bacillus psychrophilus	Pulp mill effluent	DhA	CM	Côté and Otis (1989)
β-Proteobacterium strain DhA-71[a]	Compost	DhA	CM	Yu and Mohn (1999)
β-Proteobacterium strain DhA-73[a]	ETASR	DhA	G	Yu and Mohn (1999)
Pseudomonas sp. strain DhA-92[a]	Arctic soil	DhA	G	Mohn et al. (1999b)
Pseudomonas sp. strain IpA-95[a]	Arctic soil	IpA	G	Mohn et al. (1999b)
Pseudomonas sp. strain IpA-93[a]	Arctic soil	IpA	G	Mohn et al. (1999b)
Sphingomonas sp. strain DhA-95[a]	Arctic soil	DhA	G	Mohn et al. (1999b)
Fungi				
Fomes annosus	Unknown	DhA	CM	Ekman and Sjoholm (1979)
Corticium sasakii	Unknown	Methyl DhA	CM	Brannon et al. (1968)
Mortierella isabellina	Culture collection	Resin acids	CM	Kutney et al. (1988)
Ophiostoma sp.	Softwood	Resin acids	SR	Wang et al. (1995)
Lecythophora sp.	Aspen wood	Resin acids	SR	Wang et al. (1995)
Chaetomium cochliodes	Unknown	DhA	CM	Yano et al. (1994)

[a] Identified by 16S rDNA sequence
[b] Formerly *Comamonas* sp. strain A19-6a

water. A significant amount of the overall acute toxicity of this wastewater can be attributed to the presence of resin acids (Leach and Thakore 1973; Walden and Howard 1981; Priha and Talka 1986). Thus, understanding the biodegradation process for this class of compounds in biological wastewater treatment systems is important.

This mini-review reports on the recent advances in the research on the diversity and metabolism of resin acid degraders. The various hydroxylation reactions carried out by fungi are not reviewed. However, most of these hydroxylation reactions are described in references listed in Table 1. For a general review on resin acids and their significance to the pulp and paper industry, the reader is referred to the review by Liss et al. (1997).

The ecology and diversity of resin-acid-degrading bacteria

Resin-acid-degrading microorganisms are widely distributed in the environment. This is illustrated by the fact that biodegradative activity has been found in various samples collected from natural waters (Hemingway and Greaves 1973; Côté and Otis 1989); sediments (Tavendale et al. 1997a, b); biological treatment systems for pulp mill effluents (Rogers and Mahood 1974; Servizi and Gordon 1986; Servizi et al. 1986; Taylor et al. 1988; Zender et al. 1994); activated sludge systems (Hemingway and Greaves 1973; Liver and Hall 1994); upflow anaerobic sludge bed reactors (Patoine et al. 1997); and forest, agricultural, and Arctic soils (Biellmann and Wennig 1971; Mohn et al.

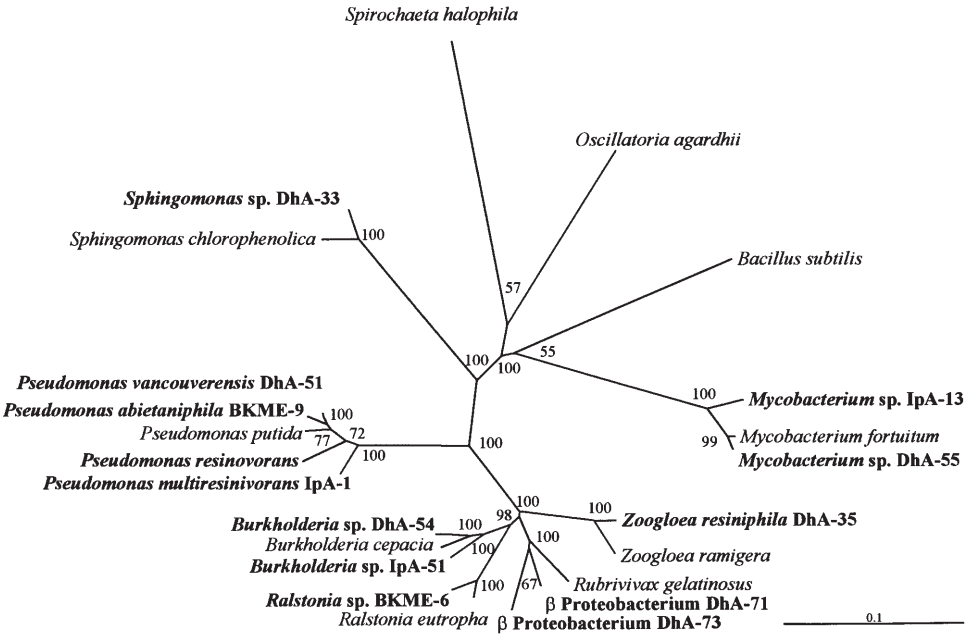

Fig. 2 Unrooted tree inferred from 16S rDNA sequence data showing the phylogenetic relationships of resin-acid-degrading bacteria and reference strains. Resin-acid-degrading strains are *in bold*. The tree was constructed by using the neighbor-joining method, and bootstrap values were calculated from 100 trees. Each *number on a branch* indicates the number of times that the node was supported by the bootstrap analysis result (*bar* 0.1 estimated nucleotide changes per sequence position)

1999 a, b). Consistent with the wide occurrence of resin acid degradation activities, varieties of pure cultures have been isolated from numerous sources after enrichment on resin acids (Table 1). Most of the bacterial isolates listed in Table 1 were isolated from enrichment cultures on mineral media supplemented with resin acids as sole organic substrates. This approach facilitated isolation of microorganisms both degrading and growing on resin acids, but excluded those transforming but not growing on resin acids as sole substrate. Undoubtedly, the latter group also exists. For example, Hemingway and Greaves (1973) have shown that among the 69 bacterial isolates from wood sources, few can use resin acids as a carbon source, but 11 strains (six *Bacillus, Escherichia coli, Flavobacterium* sp., and *Pseudomonas* sp. strains, and two unidentified ones) show some potential to degrade resin acids. Recently, Yu and Mohn (1999) have isolated from compost a bacterium that mineralizes abietane resin acids but additionally requires tryptic soy broth for growth. The wide ecological distribution of resin-acid-degrading microorganisms may be attributed to the ubiquitous nature of these compounds. Resin acids are released from terrestrial vegetation into the atmosphere or into water bodies from watershed runoffs, and are dispersed to every corner of the earth (Mazurek and Simoneit 1997).

While resin-acid-degrading microorganisms are present in many environments, in most cases they are probably found at low abundance. In an extensive survey of 21 biotreatment systems of pulp mill effluents, the two dehydroabietic acid (DhA)-degrading species, *Pseudomonas abietaniphila* BKME-9 and *Zoogloea resiniphila* DhA-35, were found in ten and three of the systems, respectively (Yu et al. 1999). However, the abundance of *P. abietaniphila* and *Z. resiniphila* in most biotreatment systems was less than 10^3 cells/ml. In most environments, resin acids are found in low concentrations and account only for a very small portion of the total organic matter present. Such low resin acid levels would not be expected to sustain large microbial populations.

Resin-acid-degrading microorganisms are phylogenetically diverse. Species of both bacteria and fungi can transform resin acids (Table 1). Aerobic bacterial isolates typically use resin acids as sole growth substrates, whereas fungal isolates only transform, especially hydroxylate, resin acids. The analysis of 16S rDNA of resin-acid-degrading bacteria illustrates their phylogenetic diversity (Fig. 2). Of the unique aerobic bacterial isolates characterized so far, most are gram-negative Proteobacteria with representatives in the α-, β-, and γ-subclasses. The gram-positive isolates include two *Mycobacterium* strains (Fig. 2) and one *Bacillus* strain (Table 1). Although most of the isolates characterized to date are mesophiles (Mohn et al. 1999 a), thermophilic (Yu and Mohn 1999) and psychrotolerant (Mohn et al. 1999 b) strains have recently been isolated.

Resin acid substrate specificity of aerobic bacteria

Consistent metabolic patterns have emerged from screening the ability of bacteria to metabolize the tricyclic structure of resin acids. These patterns are associated with differing degrees of saturation, isomers, side chains, or chlorine substituents. Several studies (Bicho et al. 1995; Mohn 1995; Mohn et al. 1999 a) have demonstrated that the ability of bacterial isolates to grow on dehydroabietic acid coincides with their ability to grow on all abietanes. Accordingly, a common biochemical pathway for the degradation of abietane-type resin acids that converges to an aromatic intermediate was proposed (Martin and Mohn 1999 a). Another deduction that can be drawn from simple substrate specificity tests is that the pathway(s) responsi-

ble for abietane degradation in gram-negative bacteria cannot accommodate pimeranes as substrates, whereas the pimerane biodegradation pathway(s) may catalyze abietane degradation (Bicho et al. 1995; Mohn 1995; Wilson et al. 1996). Bacteria isolated from enrichment cultures using either abietic or dehydroabietic acid use only abietane resin acids. The isopropyl side chain of abietanes at C-13 appears to have a critical role in determining the specificity of the biochemical pathway for this class of compounds.

Organisms isolated on pimeranes can often use abietanes. Two *Pseudomonas* strains, IpA-1 and IpA-2, isolated from isopimaric acid (IpA) enrichment cultures grew on pimeranes (isopimaric and pimaric acids) and dehydroabietic acid, although growth on the latter was slower (Wilson et al. 1996). Cell suspension induction experiments demonstrated that isopimaric acid is required to efficiently induce the degradation of abietic and dehydroabietic acids in strain IpA-1, whereas for strain IpA-2, abietanes could readily induce their own degradation. Interestingly, strain IpA-2 has a diterpenoid-inducible enzymatic system that can degrade pimeranes and abietanes, but this organism cannot grow on abietic acid and grows poorly on dehydroabietic acid. This suggests that strain IpA-2 cometabolizes abietanes in its natural habitat. Recently, a broader group of bacteria (11 isolates) were tested for their specificity for resin acids (Mohn et al. 1999a). Two gram-positive *Mycobacterium* sp. strains used both pimeranes and abietanes. One strain, *Burkholderia* sp. IpA-51, was specific for isopimaric acid, the substrate used for its enrichment and isolation.

Chlorinated derivatives of dehydroabietic acid are formed as by-products of pulp bleaching with elemental chlorine. Since these compounds are more toxic and recalcitrant than their precursors (Zanella 1983), their removal from effluents by biological treatment is important. Metabolic studies of chlorinated dehydroabietic acid have revealed that, in most instances, a chlorine substituent at the C-14 position hinders degradation of dehydroabietic acid by gram-negative bacteria, whereas a single chlorine substitution at C-12 is tolerated (Bicho et al. 1995; Mohn and Stewart 1997). It has been found that one isolate, *Sphingomonas* sp. strain DhA-33, removes both the 12- and the 14-chlorodehydroabietic acid isomers equally when growing on them. When previously induced by growth on chlorodehydroabietic acid, this strain could also remove 12,14-dichlorodehydroabietic acid. Growth of strain DhA-33 on chlorodehydroabietic acid was slower than that on dehydroabietic acid, with doubling times of 2.7 and 7 h for dehydroabietic and chlorodehydroabietic acids, respectively. Bacteria growing on chlorodehydroabietic acid left high residual levels of substrate in the culture medium and, in the case of strain DhA-33, accumulated a metabolite tentatively identified as 3-oxo-14-chlorodehydroabietin. Since chlorinated dehydroabietic acids were presumably very rare prior to the practice of bleaching pulp with chlorine, the degradation of chlorodehydroabietic acid is likely fortuitous (cometabolic) and catalyzed by enzyme systems that evolved for dehydroabietic acid degradation. As expected, pimerane-degrading bacteria are unable to degrade chlorinated dehydroabietic acid, but unexpectedly the two *Mycobacterium* sp. do not degrade chlorodehydroabietic acid even though these strains grow on dehydroabietic acid. The inability of abietane-degrading, gram-positive bacteria to metabolize chlorodehydroabietic acid combined with their ability to degrade pimeranes probably indicates divergence in the resin acid biodegradation pathways of gram-positive and gram-negative bacteria. Finally, it should be noted that bacteria that can grow on 12,14-dichlorodehydroabietic acid have yet to be isolated.

Pathways for aerobic abietane degradation

Decades ago, two studies reported on the dehydroabietic acid degradation pathways of *Flavobacterium resinovorum* (Biellmann et al. 1973a), an *Alcaligenes* sp. (Biellmann et al. 1973b), and a *Pseudomonas* sp. (Biellmann et al. 1973b). These pathways were proposed on the basis of intermediates isolated from culture media supplemented with metabolic inhibitors. Similarities emerged when these pathways were compared to one for *P. abietaniphila* BKME-9 that had recently been proposed on the basis of molecular genetic studies (Martin and Mohn 1999a). For example, in pathway **A** (Fig. 3), hydroxylation at C-7 to form the alcohol **1** followed by its oxidation to the ketone **2** was observed for all four strains. In contrast, oxidation at C-3 (pathway **B**) to form the ketone, presumably from the alcohol, followed by decarboxylation (enzymatic or spontaneous) to the corresponding 3-oxodehydroabietin **9** was observed only in *F. resinovorum*. In the case of *F. resinovorum*, it was unclear which oxidation, C-3 or C-7, proceeded first since both products were isolated from culture medium (Biellmann and Wennig 1968; Biellmann et al. 1973a). In addition, we note that in the proposed pathway for *F. resinovorum* one cannot determine if the oxidation at C-3 preceded (pathway **B**) or followed (pathway **E**) dioxygenation since neither compound **4** nor 3,7-dioxodehydroabietin was isolated from the culture supernatant.

In all four strains, dioxygenation of the aromatic ring led to the formation of a diol, presumably from the dihydrodiol **3** (Martin and Mohn 1999a), to form 3,7-dioxo-11,12-diol **6** in *F. resinovorum* (Biellmann et al. 1973a) or 7-oxo-11,12-diol **4** in the *Alcaligenes* and *Pseudomonas* strains (pathway **D**) (Biellmann et al. 1973b). The isolation of 2-isopropyl malic acid **8** from culture supernatant and the identification of a diterpenoid *meta*-cleavage dioxygenase in *P. abietaniphila* (see the genetics section) suggest that the diols **4** and **6** are degraded via an extradiol ring fission reaction, although the ring-cleavage reaction product(s) **5** and **7** have never been isolated. The proposed dioxygenolytic pathway of dehydroabietic acid is somewhat analogous to the upper degradation pathways of fused ring polycyclic aromatic hydrocarbons. In polycyclic aromatic hydrocarbon degradation, the extradiol cleavage product is unstable and aromatizes to a hemiketal that

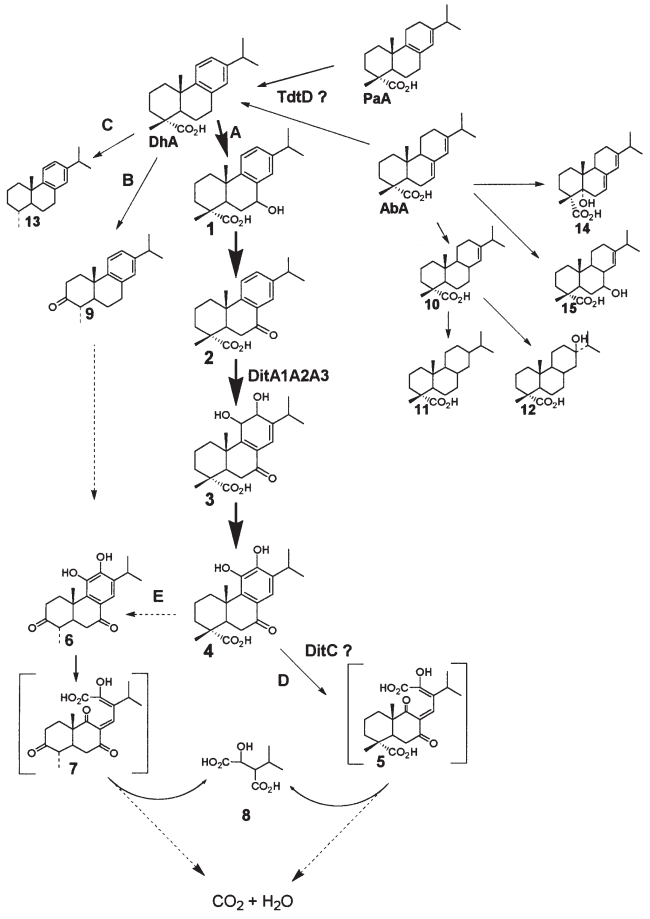

Fig. 3 Summary of proposed biochemical pathways for aerobic abietane degradation or transformation by *Flavobacterium resinovorum* (Biellmann et al. 1973a), *Pseudomonas abietaniphila* BKME-9 (Martin and Mohn 1999a), *Alcaligenes eutrophus*, a *Pseudomonas* sp. (Biellmann et al. 1973b), an *Alcaligenes* (Cross and Myers 1968), and biological pulp mill effluent treatment systems (Zender et al. 1994). Compounds *in brackets* are proposed intermediates. *Dashed arrows* represent several potential hypothetical steps in the pathway with unidentified intermediates

spontaneously or enzymatically isomerizes to a *trans-o*-hydroxyarenylidenepyruvate (Eaton and Chapman 1992). The pathway of dehydroabietic acid degradation may similarly proceed through a hemiketal intermediate, which may explain why the cleavage products (Fig. 3, compounds **5** and **7**) have not been found. However, the hypothetical hemiketal cleavage product(s) of dehydroabietic acid degradation have also never been found.

In contrast, the degradation of methyldehydroabietate by an *Arthrobacter* sp. follows a novel route of abietane catabolism (Levinson and Carter 1968; not shown). In this pathway, methyldehydroabietate is first hydrolyzed to dehydroabietic acid before being converted to 3-oxodehydroabietic acid. Ring A of the 3-oxo acid is further degraded, while the aromatic ring C remains intact, resulting in the formation of 1-carboxy-1,2-dimethyl-6-isopropyltetrahydronaphthalene (not shown). The isolation of the 3-oxo acid from *Arthrobacter* sp. suggests an enzymatic (not spontaneous) decarboxylation at C-4 (Fig. 3, compound **9**) by *F. resinovorum* since the 3-oxo acid has been shown to survive a similar purification procedure (acidification followed by organic solvent extraction and silica gel or gas-liquid chromatography).

The chemical analysis of a biological treatment system receiving effluents from a softwood bleached kraft pulp and paper mill has identified four other resin acid transformation products (Zender et al. 1994). These compounds, proposed to be hydration and hydroxylation products of abietic acid, have been identified as 13-abietenic acid **10**, abietanic acid **11**, and 13β-hydroxyabietanic acid (kinleithic acid) **12** (Fig. 3). This study has also provided evidence that abietic acid is dehydrogenated to dehydroabietic acid and further decarboxylated nonoxidatively to dehydroabietin **13**. However, these transformations were based on circumstantial evidence, and it has not been shown that they are biologically catalyzed. The transformation of abietic acid by an *Alcaligenes* sp. produced three novel compounds (Cross and Myers 1968) that were identified as 5α-hydroxyabietic acid **14**, 7β-hydroxyabietic acid **15**, and a minor product believed to be an 18→2 or an 18→6 epoxy-γ-lactone (not shown). Finally, we would like to caution the reader about the uncertainty of the pathways depicted in Fig. 3. Since many of these metabolites were isolated from culture media, sometimes in the presence of metabolic inhibitors, they might not be representative of the principal pathway(s) of resin acid degradation. Moreover, it is also conceivable that a branched, rather than a straight pathway can lead to resin acid mineralization by several routes.

Genetics of the resin acid degradation pathway(s) from two *Pseudomonas* spp.

Until recently, the extent of our knowledge of the biochemistry of resin acid biodegradation was limited to studies in which pathways were assembled from metabolites isolated from the culture broth of various organisms. Now, some of the genes encoding enzymes responsible for key steps of an abietane degradation pathway have been identified (Martin and Mohn 1999a). Consistent with the earlier report of a dehydroabietic acid diol intermediate (Biellmann et al. 1973a, b), a gene cluster encoding a diterpenoid dioxygenolytic pathway has been cloned and sequenced from *P. abietaniphila* BKME-9 (Fig. 4). This cluster encodes several genes required for abietane degradation, including an extradiol ring-cleavage dioxygenase and a novel type of aromatic ring-hydroxylating dioxygenase that hydroxylates 7-oxodehydroabietic acid **2** to 7-oxo-11,12-dihydroxy-8,13-abietadien acid **3** (Fig. 3; Martin and Mohn 1999a). The hydroxylating dioxygenase enzyme complex is formed from the α- and β-subunits of the terminal oxygenase encoded by *ditA1* and *ditA2*, the ferredoxin encoded by *ditA3*, and an unidentified putative NAD(P)H-ferredoxin oxidoreductase. Two characteristics distinguish this enzyme from previously identified multicomponent dioxygenases. The amino acid sequence of the ferredoxin shows higher similarity to

[4Fe–4S]- and [3Fe–4S]-type ferredoxins than to the [2Fe–2S] types usually found in these enzyme complexes, and the genes encoding the three components of the enzyme are located on separate transcriptional units. In *P. abietaniphila*, expression of the *ditA1A2* and *ditA3* genes is induced by a broad range of resin acids and is controlled by the IclR-type transcription regulator DitR (Martin and Mohn 1999b; Fig. 4). A common biochemical pathway for the degradation of abietane-type resin acids that converges to an aromatic intermediate, 7-oxodehydroabietic acid **2**, has been proposed (Martin and Mohn 1999a). A Tn*5* insertion in the gene encoding the α-subunit of the aromatic-ring-hydroxylating dioxygenase resulted in a mutant strain of *P. abietaniphila* BKME-9 that lost the ability to grow on dehydroabietic acid and on the nonaromatic resin acid, abietic acid. Cell suspensions of this mutant transformed the nonaromatic resin acids, palustric acid and abietic acid, to 7-oxodehydroabietic acid **2** (Fig. 3; Martin and Mohn 1999b). This suggests that in *P. abietaniphila*, the catabolism of abietane resin acids proceeds via a convergent pathway with 7-oxodehydroabietic acid **2** as the aromatic intermediate. The formation of 7-oxodehydroabietic acid followed by aromatic ring attack (Fig. 3, pathway **A**) in *P. abietaniphila* is consistent with the earlier work of Biellmann et al. (1973b) and might very well represent the most common pathway of aerobic degradation.

A convergent pathway for abietane degradation is consistent with the recent isolation of a mutant strain of *Pseudomonas* sp. A19-6a (Table 1) that can degrade dehydroabietic acid but not abietic acid (C. A. Morgan and R. C. Wyndham, Institute of Biology, Carleton University, Ottawa, Canada, personal communication). This insertional mutation was located in an open reading frame, designated *tdtD*, with similarity to genes encoding cytochrome P-450 monooxygenase enzymes. This result led to the hypothesis that a cytochrome P-450-dependent desaturation reaction may aromatize abietic acid to dehydroabietic acid, which would subsequently be degraded through a common dioxygenolytic pathway (Fig. 3). By transposon mutagenesis, C. A. Morgan and R. C. Wyndham (personal communication) cloned a cluster of genes encoding the putative P-450 monooxygenase gene *tdtD*. This cluster contained an additional six open reading frames that potentially encode a CoA ligase (*tdtL*), a putative regulator (*tdtR*), an unidentified gene product (*tdtS*), an isomerase (*tdtA*), a dehydrogenase (*tdtB*), and a thiolase (*tdtC*). Insertional inactivation of *tdtL* demonstrated that it is essential for abietic acid catabolism. Interestingly, the putative translated product of *tdtL*, the last gene of the *tdt* cluster of *Pseudomonas* sp. A19-6a, and the partial sequence of ORF1 (Fig. 4) of *P. abietaniphila* BKME-9 share 85% identity of their aligned amino acids. It is possible that the *dit* and *tdt* gene clusters are contiguous in both *Pseudomonas* spp. since the strains are closely related phylogenetically (C. A. Morgan and R. C. Wyndham, personal communication). Although there is still no biochemical evidence, we suggest that a resin acid transport system may exist in *P. abietaniphila*. This hypothesis stems from the presence of an open reading frame with sequence similarity to permeases (*ditE*) located in the *dit* gene cluster (Fig. 4).

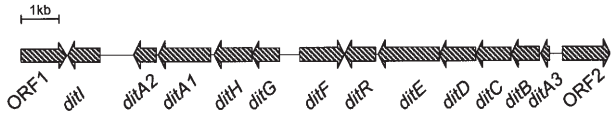

Fig. 4 Physical map of the genomic region of *Pseudomonas abietaniphila* BKME-9 encoding abietane-type resin acid degradation *dit* genes. The gene products are as follows: *ditA3* 4Fe–4S ferredoxin component of dioxygenase, *ditB* putative dehydrogenase, *ditC* extradiol ring-cleavage dioxygenase, *ditD* putative isomerase/decarboxylase, *ditE* putative permease, *ditR* IclR-type transcription regulator, *ditF* unknown, *ditG* putative dehydrogenase, *ditH* putative isomerase/decarboxylase, *ditA1* α-subunit of ring-hydroxylating dioxygenase, *ditA2* β-subunit of ring-hydroxylating dioxygenase, *ditI* putative dehydrogenase, and *ORF1* putative CoA ligase. GenBank accession no. AF119621

Transformation of resin acids in anaerobic environments

Under anoxic conditions, resin acids can be biotransformed, but there is no conclusive evidence that their carbon skeletons are degraded. Furthermore, these anaerobic transformations have been observed only in complex microbial communities such as freshwater sediments and bioreactors. Resin acids are recalcitrant under a variety of anoxic conditions (Mohn et al. 1999a), and no pure cultures have been found to use resin acids as a source of carbon or energy. Because of the hydrophobic nature of resin acids, they sorb to suspended solids and settle into environments devoid of oxygen such as sediments. In those instances, how are the resin acids ultimately degraded? Diterpenoid analysis from contemporary and "aged" samples produced evidence for an oxidative route for anaerobic transformation of resin acids (Simoneit et al. 1985), but also indicated that the transformation products are recalcitrant. The mass spectral analysis of several of these products of anaerobic metabolism with the structural features of abietane and pimerane skeletons has identified aromatized and decarboxylated transformation products. Although anaerobic transformation pathways have been pieced together from these structures, the pathway intermediates were not classified unambiguously as products from microbial activities until recently.

A biological transformation pathway for dehydroabietic acid in anoxic sediments has been described recently (Tavendale et al. 1997a). Deuterated dehydroabietic acid was incubated with anaerobic sediments and compared to a parallel autoclaved control sample. In a 264-day incubation period, dehydroabietin **16** and tetrahydroretene **17** were minor and major transformation products of dehydroabietic acid, respectively (Fig. 5). These compounds had been previously observed in lake sediments (Wakeham et al. 1980) and probably represent the principal path of anaerobic transformation of resin acids.

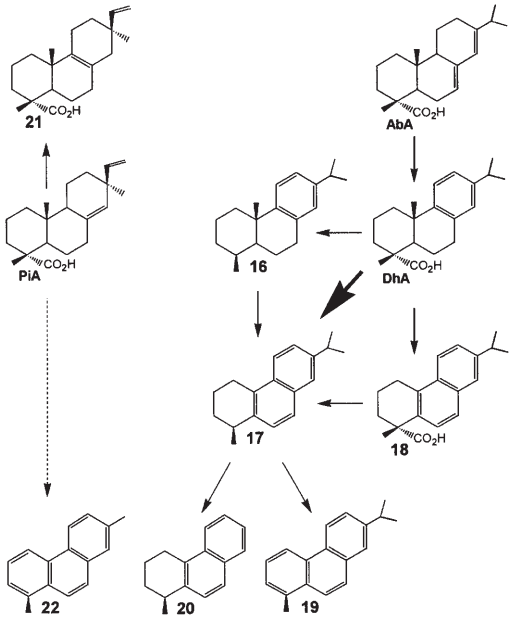

Fig. 5 Proposed pathways of anaerobic transformation of dehydroabietic, abietic, and pimaric acids, adapted principally from Tavendale et al. (1997a, b)

Tetrahydroretene may be formed in a one-step reaction or may involve the formation of 20-norabietapentaenoic acid (simonellite) **18**, a short-lived intermediate measured at very low concentration. A very small percentage of tetrahydroretene was converted to retene (1.1%) **19** and methyltetrahydrophenathrene **20**, but the majority of the tetrahydroretene was transformed to unidentified compound(s). The time scale of these experiments clearly indicates that anaerobic transformation of resin acids is slow relative to aerobic degradation. The evidence for the anaerobic biotransformation of pimerane-type resin acids is less conclusive. Anaerobic incubation of lake sediments receiving bleached kraft mill effluent has been found to significantly reduce pimaric and isopimaric acids as compared to autoclaved control samples (Tavendale et al. 1997 b). Although concentrations of 8-pimarenic **21** (Fig. 5) and 8-isopimarenic acids (not shown) increased slightly during the incubation period, they could account for only a small percentage of the pimeranes removed. The fate of pimerane-type resin acids in anoxic environments remains unresolved. Diterpenoid inventories from recent and dated environmental samples suggest an anaerobic pimerane degradation scheme similar to that for abietane resin acids, with the formation of pimanthrene **22** from pimaric acid in a multistep process (Wakeham et al. 1980). However, the biological catalysis of this proposed pathway has not been proven, and anaerobic biotransformation of resin acids remains poorly understood. The studies on the anaerobic fate of resin acids indicate that aromatization and decarboxylation to alkylated polycyclic aromatic hydrocarbons occurs and that the resulting compounds persist in the environment, as evidenced by the presence of retene and pimanthrene in dated samples. However, nitrate-reducing bacteria have recently been isolated with monoterpenes and cholesterol as sole carbon sources (Harder and Probian 1997; Foss and Harder 1998). It has been found that these organisms mineralize the organic substrates to carbon dioxide during anaerobic growth. These results suggest that resin acids may be mineralized under the appropriate anoxic culture conditions.

Conclusions

Recent studies have significantly advanced our understanding of certain aspects of the diversity of resin-acid-degrading bacteria and the metabolism of resin acids. A number of phylogenetically diverse aerobic bacteria that grow on resin acids have been characterized. Cometabolism of resin acids also appears to occur, but few organisms with this capability have been reported. The significance of cometabolism of resin acids and fungal transformation of resin acids remains unknown. Anaerobic biotransformation of resin acids has been demonstrated, but no organism capable of such a process has been identified and the mechanisms are poorly understood. A common pathway for aerobic degradation of abietanes has been further elucidated, and enzymes and genes responsible for this pathway have now been described. However, mechanisms for the biodegradation of pimeranes are almost completely unknown.

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