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Lessons learned from *Sphingomonas* species that degrade abietane triterpenoids

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Abietane terpenoid-degrading organisms include *Sphingomonas* spp which inhabit natural environments and biological treatment systems. An isolate from the high Arctic indicates that these organisms occur far from trees which synthesize abietanes and suggests that some of these organisms can occupy a niche in hydrocarbon-degrading soil communities. Abietane-degrading *Sphingomonas* spp provide additional evidence that the phylogeny of this genus is independent of the catabolic capabilities of its members. Studies of *Sphingomonas* sp DhA-33 demonstrate that biological treatment systems for pulp mill effluents have the potential to mineralize abietane resin acids. On the other hand, these studies indicate that some chlorinated dehydroabietic acids are quite recalcitrant. Strain DhA-33 grows relatively well on some chlorinated dehydroabietic acids but transforms others to stable metabolites. Using strain DhA-33, a novel method was developed to measure the metabolic activity of an individual population within a complex microbial community. Oligonucleotide hybridization probes were used to assay the 16S rRNA:rDNA ratio of DhA-33 as it grew in an activated sludge community. However, this method proved not to be sufficiently sensitive to measure naturally occurring resin acid-degrading populations. We propose that the same approach can be modified to use more sensitive assays.

Keywords: abietane; biological treatment; DhA-33; DhA-95; hybridization; RNA:DNA ratio; *Sphingomonas*; resin acid

Resin acids and their biodegradation

Resin acids are tricyclic diterpenoid compounds (Figure 1) synthesized by trees, particularly softwood trees. Abietane resin acids can be distinguished from pimarane resin acids by the isopropyl substituent at C-13 of abietanes. The abietane, dehydroabietic acid (DhA) also occurs with chlorine substituents at C-12 and/or C-14, as a result of chlorine bleaching of wood pulp or rare natural processes. Resin acids are of considerable environmental and industrial concern [reviewed in 5]. These compounds are the largest cause of toxicity of pulp mill effluents and are particularly toxic to fish in waters receiving those effluents [17,22]. These effluents are normally treated in biological systems prior to their discharge. Also, these compounds are a component of pitch which contaminates paper-making process waters, diminishing the quality of the paper product and sometimes causing costly interruptions in running paper-making machines. The amount of resin acids in the global organic carbon pool is presumably substantial, considering that resin acids constitute up to a few percent of the composition of wood and that the amount of wood on earth is vast. Biodegradation of resin acids is therefore critical in the treatment of pulp mill effluents and in the global carbon cycle. We set out to investigate resin acid biodegradation for those reasons, but along the way we also made several findings of more general microbiological interest, concerning the occurrence and nature of catabolic capabilities and the investigation of *in situ* metabolic activity of microorganisms. Here we summarize such findings from investigation of abietane-degrading *Sphingomonas* spp.

Diverse resin acid-degrading bacteria have been isolated and characterized [5,8,10–12,25]. The majority of such isolates that have been phylogenetically characterized are members of the genus *Pseudomonas*. Others include members of *Burkholderia*, *Mycobacterium*, *Ralstonia*, and *Zoogloea*. Only two resin acid-degrading members of *Sphingomonas* have been identified to date, *Sphingomonas* sp DhA-33 [8] and *Sphingomonas* sp DhA-95 [10]. Thus, resin acid degradation is by no means a unique property of members of *Sphingomonas*.

Anaerobic biotransformation of resin acids can occur [20,21], but no organism capable of this process has been identified. Most of the resin acid-degrading bacteria presently characterized are able to grow aerobically with resin acids as sole organic substrates. Of these bacteria, most are limited to use of abietanes and do not use pimaranes. The resin acid-degrading *Sphingomonas* spp so far identified share this specificity for abietanes.

Sphingomonas sp DhA-33

The first resin acid-degrading organism identified as a member of *Sphingomonas* was isolated from a laboratory sequencing batch bioreactor treating synthetic pulp mill effluent. This isolate, strain DhA-33, and numerous strains indistinguishable from DhA-33, were obtained from the bioreactor by classical enrichment and isolation with DhA as a sole organic substrate [8]. Strain DhA-33 appeared to outgrow an organism more abundant in the bioreactor, *Zoogloea resiniphila* DhA-35, which could only be obtained

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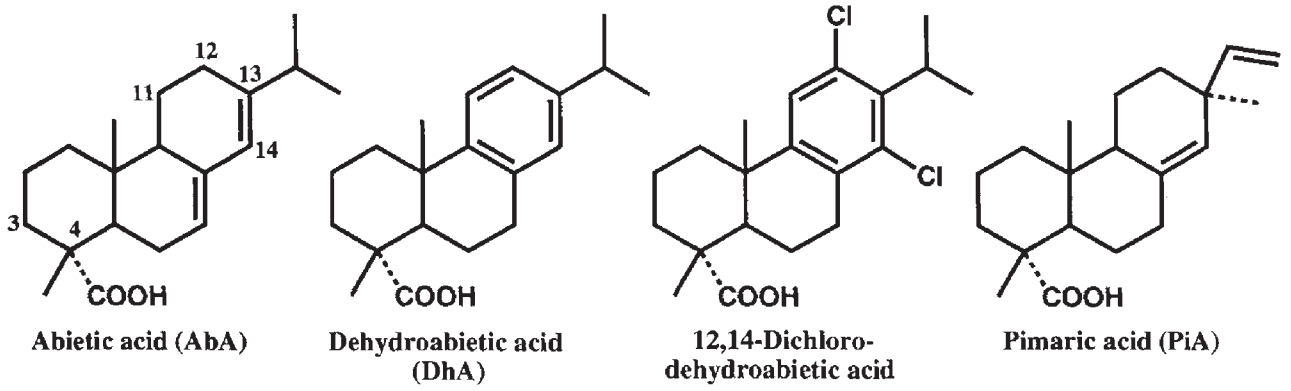

Figure 1 Resin acid structures and abbreviations, with numbering of the carbon atoms discussed in the text shown on AbA. AbA and DhA are the most abundant abietanes; PiA is an example of a pimarane.

when the enrichment inoculum was diluted to extinction. No other abietane degraders were isolated from this microbial community, but pimarane degraders were additionally isolated [23].

Strain DhA-33 has physiological characteristics typical of members of *Sphingomonas* [11]. These typical characteristics include its fatty acid profile, which included a large portion of 18:1 isomers and a substantial portion of 14:0 2OH. Strain DhA-33 is mesophilic with a maximum growth temperature of 30°C. This organism has no unusual growth characteristics or requirements. In addition to resin acids, the known range of organic substrates used by DhA-33 includes sugars, palmitic acid and certain small acids and alcohols. DhA-33 is unable to use diverse aromatic compounds and terpenoids. As for most resin acid degraders, the niche of DhA-33 does not appear to include growth on chemical analogues of resin acids.

Phylogenetic analysis of DhA-33 was based on its 16S rDNA sequence [11]. *Sphingomonas chlorophenolica* is the validly described species which appears to be the closest relative of DhA-33 (96.7% 16S rDNA sequence similarity) (Figure 2). This level of similarity would be quite low for members of the same species, strongly suggesting that DhA-33 represents a new species. Similarly, DhA-33 and *S. paucimobilis*, the type strain for the genus, have a level of similarity (92.2%) that is quite low for members of the same genus. The closest phylogenetic relatives of DhA-33 include several strains that are able to degrade diverse pollutants. There is presently no indication of a close evolutionary relationship among the enzymes responsible for degradation of these various pollutants. Rather, it appears that this phylogenetic group may be well suited to adaptation to niches involving pollutant degradation, perhaps gaining the necessary metabolic capabilities via horizontal gene transfer.

Sphingomonas sp DhA-95

The second resin acid-degrading organism identified as a member of *Sphingomonas* is strain DhA-95, isolated from soil in the high Arctic, near the northeast coast of Ellesmere Island (82°N, 62°W) [10]. Like DhA-33 above, DhA-95 was found in association with resin acid degraders from other genera that were also isolated from the same soil. This soil was contaminated with hydrocarbons, and with only one exception, resin acid degraders could not be enriched from nearby uncontaminated soils. DhA-95 was enriched and isolated on DhA. However, this strain also grows on jet fuel, which likely explains its association with hydrocarbon-contaminated soil. Of a range of alkanes tested, DhA-95 grew on only *n*-dodecane. This strain failed to use the aromatic compounds, benzene, toluene and naphthalene. Thus DhA is the only aromatic compound DhA-95 is known to use. The strain failed to use terpenoids, other than abietanes. DhA-95 is psychrotolerant, growing well at 4°C and at 30°C.

The phylogeny of DhA-95 was inferred from a preliminary sequence analysis of a 565-bp fragment of its 16S rRNA gene. Of the organisms for which 16S rDNA sequences are available in GenBank, DhA-95 appears to be most closely related to *Sphingomonas* sp UN1F1 (98.2% similarity). Strain UN1F1 is a polyaromatic hydrocarbon-degrading organism isolated from creosote-contaminated soil [13]. Relative to UN1F1, DhA-95 appears to be less closely related to the other resin acid-degrading member of *Sphingomonas*, DhA-33 (96.3% similarity). On the very limited basis of these two strains, the capacity for resin acid degradation does not seem to be associated with a particular phylogenetic group within *Sphingomonas*. This is consistent with the patchy distribution of that capacity among groups at higher taxonomic levels [11].

Resin acid metabolism by strain DhA-33

Strain DhA-33 grows on abietanes, such as abietic acid (AbA), palustric acid (Figure 3) and DhA, but cannot grow on pimaranes, such as pimaric acid and isopimaric acid [8]. Resin acid degradation activity is inducible in DhA-33 (and is prevented by a protein synthesis inhibitor), and growth on DhA also induces degradation of AbA (suspensions of cells grown on DhA degrade AbA, while suspensions of cells grown on glucose do not). This specificity for abietanes and induction pattern of abietane degradation activity is consistent with a convergent, dioxygenolytic pathway for abietane degradation (Figure 3), proposed on the basis of studies with *Pseudomonas abietaniphila* BKME-9 [6,7]. In this pathway, nonaromatic abietanes are first transformed to the aromatic intermediate, DhA. Abietanes are removed

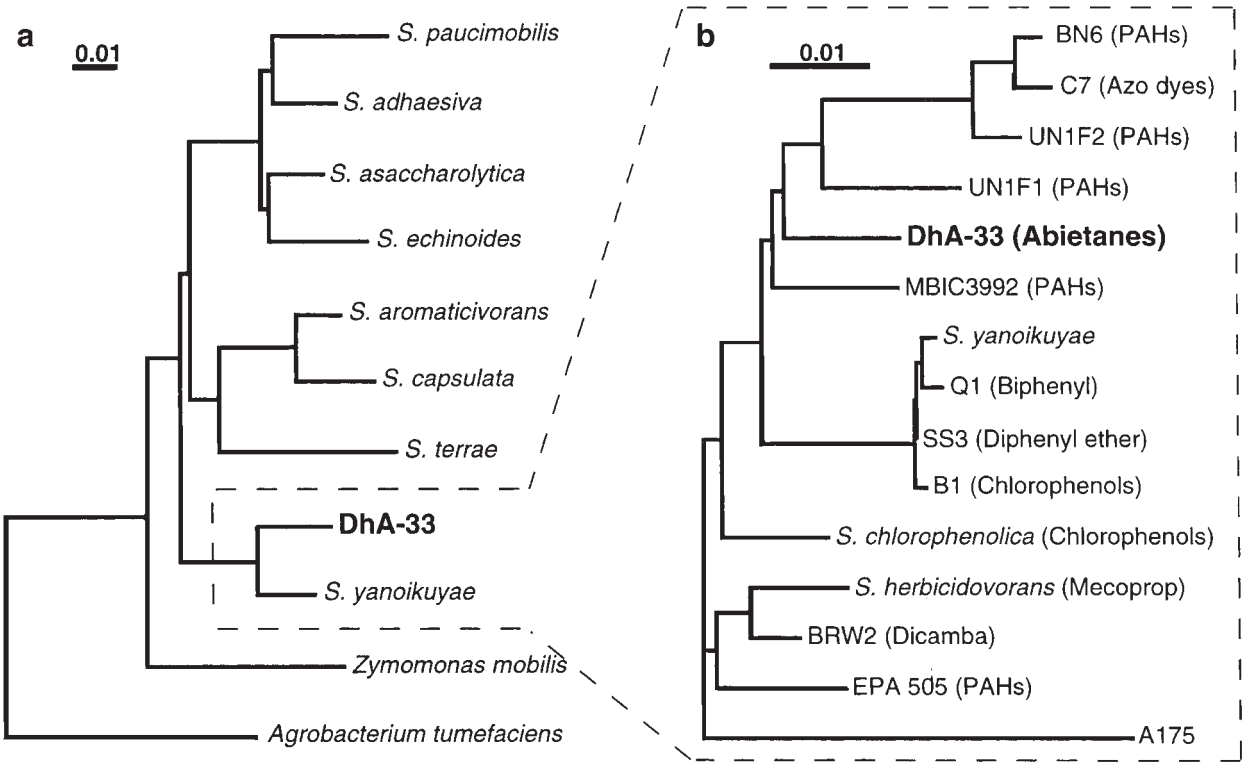

Figure 2 (a) The relative phylogenetic position of *Sphingomonas* sp DhA-33, as estimated from 16S rRNA gene sequence comparisons, among species of the different intrageneric lineages of the genus. (b) An unrooted dendrogram of the phylogenetic relationships of *Sphingomonas* sp DhA-33 with respect to validly published species and strains, of the *S. yanoikuyae*/DhA-33 evolutionary lineage, which have been described to degrade or transform various pollutants (shown in parentheses). The scale bars correspond to an estimated 0.01 mutations per nucleotide from the point of branching. The 16S rRNA gene sequences used for these analyses were previously deposited with the EMBL/GenBank/DDJB databases and were obtained with the following accession numbers: *Sphingomonas* sp DhA-33, AJ011505; *S. paucimobilis*, X72722; *S. adhaesiva*, X72720; *S. aromaticivorans*, U20756; *S. asaccharolytica*, Y09639; *S. capsulata*, D16147; *S. chlorophenolica*, X87161; *S. echinoides*, AJ012461; *S. herbicidovorans*, AB022428; *S. terrae*, D13727; *S. yanoikuyae*, X72725; *Sphingomonas* sp B1, X94099; *Sphingomonas* sp BN6, X94098; *Sphingomonas* sp BRW2, AF025350; *Sphingomonas* sp C7, L22759; *Sphingomonas* sp EPA 505, X94100; *Sphingomonas* sp MBIC 3992, AB025572; *Sphingomonas* sp Q1, X87167; *Sphingomonas* sp SS3, X87165; *Sphingomonas* sp UN1F1, U37345; *Sphingomonas* sp UN1F2, U37346; *Agrobacterium tumefaciens*, M11223; *Zymomonas mobilis*, U63733.

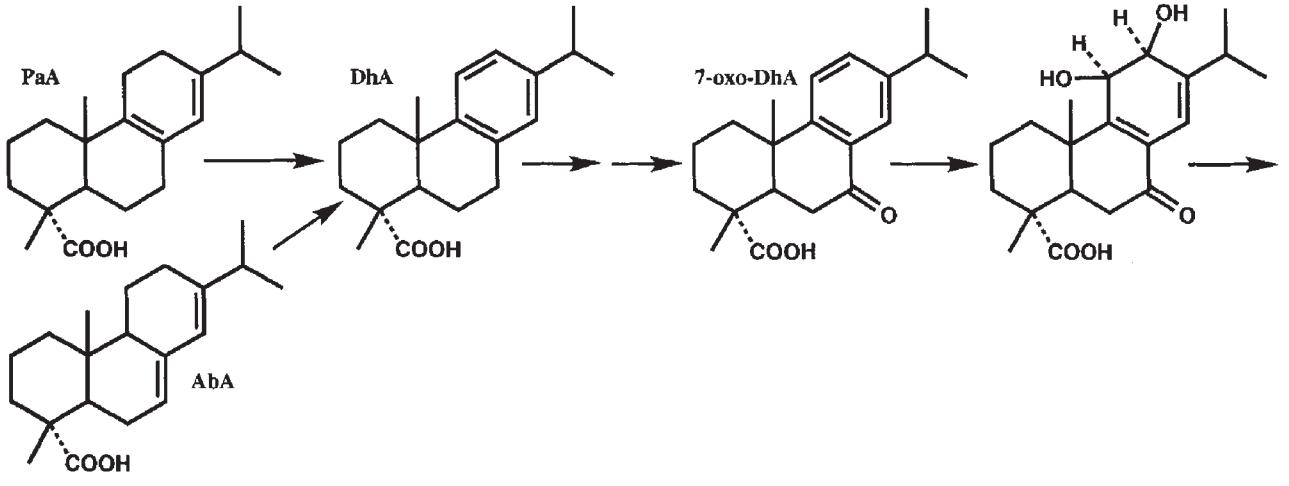

Figure 3 Proposed convergent, dioxygenolytic pathway for degradation of abietanes based on studies of *Pseudomonas abietaniphila* BKME-9 [6,7]. PaA, palustric acid.

by DhA-33 from its growth medium to undetectable levels [8]. DhA-33 can grow on DhA with a doubling time of 2.7 h and a growth yield of 0.30 g of protein per g of DhA. This growth yield corresponds to an estimated incorporation of 40% of DhA carbon into biomass, which is consistent with complete mineralization of DhA. DhA-33 does not form detectable metabolites from any abietanes tested. These characteristics suggest that biological treatment of abietanes in effluents can be highly effective.

Strain DhA-33 is unique among the Gram-negative bacteria examined for the ability to metabolize chlorinated DhAs (strain DhA-95 has not been examined). All Gram-negative bacteria examined (10 strains) which grow on abietanes can also grow on monochlorodehydroabietic acids (Cl-DhAs) [9]. However, DhA-33 is distinct in that it grows substantially faster and to a higher cell density on Cl-DhAs than does any other strain tested. The Cl-DhAs, 12-Cl-DhA and 14-Cl-DhA, are not commercially available as pure compounds, so they were tested as growth substrates in an equimolar mixture. Gram-negative bacteria growing on Cl-DhAs do not completely remove those compounds from their medium, and in particular, high residual levels of 14-Cl-DhA are typically found. However, DhA-33 is distinct in that it removes both Cl-DhA isomers to similar residual levels, which are relatively low. The 14-Cl-DhA removed by DhA-33 is not necessarily used for growth, as much of it appears to be transformed to 14-chlorodehydroabietin via oxidation to a ketone at C-3 and decarboxylation, potentially spontaneous, at C-4 (Figure 4). We have no evidence that 14-Cl-DhA is further transformed by DhA-33. It appears likely that, of the Cl-DhAs, 12-Cl-DhA is the primary substrate used for growth. The growth yield suggests that 12-Cl-DhA may be mineralized, but there is no further evidence for this possibility. Overall, approximately 30% of the carbon in Cl-DhAs removed by DhA-33 is transformed to biomass and CO_2. Thus, despite the fact that DhA-33 grows relatively well on Cl-DhAs, it does not completely degrade these compounds. In light of this observation, it may not be reasonable to assume that Cl-DhAs are effectively mineralized in biological treatment systems.

Strain DhA-33 is the only bacterium known to degrade 12,14-dichloro-DhA (diCl-DhA) [9]. This degradation appears co-metabolic, as DhA-33 will not grow on diCl-DhA and requires induction with another compound, such as Cl-DhA, before it will degrade diCl-DhA. High residual levels of diCl-DhA (approximately 100 μM) remained after metabolism by DhA-33. Probable metabolites of diCl-DhA were detected, but were not identified. Thus, as might be expected, diCl-DhA appears even more recalcitrant than Cl-DhAs.

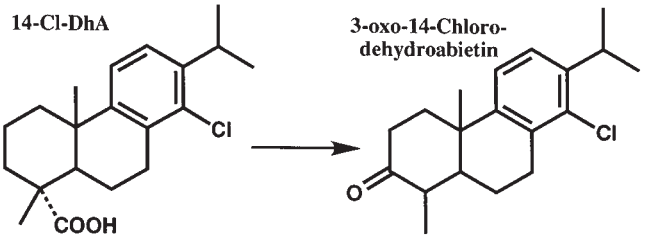

Figure 4 Formation of a metabolite from 14-Cl-DhA, which is not further degraded, by *Sphingomonas* sp DhA-33 based on [9].

The exceptional ability of DhA-33 to transform chlorinated DhAs suggests that its enzyme system for resin acid degradation differs from those of other bacteria studied. Clearly, certain enzymes of DhA-33 are relatively tolerant of a chlorine substituent at C-14, allowing its relatively great removal of 14-Cl-DhA and its production of a metabolite from that compound (Figure 4). Abietane degraders, including DhA-33, may be able to grow on 12-Cl-DhA by virtue of oxidative dechlorination activity of an aromatic ring-hydroxylating dioxygenase which forms a *cis*-dihydrodiol by hydroxylating C-11 and C-12 (Figure 3) [7]. Such oxidative dechlorination was demonstrated with biphenyl 2,3-dioxygenase of *Burkholderia cepacia* LB400 [2]. Interestingly, PCR primers designed to screen for homologues of the gene encoding the large subunit of the hydroxylating dioxygenase of *P. abietaniphila* BKME-9, detected putative homologues in some strains of the genera *Pseudomonas*, *Zoogloea*, *Burkholderia* and *Mycobacterium* as well as members of the *Rubrivivax* subgroup of the beta-*Proteobacteria* [24]. However, such a homologue was not detected in DhA-33, suggesting that any homologous dioxygenase of DhA-33 is relatively distantly related to that of the others. The unique enzymology of DhA-33 may or may not cause abietane degradation by a pathway different from that of *P. abietaniphila* BKME-9. It is interesting that DhA-33 is phylogenetically related to other organisms which degrade chlorinated aromatic compounds (Figure 2); however, there is presently no evidence that the degradative enzymes for the different chlorinated compounds are closely related.

A novel method to monitor population and metabolic dynamics

Using DhA-33, we have developed a novel molecular assay that quantifies both the population and the metabolic activity of a species population within a complex microbial community [14,15]. This method involves two complementary oligonucleotide hybridization probes that are used to assay nucleic acids extracted from the microbial community. In this case, species-specific probes were designed for DhA-33. The first probe hybridizes to the 16S rRNA gene (rDNA) of DhA-33. The second, complementary probe hybridizes to the 16S rRNA (as well as the less-abundant coding strand of the rDNA). To avoid hybridization with the coding strand and to simplify the hybridization mixtures, the nucleic acid extract was separated into two aliquots for hybridization. One aliquot was treated with RNase and assayed with the rDNA probe. The second aliquot was treated with DNase and assayed with the rRNA probe. Hybridization was quantified by labeling the probes with ^{32}P and performing slot blot assays. The population of DhA-33 was monitored by quantifying its rDNA. The metabolic activity of DhA-33 was monitored by quantifying its rRNA:rDNA ratio.

In preliminary experiments with pure cultures of DhA-33, we validated assumptions about the relationships between population size, metabolic activities and amounts of cellular rRNA and rDNA [14,15]. We observed good agreement between DhA-33 biomass and DNA concentrations. We found that the cellular 16S rRNA:rDNA ratio

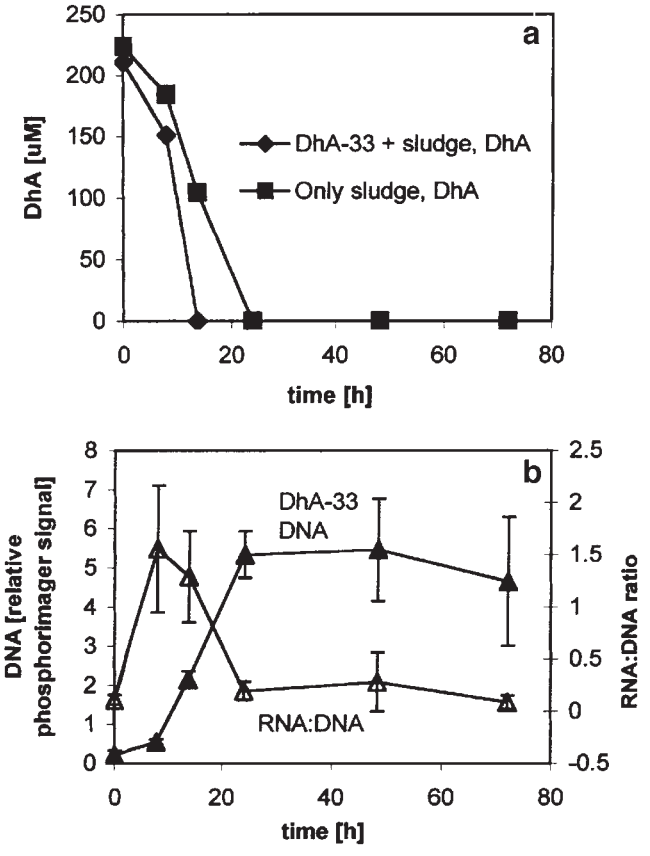

Figure 5 Removal of DhA by a laboratory pulp mill effluent treatment system (a) and population growth (DNA) and metabolic activity (RNA:DNA ratio) of strain DhA-33 within the complex microbial community of that treatment system (b); $n = 3$; error bars show standard deviation of DNA and variance of the RNA:DNA ratio; standard deviation bars for DhA are smaller than the symbols.

of DhA-33 was proportional to the exponential growth rate constant in batch cultures and the steady-state specific growth rate in chemostat cultures. It is often assumed that cellular nucleic acid pools are indicative of metabolic activity or growth rate; however, until recently, this had only been demonstrated for a few organisms [1,16,19]. The results with DhA-33 and other resin acid degraders [15] contribute to a growing body of evidence [3,4,18] that this relationship is general and is useful in studies of microbial ecology.

A laboratory culture simulating a treatment system for pulp mill effluent was amended with DhA-33 [14]. The microbial community was activated sludge from a pulp mill treatment system, and the medium contained bleached kraft pulp mill effluent with added DhA. Inoculation of the culture with DhA-33 stimulated removal of DhA (Figure 5). Thus, inoculation of such communities appears to be an effective strategy for increasing resin acid removal capacity in treatment systems. Further studies are required to determine if this strategy is practical and economical.

The hybridization method successfully monitored the population and metabolic activity of DhA-33 as it grew within the complex microbial community of the laboratory treatment system for pulp mill effluent [14]. Growth of DhA-33 was concurrent with DhA removal (Figure 5). DhA-33 grew faster in pure culture on the same medium (not shown). Metabolic activity, as indicated by the rRNA:rDNA ratio, peaked during early exponential growth and quickly returned to the initial level in early stationary phase. Thus, the rRNA:rDNA ratio had the expected relationships with growth and metabolism of DhA. The rRNA:rDNA ratio was very responsive, changing quickly as growth of the culture changed. It may be difficult to calibrate this method for determination of growth rate values in batch cultures, but we propose that it should be possible to determine specific growth rates under steady-state conditions, as in continuous biological treatment systems.

The above laboratory treatment system for pulp mill effluent was inoculated with a relatively large population of DhA-33. Approximately equal amounts of activated sludge and DhA-33, measured as optical density, were initially present. The detection limit for DhA-33, using the rDNA probe was 10^7 cells ml^{-1}. Thus, the hybridization method was effective for this experiment, but would probably not be sensitive enough to monitor normal populations of resin acid-degrading species in treatment systems. We have determined that some of these populations are as low as 100 cells ml^{-1} in actual pulp mill effluent treatment systems [24]. Thus, the current hybridization method is limited to assaying relatively abundant species or broader phylogenetic groups which are relatively abundant. We propose that the rDNA and the rRNA:rDNA ratio of a species can be measured with greater sensitivity using other methods, such as PCR.

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