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Biochemistry and Ecology of Resin Acid-Degrading Bacteria

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Biochemistry and Ecology of Resin Acid-degrading Bacteria

SFM Network Project: Microbial Ecology of Toxic Compound Removal in Biological Treatment Systems

by

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EXECUTIVE SUMMARY

Resin acid biodegradation was examined in detail because of the importance of these compounds as toxic components of pulp and paper mill effluents. Seventeen isolated strains of bacteria which grow on resin acids as sole organic substrates were characterized. Such organisms are rare but are found in diverse phylogenetic groups. Accordingly, these organisms are physiologically diverse and adapted to a variety of habitats. For example, some of these isolates are adapted to growth at temperatures as low as 4°C, while others are adapted to growth at temperatures as high as 60°C. Organisms capable of anaerobic growth on resin acids were not found. The diversity of resin acid degraders suggests that it is possible to design biotreatment systems which effectively remove resin acids under unconventional conditions, including low and high temperatures.

Most isolates examined completely degrade (mineralize) resin acids, indicating that biotreatment is very effective for these compounds. Pimaranes, including pimaric and isopimaric acids, appear to be more recalcitrant than abietanes, including abietic and dehydroabietic acids. Chlorinated dehydroabietic acids, particularly 14-chloro and 12,14-dichloro congeners, appear to be very recalcitrant and may not be effectively treated in biological systems. Individual organisms have specificities for particular resin acids, and a complex population may be required to effectively degrade a mixture of resin acids, as normally occurs in effluents or process waters.

A partial biochemical pathway for abietane degradation by *Pseudomonas abietaniphila* BKME-9 was elucidated. This convergent pathway is consistent with the most typical substrate specificity of resin acid degraders, use of abietanes but not pimaranes. Several enzymes in this pathway require oxygen which is consistent with the apparent recalcitrance of resin acids under anaerobic conditions. The abietane degradation enzymes are genetically regulated and are specifically induced by abietane and pimarane resin acids. The system which allows BKME-9 to detect resin acids might be exploited to make a sensitive, specific biosensor for these compounds. Certain of these enzymes might also be exploited for synthesis of valuable chemicals from resin acids or structurally related compounds, such as steroids.

Molecular assays were developed to study resin-acid-degrading populations living within the complex microbial communities of biotreatment systems. A new method for in situ measurement of the metabolic activity of such populations was also developed. Particular species of resin acid degraders were found to comprise very small fractions of microbial communities in biotreatment systems. It appears that these small populations may be important for detoxication of effluents. It is be possible to inoculate biotreatment systems with resin acid degraders to establish or restore detoxication activity. The efficacy and economy of this strategy needs to be examined.

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INTRODUCTION

Background

Resin acids were the focus of this project, because of their importance as toxic components of pulp mill effluents and because of their importance as contaminants of process waters for pulp and paper manufacturing. Resin acids are diterpenoid compounds produced by trees (Fig. 1). These compounds are the major cause of acute toxicity of pulp mill effluents (Leach and Thakore, 1973; Priha and Talka, 1986; Walden and Howard, 1981). As such, resin acids must be successfully removed from effluents prior to their discharge. Failure to do so results in substantial fines and deterioration of the public perception of the pulp and paper industry. Generally pulping effluents are treated with biological systems, which are normally effective. However, stresses, such as chemical spills, or periods of system shutdown can lead to failure of resin acid removal and to toxicity breakthrough, in some cases. Improved dependability of treatment systems and the ability to quickly correct failure of toxicity removal are desirable. The microbiology of resin acid biodegradation is at the core of accomplishing these goals.

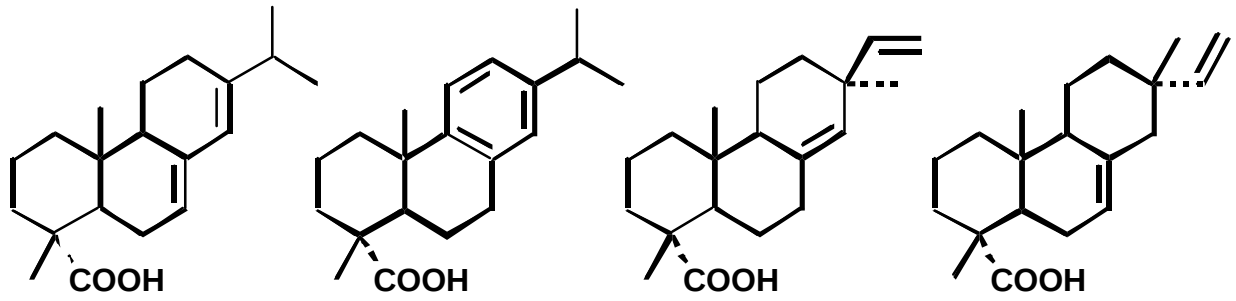

Fig. 1. Structures of resin acids.

Resin acids also are a component of pitch which interferes in the papermaking process. Pitch is particularly problematic when process waters are reused, because such impurities are thus concentrated. A general objective of current environmental management efforts is to reduce the use and discharge of water. Thus, new ways to purify water for reuse in both pulping and papermaking are desired. Biological treatment is recognized as one option for removal of organic contaminants during the reuse of process waters. Biological treatment has previously been shown to meet important requirements of being effective, inexpensive and simple. Experience with treatment of effluents suggests that resin acids and other organic contaminants can be biologically removed from process waters. However, there are numerous uncertainties and challenges associated with this approach, which has not yet been demonstrated on a large scale. Such challenges include treatment at relatively high temperatures, treatment of waters with new physicochemical properties and avoiding biofouling of the processes in which the water is

reused. Again, the microbiology of resin acid biodegradation is one key to designing such processes.

Previously, very little research on the microbiology of resin acid biodegradation was reported. Studies demonstrated that some resin acids can support bacterial growth and, using now-outdated methods, provided some indication of biodegradation pathways (Biellmann et al., 1973a; Biellmann et al., 1973b; Biellmann et al., 1968; Raynaud and Daste, 1962). Other studies indicated the ability of bacteria to remove resin acids from aqueous media (Côté and Otis, 1989; Hemingway and Greaves, 1973; Rogers and Mahood, 1974). However, in most cases the fate of the resin acids was not determined. Fungi were shown to hydroxylate resin acids (Servizi et al., 1986). Thus, at the outset of this project it was clear that microorganisms have the potential to transform resin acids, and it seemed likely that microorganisms could degrade at least some resin acids. However, there was no conclusive evidence for the fate of resin acids in effluent treatment systems. Further, there was no ecological understanding of resin acid degraders in treatment systems. Thus, engineering of these systems could not take into account the effect of design parameters on detoxifying organisms.

Objectives and rationale

A general objective of this project was to characterize resin acid degraders. Such organisms were enriched and isolated from diverse environments, including ones with extreme conditions, such as high temperature, which might be found in novel biological treatment systems currently being contemplated. The physiology and phylogeny of the organisms was investigated, with particular emphasis placed on the metabolic capabilities of the organisms. Understanding these organisms facilitates rationale design of treatment systems, as the systems can be designed to select and maintain organisms with desired detoxication activities. This work establishes the range of physicochemical conditions under which resin acids can be biologically treated and leads to the discovery of organisms which may be used in novel systems.

A second general objective was to improve our fundamental understanding of the mechanism(s) by which resin acids are biodegraded. This objective was addressed by using, for the first time, powerful new molecular biology techniques to examine the biochemical pathway and enzymology of abietane biodegradation by a selected isolate, *Pseudomonas abietaniphila* BKME-9. The resulting information helps us to understand the fate of resin acids in treatment systems (i.e., Are they partly or completely degraded? Are there potentially harmful degradation products?) and to predict the effects of treatment system operating conditions on resin acid biodegradation (i.e., Will certain changes in operating conditions promote or inhibit resin acid biodegradation?). Such understanding allows us to change from a strictly empirical approach to a more rational approach to optimizing desirable detoxication activities in treatment systems.

While characterizing isolated bacteria yields much useful information, a comprehensive ecological understanding of a microorganism can only be achieved by observing that organism as is lives within a complex microbial community interacting with very many other microbial species. For that reason, a third objective of this project was to develop molecular techniques

allowing us to observe resin acid degraders in the complex communities of treatment systems. The approach used was to develop quantitative assays for DNA and RNA sequences unique to particular resin acid degraders. An important goal was to go beyond existing methods to measure populations and to develop novel methods to quantify the metabolic activity of those populations. A use of these molecular techniques will be to determine the effects on resin acid-degrading populations of stresses which occur in treatment systems. These techniques will also be used to evaluate attempts to manipulate resin acid-degrading populations in treatment systems, either by inoculation with those populations or by alteration of treatment system operating conditions.

SUMMARY OF DATA ANALYSIS

Much of our work on this project has been summarized and related to research by others in a recent review paper (Martin et al., 1999) and the complete publications and abstracts reporting this project are listed in the appendix.

Isolation and characterization of resin acid degraders

During the course of this project we have isolated numerous resin-acid-degrading bacteria, and we have extensively characterized over 17 strains (Table 1). These organisms are widely distributed in natural and human-made environments, and we obtained isolates from full-scale and laboratory pulp and paper mill effluent treatment systems, forest soil, Arctic soil and woody compost. Some of the isolates are adapted to growth at low temperature (4°C); while, others are adapted to high temperature (60°C). All of the organisms found are aerobes, and a concerted effort to find anaerobic resin acid degraders was unsuccessful. The organisms isolated were selected for the ability to grow on resin acids. To obtain sufficient energy for growth, these organisms mineralize (completely degrade) or extensively degrade these compounds. There are other organisms able to transform resin acids, but unable to grow on those compounds, which were not selected by the methods used.

The resin acid degraders we have characterized are phylogenetically diverse. We analyzed 16S rRNA gene sequences to determine phylogeny. These analyses indicated that resin acid degraders are widely distributed among several distantly related groups, including at least six genera within three subclasses of the *Proteobacteria* and one gram-positive genus, *Mycobacterium* (Table 1). Resin acid degraders are sparse within each genus (i.e., most members of each genus do not use resin acids). This strongly suggests that the genes enabling organisms to degrade resin acids have spread horizontally through phylogenetic groups, rather than having originated from a common ancestor of all groups containing resin acid degraders. This phylogenetic diversity of resin acid degraders is consistent with the fact that these organisms are adapted to diverse environments.

Table 1. Resin-acid-degrading bacteria characterized during this project

Name	Source	Resin acids used	Reference
Sphingomonas sp. DhA-33	Bioreactor	Abietanes	(Mohn, 1995)
Sphingomonas sp. DhA-96	Arctic soil (7°C)	Abietanes	(Mohn et al., 1999b)
Zoogloea resiniphila DhA-35	Bioreactor	Abietanes	(Mohn, 1995)
Ralstonia sp. BKME-6	Bleached Kraft Mill Effluent	Abietanes	(Bicho et al., 1995)
Burkholderia sp. IpA-51	Forest soil	IpA[1]	(Mohn et al., 1999c)
Burkholderia sp. DhA-54	Forest soil	Abietanes	(Mohn et al., 1999c)
Beta-Proteobacterium DhA-71	Woody compost (50°C)	Abietanes	(Yu and Mohn, 1999a)
Beta-Proteobacterium DhA-73	High-temp. bioreactor (55°C)	Abietanes	(Yu and Mohn, 1999a)
Pseudomonas vancouverensis DhA-51	Forest soil	Abietanes	(Mohn et al., 1999c)
Pseudomonas multiresinivorans IpA-1	Bioreactor	Pimaranes & abietanes	(Wilson et al., 1996)
Pseudomonas sp. IpA-2	Bioreactor	Pimaranes & DhA[2]	(Wilson et al., 1996)
Pseudomonas abietaniphila BKME-9	Bleached Kraft Mill Effluent	Abietanes	(Bicho et al., 1995)
Pseudomonas sp. DhA-91	Arctic soil (7°C)	Abietanes	(Mohn et al., 1999b)
Pseudomonas sp. DhA-93	Arctic soil (7°C)	Abietanes	(Mohn et al., 1999b)
Pseudomonas sp. DhA-95	Arctic soil (7°C)	Abietanes	(Mohn et al., 1999b)
Mycobacterium sp. DhA-55	Forest soil	Abietanes & pimaranes	(Mohn et al., 1999c)
Mycobacterium sp. IpA-13	Bioreactor	Abietanes & pimaranes	(Wilson et al., 1996)

[1]Isopimaric acid
[2]Dehydroabietic acid

Most of the resin acid degraders examined are, to some degree, relatively limited in their specificity for organic substrates. The vast majority of the isolates use abietanes, such as abietic and dehydroabietic acids, and can not use pimaranes, such as pimaric and isopimaric acids (Table 1, Fig. 1). Very few of the isolates use pimaranes, and most of those use abietanes poorly. An exception is the ability of two gram-positive *Mycobacterium* strains to use both abietanes and pimaranes well. Abietane degraders are typically able to use 12-chlorodehydroabietic acid, but they typically can not use 14-chlorodehydroabietic acid or 12,14-dichlorodehydroabietic acid (Mohn and Stewart, 1997). The different substrate specificities indicate that there must be differing enzymatic systems used for resin acid degradation. Most resin acid degraders can also use fatty acids. These organisms typically do not use other compounds that are structurally similar to resin acids, such as monoterpenes, betulin (a triterpenoid from trees) or sitosterol (a sterol from trees). These organisms typically do not use simple aromatic compounds or alkanes. Use of sugars (mainly wood sugars were tested) is variable, with many strains not using any sugars tested. The picture which emerges from these studies is one of organisms nutritionally specialized and adapted to niches in which they primarily use resin and fatty acids as organic substrates under aerobic conditions.

Biochemical pathway for abietane degradation by *Pseudomonas abietaniphila* BKME-9

Pseudomonas abietaniphila BKME-9 was chosen for investigation of the biochemical mechanism of resin acid degradation. In retrospect, the abietane degradation enzyme system of

this organism appears to be representative of that of most of our gram-negative isolates. We used a molecular biology approach to study the degradation pathway. We isolated and sequenced a cluster of 13 genes which appear to encode enzymes involved in the catabolic pathway (Martin and Mohn, 1999a; Martin and Mohn, 1999b). Gene knock-outs were constructed and demonstrated that at least 8 of the genes are essential for growth on dehydroabietic acid. Analysis of mutant phenotypes and enzymes cloned and expressed in *E. coli* were used to elucidate the initial steps in abietane degradation by BKME-9 (Fig. 2). The pathway is convergent at its beginning, with non-aromatic abietanes being transformed to dehydroabietic acid. We have not identified the enzyme(s) involved in this aromatization process but we have observed this transformation in cell suspensions. Next, C-7 is oxidized to a ketone. Again we have not isolated the responsible enzymes, but they are likely a monooxygenase and a dehydrogenase. A mutant which accumulates the 7-oxo-dehydroabietic acid intermediate was produced. The next step is dihydroxylation of the aromatic ring at C-11 and C-12. This reaction is catalyzed by a complex dioxygenase composed of four different proteins. We cloned this enzyme and expressed it in *E. coli*, thereby demonstrating its catalytic function. The next step is almost certainly catalyzed by a dehydrogenase, by analogy with other degradation pathways involving ring-hydroxylating-dioxygenases. We have found a gene possibly encoding the dehydrogenase, but have not conclusively demonstrated its function. The final step shown is meta-cleavage of the aromatic ring. We have cloned and expressed an extradiol cleavage dioxygenase with this general activity (demonstrated with the substrate analog, 2,3-dihydroxy biphenyl), but we lack the natural substrate to conclusively demonstrate the ring-cleavage reaction. Thus, the structure of the cleavage product is somewhat speculative.

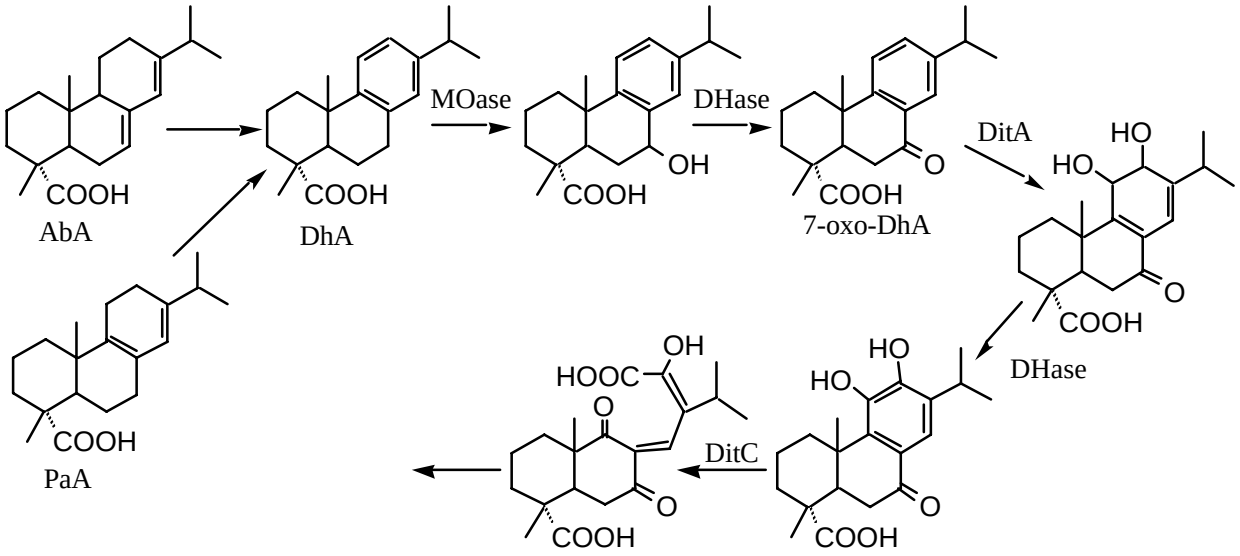

Fig. 2. Proposed initial steps in abietane degradation by *Pseudomonas abietaniphila* BKME-9; AbA, abietic acid; PaA, palustric acid; DhA, dehydroabietic acid; MOase, monooxygenase; DHase, dehydrogenase; DitA, ring-hydroxylating dioxygenase; DitC, extradiol ring-cleavage dioxygenase.

In the cases examined, resin acid degradation enzymes are inducible (Bicho et al., 1995; Mohn, 1995; Wilson et al., 1996). Various resin acids, including pimaranes and chlorinated dehydroabietic acids that are not degraded, induce the pathway in BKME-9. Other substrate analogs, such as isopropyl benzene, do not act as inducers. A regulatory protein, DitR, was shown to modulate expression of some of the genes encoding abietane degradation by BKME-9.

Ecological investigations using molecular assays for resin acid degraders

We developed polymerase chain reaction (PCR) assays to detect and quantify resin-acid-degrading populations with great sensitivity and specificity (Yu et al., 1999; Yu and Mohn, 1999b). These assays target DNA sequences specific to the organisms of interest, such as 16S rRNA gene sequences (specific for resin-acid-degrading species) or sequences of genes encoding resin-acid-degradation enzymes. These assays are extremely sensitive (detect 1 cell in 10^7 cells) and are very specific. We assayed most of the effluent treatment systems at pulp and paper mills in British Columbia. *Pseudomonas abietaniphila* BKME-9 was found in 9 of 18 systems; while, *Zoogloea resiniphila* DhA-35 was found in only 3 of 18 systems. The former was also found in laboratory treatment systems; while, the latter was found in municipal sewage treatment systems (which did have detectable resin acid concentrations). Strain BKME-9 was detected in the same treatment system from which it was originally isolated 4 years prior to this study, suggesting that it is a stable member of the community. The *ditA1* gene, encoding part of the aromatic-ring-hydroxylating dioxygenase found in BKME-9 was found in 9 of the 18 systems. Interestingly, BKME-9 and *ditA1* were not always in the same systems, suggesting that we detected resin acid degraders other than BKME-9 that have a gene homologous to *ditA1*. Quantitative analyses indicated that populations containing *ditA1* were very small, on the order of 10 to 10^3 cells/ml or, as a fraction of the total populations, 1 out of approximately 10^7 cells. In all cases, the resin-acid-degrading populations examined appeared to be very small components of their communities.

We examined the spatial and temporal distribution of the population of resin acid degraders containing *ditA1* (probably equivalent to the population of BKME-9) in an aerated lagoon treatment system (Mohn et al., 1999a). The system is at the Kamloops, BC, mill of Weyerhaueser Canada. The system has a 6-day hydraulic retention time and was sampled at 8 locations approximately evenly spaced in the lagoon. We sampled the system in September 1998 and January and February 1999. Surprisingly, the population did not vary beyond 11 to 30 cells/ml. The only exceptions were the samples taken from the influent and the effluent of the system, where the population was lower in September 1998 and was undetectable in January and February 1999. This spatial consistency of the population was in spite of substantial gradients through the system of temperature, pH, dissolved oxygen and total organic carbon. This temporal consistency of the population was in spite of changes between samplings of the temperature, due to weather, and of the chemical environment, due to the pulping process of the mill.

We developed a method to measure the metabolic activity of a resin-acid-degrading population within a complex microbial community (Muttray and Mohn, 1998; Muttray and Mohn, 1999). This method is a substantial advance beyond measuring only the size of

populations and allows a much more comprehensive examination of organisms in their normal, typically complex microbial communities, as in biological treatment systems. The method uses species-specific oligonucleotide hybridization probes to measure the rRNA and the rDNA of the species. Because the cellular rRNA content of bacteria is modulated according to metabolic activity, this method allows measurement of activity based upon the rRNA:rDNA ratio. This approach was first validated by chemically measuring RNA:DNA ratios of pure cultures of several strains growing at different rates. The approach was then further validated and the hybridization method proven with pure batch and chemostat cultures of *Sphingomonas* sp. DhA-33 in which growth rates were varied (Fig. 3). Finally, DhA-33 was examined when it was grown in a complex activated sludge community on a complex substrate, bleached kraft mill effluent with additional dehydroabietic acid (Fig. 4). The effects of competition from other resin acid degraders in the activated sludge community were observed. The presence of the activated sludge community caused faster dehydroabietic acid removal, indicating that the community included DhA degraders other than DhA-33. The maximum growth rate of DhA-33 (measured as rDNA) and the maximum metabolic activity of DhA-33 (measured as rRNA:rDNA) were unchanged by the presence of competitors. However, in the presence of the competitors, the dehydroabietic acid was depleted faster and growth of DhA-33 therefore ended sooner. Thus, competition decreased the cell yield of DhA-33. The hybridization method could measure rRNA:rDNA ratios of populations as small as 10^6 cells/ml within a community with an approximate density of 10^9 cells/ml. A PCR assay for the rRNA:rDNA ratio is being developed in order to reduce the lower detection limit.

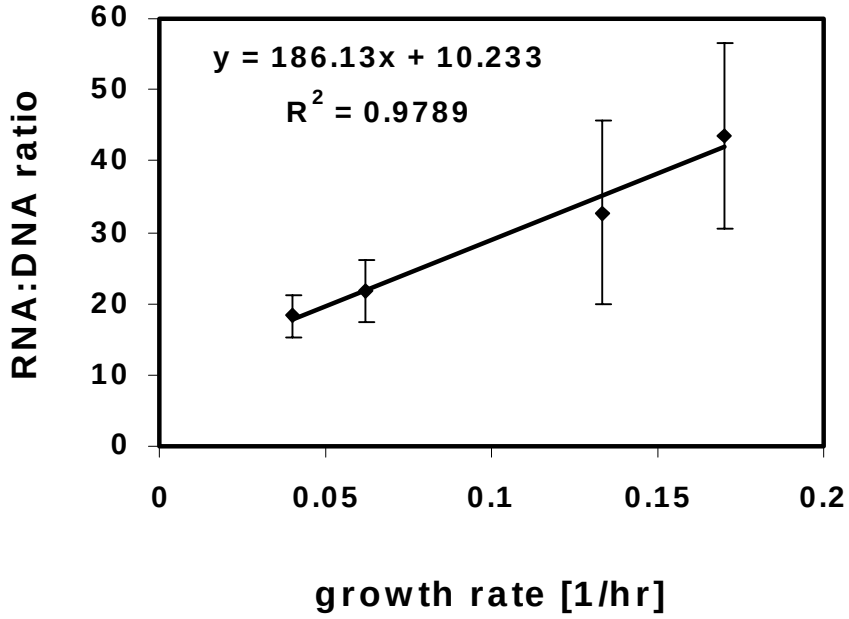

Fig. 3. For *Spingomonas* sp. DhA-33 grown at steady state in a chemostat, the relationship between specific growth rate and the 16S rRNA:rDNA ratio measured with oligonucleotide hybridization probes.

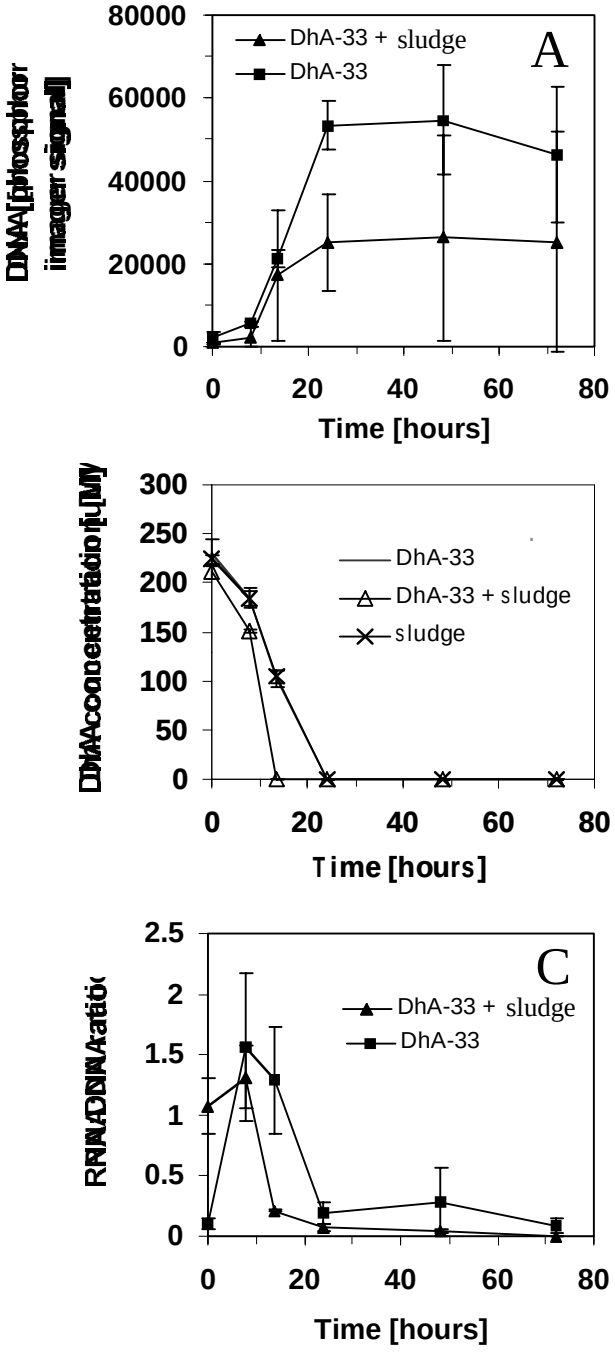

Fig. 4. Growth of *Sphingomonas* sp. DhA-33, axenically or with a complex activated sludge microbial community, on bleached kraft mill effluent plus 60 mg/l dehydroabietic acid (n = 3); **A**, DhA-33 growth measured as DNA (bars indicate standard error); **B**, dehydroabietic acid removal (bars indicate standard error); **C**, metabolic activity of DhA-33 measured as 16S rRNA:rDNA ratio (bars indicate variance of the ratio).

MANAGEMENT APPLICATIONS

Limits of biological treatment

The physiological and phylogenetic diversity of resin-acid-degrading bacteria suggests that biological destruction of resin acids is possible under a broad range of physicochemical conditions. The temperature range for resin acid biodegradation is at least 4°C to 60°C. The pH range is at least 5.5 to 8.5 (Werker and Hall, 1999) but has not been thoroughly examined. High resin acid concentrations do not appear to be inhibitory to organisms which degrade these compounds. Gram-positive resin acid degraders, such as *Mycobacterium* sp. DhA-55, are probably relatively resistant to desiccation, a common trait of such soil actinomycetes. There exist resin acid degrading bacteria which grow in flocs and settle well (e.g., *Zoogloea resiniphila* DhA-35) as well as others which grow as motile, suspended cells (e.g., *Pseudomonas abietaniphila* BKME-9). Others likely are adapted to attached growth, but these have not been characterized. Thus, treatment systems can potentially be engineered to exploit resin acid degraders under almost any physicochemical conditions known to permit microbial growth, including relatively extreme conditions. A few examples are given below, but it would be useful for engineers and system operators to reflect upon how perceived limitations to the tolerance of biological systems may have limited their design and operation of treatment systems. Perhaps the greatest potential benefit from this design strategy could be the simplification of systems which presently have complex mechanisms to achieve what are considered "normal" operating conditions (e.g., water cooling or heating systems).

One promising application is use of treatment systems that operate at above what are considered normal operating temperatures (40°C to 60°C). Such systems would reduce the expense of water cooling. This would be especially advantageous in recycling process waters. Studies have demonstrated the effective removal of organic matter from whitewaters at high temperatures (Duff, ; Tardif and Hall, 1997). This project indicates that resin acid destruction is possible in such systems.

Whitewaters can also have low pH values, so the pH range of resin acid biodegradation should be better explored. As well, a separate topic which warrants further investigation is tolerance of transient pH shock (as opposed to growth at extreme pH values). It may be possible to engineer existing and future treatment systems to be more stable and reliable by insuring that resident resin acid degraders tolerate transient pH shocks.

Another possibility resulting from the physiological and phylogenetic diversity of resin acid degraders are systems that operate or survive at low temperature. In regions where ambient temperatures can be very low in winter, it should be possible to have treatment lagoons which perform well at temperatures previously considered too low (0°C to 30°C). Also, it should be possible to engineer intermittently-operating treatment systems such as constructed wetlands or soil filters which would effectively detoxify wood-extractive-containing runoff yet would not

lose their treatment potential during dry or freezing weather. Such constructed wetlands are under investigation (Duff, pers. comm.).

Fate of resin acids in treatment systems

Many previous studies have demonstrated effective removal of organic compounds from pulp and paper mill effluents. However, the fate of resin acids, a small but important fraction of the total organic compounds, is much less certain. The work described in this report generally supports the conclusion that unchlorinated resin acids are completely destroyed in properly-operating biological treatment systems. Resin acids can be regarded as readily-used organic substrates for certain aerobic bacteria. Such bacteria exist in all pulp and paper mill effluent treatment systems which we have examined. The resin acid degraders which we have studied use resin acids as organic substrates and generally mineralize (completely destroy) these compounds. Thus, the fate of carbon in these compounds is complete degradation to carbon dioxide, or incorporation into microbial biomass. There are no harmful metabolites resulting from this process. While other organisms do not completely destroy resin acids, we can expect natural selection to favor those which do so in properly-operating treatment systems. Therefore, biological treatment is a very effective strategy for resin acid detoxication.

Relative to abietanes (e.g., abietic and dehydroabietic acids, Fig. 1), pimaranes (e.g., pimaric and isopimaric acids) appear to be more recalcitrant. Pimarane-degrading bacteria are harder to isolate from treatment systems or natural environments. This observation could, but does not necessarily, indicate that pimarane degraders are less abundant. Also, the evidence that biodegradation completely destroys pimaranes is less clear that for abietanes. The mechanism(s) of pimarane biodegradation are not well understood. Thus, we can be less certain of the fate of pimaranes in treatment systems, and we might expect pimaranes to be more problematic than abietanes. A better understanding of degradation of pimaranes is desirable.

The fate of some chlorodehydroabietic acids is uncertain. In particular, 12-chloro- and 12,14-dichlorodehydroabietic acid have not been shown to be effectively biodegraded. These compounds must be considered recalcitrant and cannot be assumed to be destroyed during biological treatment. Even if these compounds are "removed" by biological systems, it is very possible that they are simply being retained in the system by mechanisms such as sorption to biomass and fines. It is necessary to evaluate whether such a removal mechanism is acceptable or whether it will lead to undesirable consequences at a later time. Fortunately, changes in bleaching processes have dramatically reduced the occurrence of chlorodehydroabietic acids.

Importance of small populations

Studies so far completed suggest that the populations in treatment systems responsible for key detoxication processes may be relatively small fractions of the total microbial population. This conclusion is consistent with the fact that the toxic compounds degraded by these populations are a small fraction of the total organic matter in the effluents being treated. The

available data are not conclusive for all detoxifying populations, but particular resin acid degrading populations examined in pulp and paper mill effluent treatment systems were very small (e.g., 10 out of 10^9 cells/ml). Designers and operators should take this fact into consideration: the status of the general microbial population will not necessarily indicate the capacity of a system for detoxication. To insure adequate detoxication activity in a system, it is desirable to monitor and maintain the detoxifying population. Methods, such as those developed in this study, are becoming available to study and monitor such populations and can be exploited in order to better understand the function and status of these populations in treatment systems.

Inoculation with detoxifying populations

There now exists the possibility of inoculating systems having a broad range of physicochemical conditions with resin acid degraders having the appropriate characteristics to survive and degrade resin acids in those systems. Such inocula might be prepared in a freeze-dried form which is easily transported and has a long shelf life. This strategy may be an effective response to accidents, such as chemical spills, which inactivate treatment systems. This strategy may also be an effective way to establish or restore detoxication activity in systems after periods of shut down or changes in the pulping process. It remains to determine the efficacy and the cost of this strategy on a large scale. The molecular assays developed in this project for resin-acid-degrading populations would be useful to monitor the fate and activity of inocula in biotreatment systems.

Since most resin acid degraders do not degrade all resin acids, it is likely that multiple organisms will be required for effective removal of the complex mixtures of resin acids which typically occur in effluents and process waters. Also, a population well adapted to degradation of a mixture of resin acids may not be able to degrade a new mixture resulting from some operational change, such as changing the wood furnish. This is another reason why it would be desirable to monitor resin-acid-degrading populations and why it may be advantageous to augment them.

Biosensors

It is possible to exploit the molecular system used by resin acid degraders to recognize the toxic compounds which these organisms use as growth substrates. One resin acid degrader was found to genetically regulate its abietane-degradation enzyme system in response to abietanes and pimaranes (i.e., all resin acids) but does not respond to other similar chemical structures. It would be possible to genetically engineer the organism to respond to the stimulus with a measurable output such as bioluminescence. Currently the technology is being developed to detect such output with electronic microsensors (small electronic chips). It would be possible to detect resin acids by interfacing the above engineered bacterium and a microsensor. This biosensor would be very specific for resin acids (all types), very sensitive (probably with a detection limit in the µg/l range) and inexpensive (probably disposable after a predetermined

lifespan). This biosensor would likely not be affected by other compounds in effluent or process water and could allow continuous monitoring of those compounds in real time.

Biosynthesis of valuable compounds

The resin-acid-degrading enzymes discovered during the course of this project may be useful for the synthesis of valuable chemicals. The types of reactions elucidated, including desaturation, hydroxylation, *cis*-dihydroxylation and oxidation, can all be useful in such syntheses. Enzymes are advantageous for syntheses because of their high efficiency, high specificity and lack of environmentally harmful byproducts. Enzymes capable of such transformations of simple aromatic compounds and monoterpenes have been examined and exploited for this purpose. However, this project identified, for the first time, such enzymes which modify diterpenoids. Thus, these enzymes may have novel activities useful for chemical syntheses using resin acids or structurally related compounds, such as steroids. This possibility should be explored.

CONCLUSIONS

1. Resin acid degraders include physiologically and phylogenetically diverse bacteria.
2. Anaerobic resin acid degraders have not been identified.
3. Resin acid degraders are adapted to a wide range of physicochemical conditions.
4. Resin acid degraders examined tend to be nutritionally specialized and most are specific for use of abietanes but not pimaranes.
5. Chlorinated dehydroabietic acids appear to be recalcitrant.
6. A biochemical pathway for resin acid degradation has been partially elucidated (Fig. 2).
7. Expression of abietane degradation genes in at least one strain is induced by resin acids.
8. Resin acid degrading populations in biotreatment systems appear to be small fractions of total microbial populations and can be monitored with PCR assays.
9. The metabolic activity of such populations can be measured by determining their rRNA:rDNA ratios.

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APPENDIX:
PUBLICATIONS AND ABSTRACTS RESULTING FROM THIS PROJECT

Publications

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