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Growth, Induction, and Substrate Specificity of Dehydroabietic Acid-Degrading Bacteria Isolated from a Kraft Mill Effluent Enrichment

P. A. BICHO, V. MARTIN, AND J. N. SADDLER*

Department of Wood Science, Faculty of Forestry, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

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We investigated resin acid degradation in five bacteria isolated from a bleach kraft mill effluent enrichment. All of the bacteria grew on dehydroabietic acid (DHA), a resin acid routinely detected in pulping effluents, or glycerol as the sole carbon source. None of the strains grew on acetate or methanol. Glycerol-grown, high-density, resting-cell suspensions were found to undergo a lag for 2 to 4 h before DHA degradation commenced, suggesting that this activity was inducible. This was further investigated by spiking similar cultures with tetracycline, a protein synthesis inhibitor, at various times during the DHA disappearance curve. Cultures to which the antibiotic was added prior to the lag did not degrade DHA. Those that were spiked with the antibiotic after the lag phase (4 h) degraded DHA at the same rate as did controls with no added tetracycline. Therefore, de novo protein synthesis was required for DHA biodegradation, confirming that this activity is inducible. The five strains were also evaluated for their ability to degrade other resin acids. All strains behaved in a similar fashion. Unchlorinated abietane-type resin acids (abietic acid, DHA, and 7-oxo-DHA) were completely degraded within 7 days, whereas pimarane resin acids (sandaracopimaric acid, isopimaric acid, and pimaric acid) were poorly degraded (25% or less). Chlorination of DHA affected biodegradation, with both 12,14-dichloro-DHA and 14-chloro-DHA showing resistance to degradation. However, 50 to 60% of the 12-chloro-DHA was consumed within the same period.

Resin acids are tricyclic carboxylic acids found in many softwoods, particularly pines (28) (Fig. 1). These compounds are of environmental concern because they contribute to the overall toxicity of pulp mill effluents (7, 11, 18, 22, 28, 31). Conventional aerobic biological treatment of pulp mill effluents generally reduces resin acid concentrations to below toxic levels (25, 28), with biodegradation being an important mechanism of this removal (20, 28). However, resin acid removal is not always consistent, and toxicity failures do occur (24, 28). Although research into resin acid removal by effluent treatment has been well documented, there is a lack of information on the microbiology of resin acid metabolism. The study of resin acid biodegradation by microorganisms inherent to effluent treatment systems should aid in improving reactor performance.

Bacterial degradation of dehydroabietic acid (DHA), a common resin acid found in pulp mill effluents and receiving waters (5, 28), has been found in *Arthrobacter* sp. (19), *Bacillus* sp. (10), *Alcaligenes eutrophus*, *Pseudomonas* sp. (3), and *Flavobacterium resinovorum* (2). Studies on the metabolism of DHA have identified several oxidized DHA analogs and have outlined several possible pathways for metabolism (2, 3). However, neither the enzymatic activities nor the genes involved in resin acid degradation by these microorganisms have been studied. Also, as these microorganisms were not isolated from pulp effluent treatment systems, their resin acid-degrading capabilities may not be representative of the microbial populations found in treatment environments.

* Corresponding author. Mailing address: Department of Wood Science, Faculty of Forestry, 270-2357 Main Mall, University of British Columbia, Vancouver, B.C., Canada V6T 1Z4. Phone: (604) 222-3200 ext. 420. Fax: (604) 222-3207. Electronic mail address: bicho@unixg.ubc.ca.

Recently, five bacteria that degrade DHA were isolated from enrichments of bleached kraft mill effluent (1). These bacteria share some similarities (gram-negative, rod-shaped aerobes). However, their physiology, cell size, and other morphological features indicated that the strains are different. Further characterization of these microorganisms is required to resolve mechanisms of resin acid metabolism and to establish their role in resin acid biodegradation within effluent treatment systems. One way of characterizing the metabolic enzymes involved in this activity is to examine the effects of substrate modification on biodegradation. Fortunately, several resin acids with a similar carbon skeleton are found in nature. These compounds are classified as either abietane- or pimarane-type resin acids (Fig. 1). The former have an isopropyl moiety at the C-13 carbon, while the latter have two substituents, a vinyl and a methyl moiety, at this position. Substituents at other positions are also possible. These differences can be exploited to determine sites that are essential for biodegradation.

The work in this paper establishes that the five bacteria grow on DHA as the sole carbon source and that this activity is inducible. Substrate specificities of the five strains using several resin acids are also reported. These included abietane and pimarane resin acids, as well as chlorinated and oxygenated DHA analogs.

MATERIALS AND METHODS

Bacterial strains. The five bacteria previously described by Bicho et al. (1) were used in this study. These strains were originally designated 5s, 6lb, 7s, 9, and 11. In this paper, they are renamed BKME 5, BKME 6, BKME 7, BKME 9, and BKME 11, respectively. The strains were maintained on nutrient agar supplemented with 50 mg of DHA liter^{-1}.

Chemicals. All resin acids, including *O*-methyl podocarpic acid (surrogate standard), were purchased from Helix Biotech, Richmond, B.C., Canada, and

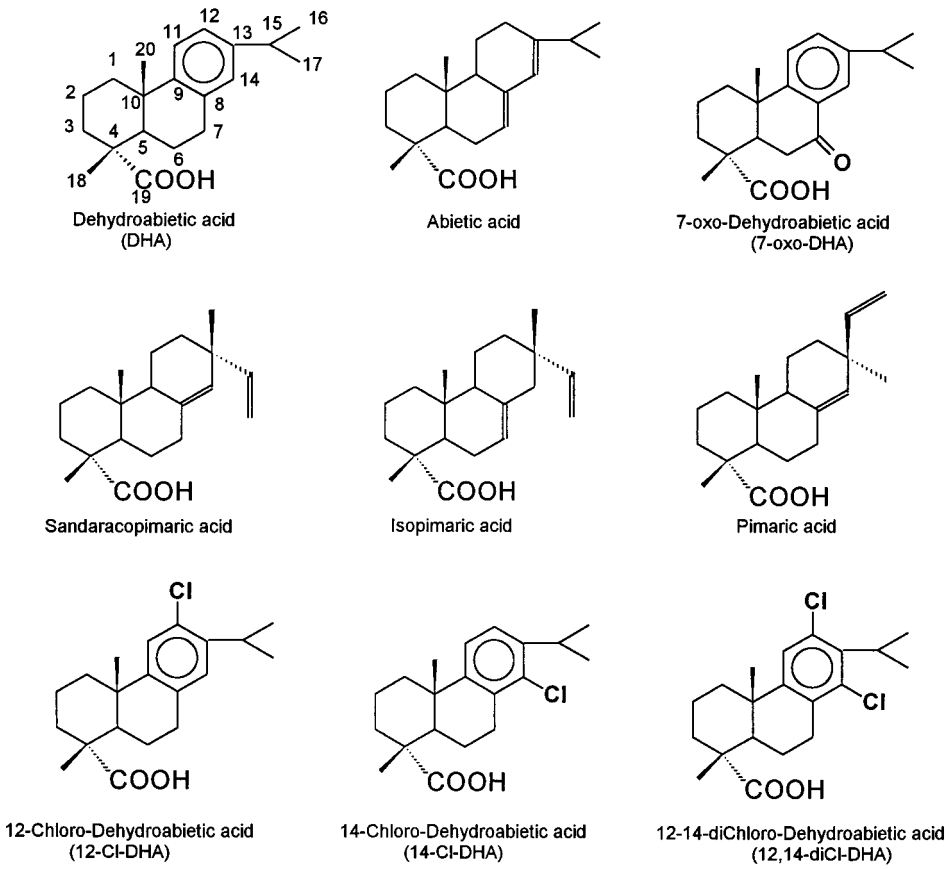

FIG. 1. Resin acids used in this study. The carbon skeleton numbering scheme is presented on DHA.

were of high purity (90 to 99%). Methyl heneicosanoic acid (internal standard) was purchased from Aldrich, Milwaukee, Wis., and was also of high purity (99%).

Media. A defined medium with DHA as the sole carbon source was used in all growth and high-density resting-cell suspension experiments. This final medium contained (per liter) 1.15 g of Na_2HPO_4, 0.26 g of NaH_2PO_4, 4.1 g of NH_4Cl, and 1.7 g of yeast nitrogen base, without nitrogen or amino acids (8) and with 100 mg DHA (added as a 20,000-mg liter^{-1} concentrate in methanol). Filter-sterilized NH_4Cl-yeast nitrogen base concentrate was added to autoclaved phosphate salts-DHA solution to give a final pH between 7.2 and 7.6. In some cases, alternative carbon sources, such as other resin acids, glycerol, methanol, or acetate, were used.

Growth experiments. Bacteria were grown on the defined medium described above with 100 mg of DHA (99% purity) liter^{-1} used as the carbon source. A 100-ml volume of DHA medium was inoculated with a 3% volume of the appropriate strain, which had previously been transferred twice on DHA–defined-salts medium (4 days at 30°C). Flasks were incubated at 30°C with agitation (150 rpm) on a gyratory shaker. Because resin acid solutions (>50 mg liter^{-1}) formed turbid suspensions that preclude measurement of biomass by observing optical density, cell concentrations were monitored by the standard plate count method. Cultures were plated on nutrient agar and incubated for 48 h at 30°C. DHA concentrations were monitored by capillary gas chromatography as described below. When glycerol, acetate, or methanol was used as the carbon source, growth was monitored by measuring optical density at 600 nm.

High-density resting-cell suspension experiments. A 300-ml portion of defined medium, with 10 g of glycerol liter^{-1} as the carbon source, was inoculated with 25 ml of a 2- to 3-day-old culture grown on glycerol. Cultures were incubated for 24 h at 30°C on a gyratory shaker (150 rpm). Late-log-phase cultures were harvested by centrifugation for 20 min at 8,000 × *g* and 15°C, washed in 10 mM phosphate buffer, and resuspended in three 25-ml portions of sterile defined medium without a carbon source. Experiments were started by spiking the medium with DHA (20,000-mg liter^{-1} stock in methanol) to give a final concentration of 50 mg liter^{-1}. Each flask was treated in one of the following ways: (i) 40 μl of methanol was added at time 0; (ii) 40 μl of 32-mg liter^{-1} tetracycline in methanol was added at time 0; or (iii) 40 μl of 32-mg liter^{-1} tetracycline was added 4 h into the incubation. Cell suspensions were incubated at 30°C with vigorous agitation (250 rpm) for 9 h. Periodically, 0.5-ml aliquots were aseptically removed and immediately extracted for DHA analysis. At the end of the 9-h incubation period, aliquots from each flask were plated on nutrient agar to ensure culture purity and viability.

Utilization of individual resin acids. Defined medium was prepared as described above, except that DHA was substituted by one of a number of different resin acids as the sole carbon source. These resin acids were all of analytical grade (90 to 99% purity) and included DHA, abietic acid, 12-monochloroabietic acid (12-Cl-DHA) plus 14 monochloroabietic acid (14-Cl-DHA) (1:1 mixture of each isomer), 12,14-dichlorodehydroabietic acid (12,14-diCl-DHA), 7-oxodehydroabietic acid (7-oxo-DHA), pimaric acid, isopimaric acid, and sandaracopimaric acid. A 5-ml portion of medium with individual resin acids (50 mg liter^{-1}) was inoculated with 0.5 ml of DHA-grown cultures, loosely capped, and incubated for 7 days in the dark with daily mixing to resuspend cells. Abiotic controls were run in parallel. After incubation, tube contents were extracted and resin acids were quantified by gas chromatography as described below. Initial and residual resin acid concentrations were used to calculate the percentage of resin acid consumed by each strain. Each value reported is an average of results from three independent replicate determinations.

Resin acid analysis. Resin acids were extracted by the procedure of Kutney et al. (12–17). This method was used to extract resin acids and various hydroxylated derivatives from microbial medium systems. Aliquots were spiked with 50 μg of surrogate standard, acidified with 2 to 3 drops of 10 N HCl or H_2SO_4, and extracted twice with 1 to 1.5 ml of ethyl acetate. Extracts were pooled, dried over nitrogen, and spiked with 50 μg of internal standard prior to methylation with diazomethane as described by Voss and Rapsomatiotis (30). Resin acid identification and quantification were performed by capillary gas chromatography with model 5890 gas chromatograph flame ionization detector equipped with an HP 7673 autoinjector (Hewlett-Packard). Helium and nitrogen were used as carrier and makeup gases, respectively. Analytes were resolved on a DB-5 column with one of two temperature programs. The first temperature program resolved all resin acids used in this study (70°C for 2 min, 30°C/min to 170°C, 0.6°C/min to 200°C, 10°C/min to 280°C, hold for 10 min). The second program was a rapid method for DHA quantification only (150°C for 2 min, 4°C/min to 260°C, 20°C/min to 280°C, hold for 3 min). Injector and detector temperatures for both temperature programs were 260 and 290°C, respectively. Surrogate recoveries were consistently between 85 and 105%, and the lower detection limit for all of the resin acids was 0.5 mg liter^{-1} for both temperature programs.

Identification of 12-Cl-DHA and 14-Cl-DHA. Two monochloro-DHA isomers

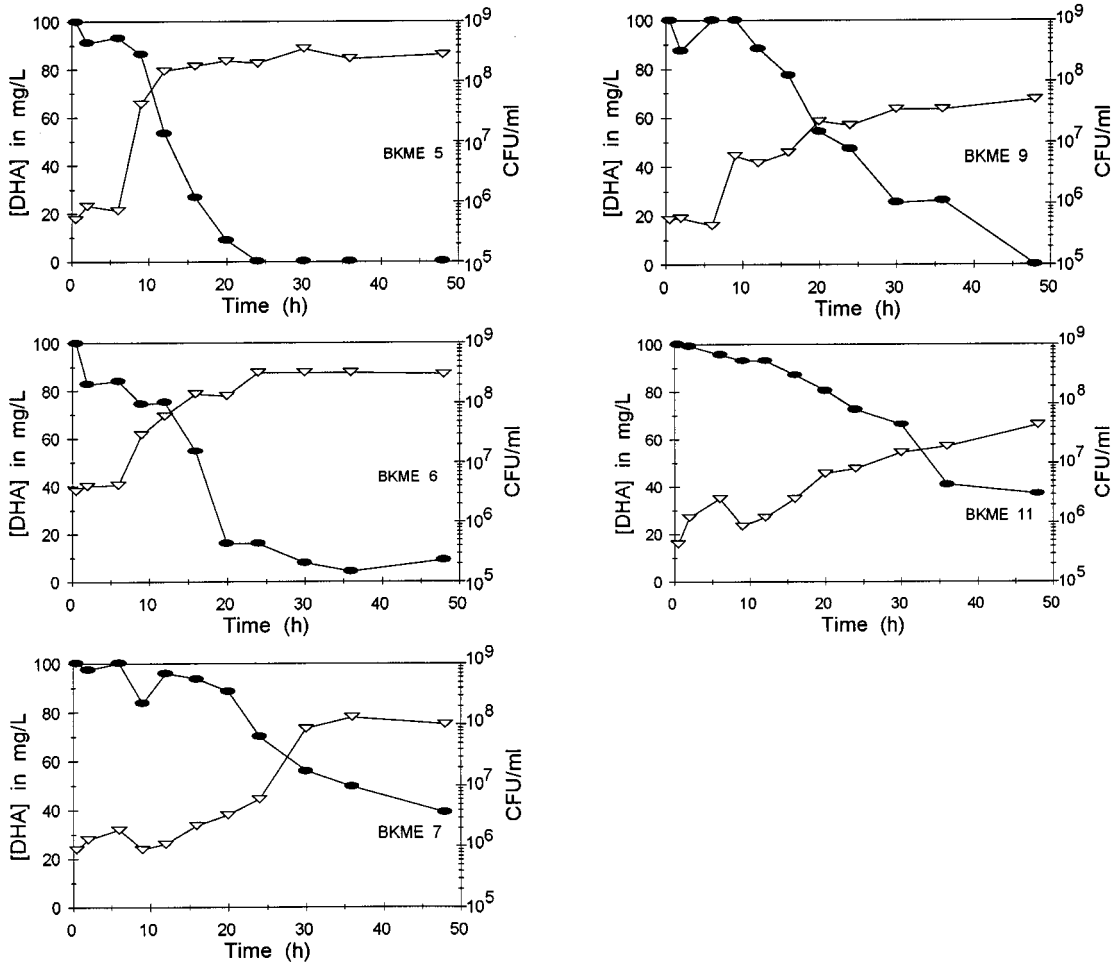

FIG. 2. Time courses of DHA concentration (in milligrams per liter) (●) and cell concentration (in CFU per milliliter) (▽) for the five bacteria over time.

were obtained commercially as a 1:1 mixture and were resolved by capillary gas chromatography as described above. Isomers were partially purified from 200 mg of mixture by the selective crystallization of the imidazole derivatives as described by Kutney et al. (13). Chlorinated resin acid preparations were cleaned up by the solid-phase procedure of Chen et al (6). In this way, two fractions were obtained, one enriched with 14-Cl-DHA (22.5 mg) and the other enriched with 12-Cl-DHA (79 mg). Isomer identity was confirmed by ^{1}H nuclear magnetic resonance spectroscopy as described by Kutney et al (13), with a Bruker 400-MHz spectrometer.

RESULTS AND DISCUSSION

Growth on DHA. The five strains were grown on DHA as the sole carbon source. Cell number and DHA concentration were monitored over time (Fig. 2). All five strains exhibited an increase in cell concentration of at least 2 log units that coincided with a decrease in DHA concentration, thus confirming growth on this substrate. Three strains, BKME 5, 6, and 9, consumed more than 95% of the available DHA within 48 h, with the maximum growth rate occurring during this period. The rate of DHA utilization decreased as growth slowed. Similar results were obtained with strains BKME 7 and 11. However, DHA utilization was incomplete, with only 60 and 65% of the DHA consumed, respectively. Again, growth was concurrent with DHA utilization.

These results are in agreement with previous studies in which growth on low-grade DHA (60% purity) had been demonstrated (1). As this previous work used a technical grade of resin acid, it was possible that impurities in the DHA sample also supported growth. However, growth on the analytical grade DHA (99% purity) used in this study confirmed that DHA is utilized as the sole carbon source for the growth of all five strains.

Similar experiments with acetate, methanol, or glycerol as the sole carbon source were also carried out (data not shown). These three carbon sources were selected as possible growth substrates for use in high-density resting-cell suspensions. Also, acetate and methanol are known to be major contributors to biological oxygen demand in pulping effluents (27). It is possible that these compounds, if metabolized by the five strains, will affect resin acid biodegradation. Glycerol was utilized for growth by all five strains, but acetate and methanol were not. As a small amount of methanol was routinely used as a carrier solvent to aid in DHA addition to the media, these results also confirmed that the growth in Fig. 2 was due solely to DHA utilization.

In earlier work, Hemingway and Greaves (10) reported that more than half of 69 bacteria screened for resin acid-degrading capabilities were completely inhibited by resin acid concentrations of 40 mg liter^{-1} or higher. In contrast, our results (1) indicate that all five isolates could grow on DHA at concentrations as high as 100 mg liter^{-1} and could tolerate concentrations as high as 180 mg liter^{-1}. These levels are comparable to concentrations found in many untreated pulp mill effluents

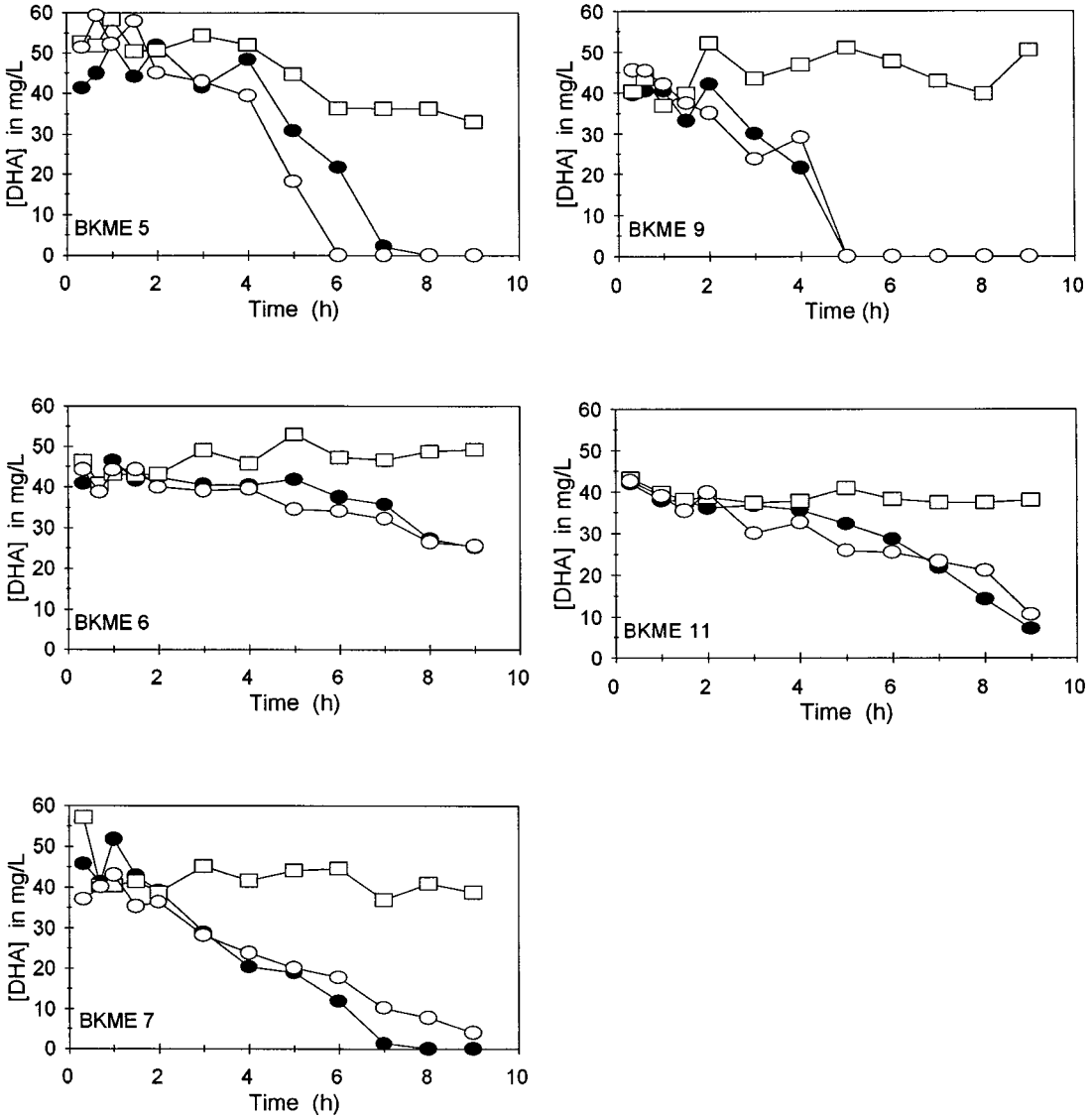

FIG. 3. Time course of DHA biodegradation (in milligrams per liter) by glycerol-grown cells at high density that were not exposed to tetracycline (●), spiked with tetracycline at time 0 (□), or spiked with tetracycline after 4 h (○).

(24, 28). Another indication of the relevance of these isolates is that high concentrations of DHA could be completely removed within a period comparable to the hydraulic retention time of a typical aerated lagoon if sufficient biomass is present.

Induction of DHA biodegradation. It was observed that DHA biodegradation was preceded by a lag of 2 to 4 h in high-density resting-cell suspensions (data not shown). Reports of similar lags in resin acid degradation by bacteria isolated from receiving waters (10) and sludges (20) have also been noted. Several physical and biological factors may be responsible for this lag, including a cofactor or nutrient requirement essential for biodegradation, slow transport of DHA, or the need for enzyme induction. Because the need for induction of resin acid metabolic activity was considered to be the more likely reason for the observed lag, this was further investigated. To determine whether DHA biodegradation is an inducible activity, glycerol-grown high-density cultures were spiked with DHA in the presence or absence of tetracycline. Tetracycline, which is known to inhibit de novo protein synthesis at the translational level (4), halted growth of the five strains at concentrations of 20 to 30 mg liter^{-1}.

Induction experiments demonstrated a 2- to 4-h lag period prior to the onset of DHA degradation (Fig. 3). After this lag, strains BKME 5, 7, and 9 were able to completely degrade the DHA within 8 h while BKME 6 and 11 consumed only 50 and 80%, respectively, of the DHA. It is possible that this reflects variations in cell density rather than differences in DHA metabolism. The addition of tetracycline at the start of the time course prevented DHA degradation by four of the strains (BKME 6, 7, 9, and 11) and severely inhibited DHA degradation by BKME 5. This suggests that de novo protein synthesis is required for DHA biodegradation. The BKME 5 isolate consumed 25% of the DHA within a 6-h period, indicating incomplete inhibition of protein synthesis.

Tetracycline added to the cultures after the 4-h lag time did not inhibit DHA biodegradation in any of the strains. The rate of DHA biodegradation in this treatment was comparable to that of controls to which no tetracycline had been added (Fig.

TABLE 1. Percent consumption of eight resin acids by the five isolates after a 7-day incubation

Resin acid[a]	% Consumption[b] by:					
	Abiotic control	BKME 5	BKME 6	BKME 7	BKME 9	BKME 11
Abietic acid	24.7 (2.1)	**100.0**[c] (0.0)	**87.0** (18.4)	**99.3** (0.9)	**100.0** (0.0)	**100.0** (0.0)
DHA	0.0 (0.0)	**97.3** (3.1)	71.0[d]	**100.0** (0.0)	**95.7** (4.8)	**95.7** (4.8)
7-Oxo-DHA	0.0 (0.0)	**100.0** (0.0)	**100.0** (0.0)	**100.0** (0.0)	**100.0** (0.0)	**100.0** (0.0)
12-Cl-DHA[e]	0.0 (0.0)	**60.0** (5.9)	57.0[d]	**56.0** (2.2)	**56.3** (2.1)	**57.3** (5.4)
14-Cl-DHA[e]	0.0 (0.0)	9.0 (6.5)	5.7 (4.5)	3.7 (2.9)	2.7 (1.9)	2.7 (2.5)
12,14-DiCl-DHA	0.0 (0.0)	2.0 (1.4)	2.7 (1.9)	5.3 (2.9)	2.3 (1.7)	1.3 (1.9)
Pimaric acid	0.0 (0.0)	3.4 (3.4)	3.8 (2.7)	1.3 (0.9)	6.0 (7.0)	4.0 (5.9)
Isopimaric acid	6.0 (9.3)	22.3 (9.5)	2.0[d]	25.0[d]	23.0[d]	15.0 (6.2)
Sandaracopimaric acid	0.0 (0.0)	0.0 (0.0)	0.7 (0.5)	5.7 (5.0)	4.3 (6.1)	2.3 (3.3)

[a] Initial concentration, 50 mg liter^{-1}.
[b] Values are means of three separate determinations. Standard deviations are in parentheses.
[c] Boldface values are statistically significant from the corresponding abiotic control (99% confidence level).
[d] Mean of duplicate determinations.
[e] Evaluated as a 50:50 mixture of two monochloro isomers.

3). This demonstrated that tetracycline did not affect any physiological functions, other than protein synthesis, necessary for DHA biodegradation.

Utilization of different resin acids. The ability of the five strains to degrade a range of resin acids commonly found in kraft mill effluent was evaluated. These included abietanes, pimaranes, and several chlorine-substituted resin acids (Fig. 1). It has been shown that chlorinated resin acids are found in effluents from kraft mills in which chlorine or chlorine dioxide is used during bleaching (27, 33) and that these types of resin acids are more biologically recalcitrant than the unchlorinated parent molecule (9, 22). The 7-oxo-DHA analog was also evaluated, because it has been found in pulp mill effluents (33) and receiving waters (5) and is a common product of DHA oxidation (26). It has also been identified as a possible intermediate in the biodegradation of DHA by *F. resinovorum* (2, 3).

An assessment of the individual consumption of eight resin acids by the five strains indicated that there was a decrease in the level of abietic and isopimaric acids as a result of abiotic phenomena (Table 1). All of the other resin acids were stable over the incubation period. The instability of several resin acids including abietic, palustric, and neoabietic acids has been described previously (26, 28). In particular, abietic acid can be oxidized or isomerized upon exposure to air, heat, or high acidity (23, 26). These abiotic phenomena may have resulted in the losses observed with the uninoculated controls. However, in general, other resin acids are considered stable, as exemplified by their survival during kraft cooking, bleaching, and often biological treatment as well. Biodegradation was defined as a statistically significant drop in resin acid concentration relative to abiotic controls that had been incubated in parallel.

All unchlorinated abietanes were utilized by the five strains. Abietic acid and 7-oxo-DHA consumption was 87% or greater (Table 1). Utilization of DHA was also extensive, with the exception of BKME 6, which consumed only 71% of the available DHA. Microscopic examination confirmed cell growth in tubes where resin acid consumption had occurred. In contrast to the extensive biodegradation of the unchlorinated abietanes, consumption of the pimaranes was poor. Little or no biodegradation of pimaric, isopimaric, and sandaracopimaric acids was noted (Table 1). In previous work with resin acid mixtures, BKME 6 and 9 degraded mixtures of both abietane- and pimarane types-resin acids (1). The contrast between the biodegradation of abietane-type and pimarane-type resin acids, which differ primarily at the C-13 side group, denotes the importance of this moiety in resin acid metabolism. The C-13 side group appears to be important in either the induction or specificity of the enzymes involved in resin acid biodegradation.

The nature of the chlorine substitution on the DHA also affected its biodegradation by the five isolates (Table 1). No significant biodegradation of 12,14-diCl-DHA by any of the strains was observed. This is in agreement with results of earlier experiments (1), which found that the five strains consumed DHA but little or no 12,14-diCl-DHA when the two resin acids were present as a mixture. These observations also agree with reports that 12,14-diCl-DHA is relatively difficult to degrade biologically and often survives biological treatment (9, 22).

The gas chromatography protocol employed could resolve but not identify the two monochlorinated DHA isomers. It has been reported that 14-Cl-DHA elutes before 12-Cl-DHA (21). However, mass spectrometry used for peak identification could not differentiate between the two isomers. To establish their elution order, the partially purified monochlorinated DHA isomers were subjected to gas chromatography analysis and ^{1}H nuclear magnetic resonance spectroscopy. The 12-Cl-DHA fraction was enriched in the faster-eluting peak (area ratio, 2:1), while the 14-Cl-DHA fraction corresponded to the more slowly eluting peak (area ratio, 1:4). ^{1}H nuclear magnetic resonance spectroscopy was used to identify the two monochloro-DHA isomers, as previously reported (14, 29). The ^{1}H nuclear magnetic resonance spectrum of the faster-eluting fraction had two signals at 6.9 and 7.15 ppm, indicative of the 12-Cl-DHA isomer. The spectrum of the more slowly eluting fraction had two doublets at 7.15 ppm (J = 8), which is characteristic of the 14-Cl-DHA isomer. Therefore, the elution order of the isomers was established as 12-Cl-DHA followed by the 14-Cl-DHA. Having identified the two monochloro-DHA isomers, it was evident that no significant degradation of 14-Cl-DHA had occurred (Table 1). However, 55 to 60% of the 12-Cl-DHA was degraded by all strains. Therefore, although chlorination of the C-12 position may impede DHA degradation, the C-14 position must remain unchlorinated for biodegradation to occur. From the literature, no modification of the C-14 position during DHA metabolism has been reported in bacteria (2, 3) or fungi (12, 14, 32). However, the selective degradation of one monochlorinated isomer over the other indicates the importance of the C-14 position in one or more enzymatic steps in DHA biodegradation by these strains.

This paper has demonstrated that the five strains isolated from kraft-pulping effluents can utilize DHA as the sole carbon source and that this phenomenon is an inducible activity. It was

apparent that resin acid biodegradation was affected by the nature and position of various substituents. This selective degradation of DHA analogs is currently being used as a tool for the elucidation of resin acid metabolism in the five strains under investigation.

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