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Bacterial Metabolism of Chlorinated Dehydroabietic Acids Occurring in Pulp and Paper Mill Effluents

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Chlorinated dehydroabietic acids are formed during the chlorine bleaching of wood pulp and are very toxic to fish. Thus, destruction of these compounds is an important function of biological treatment systems for pulp and paper mill effluents. In this study, 12 strains of diverse, aerobic resin acid-degrading bacteria were screened for the ability to grow on chlorinated dehydroabietic acids as sole organic substrates. All seven strains of the class *Proteobacteria* able to use dehydroabietic acid were also able to use a mixture of 12- and 14-chlorodehydroabietic acid (Cl-DhA). None of the strains used 12,14-dichlorodehydroabietic acid. *Sphingomonas* sp. strain DhA-33 grew best on Cl-DhA and simultaneously removed both Cl-DhA isomers. *Ralstonia* sp. strain BKME-6 was typical of most of the strains tested, growing more slowly on Cl-DhA and leaving higher residual concentrations of Cl-DhA than DhA-33 did. Strains DhA-33 and BKME-6 mineralized (converted to CO_2 plus biomass) 32 and 43%, respectively, of carbon in Cl-DhA consumed. Strain DhA-33 produced a metabolite from Cl-DhA, tentatively identified as 3-oxo-14-chlorodehydroabietin, and both strains produced dissolved organic carbon which may include unidentified metabolites. Cl-DhA removal was inducible in both DhA-33 and BKME-6, and induced DhA-33 cells also removed 12,14-dichlorodehydroabietic acid. Based on activities of strains DhA-33 and BKME-6, chlorinated DhAs, and potentially toxic metabolite(s) of these compounds, are relatively persistent in biological treatment systems and in the environment.

Dehydroabietic acid (DhA) belongs to a group of tricyclic diterpenes, collectively called resin acids (Fig. 1). Resin acids occur naturally in trees, particularly softwoods. Pine wood can be composed of up to a few percent resin acids. Chlorobleaching (using Cl_2, ClO_2, or other chlorine compounds) of wood pulp causes formation of chlorinated DhAs (7, 23), the major congeners being 12-chlorodehydroabietic acid (12-Cl-DhA), 14-chlorodehydroabietic acid (14-Cl-DhA), and 12,14-dichlorodehydroabietic acid (diCl-DhA) (Fig. 1). Resin acids, including chlorinated DhAs, are commonly found in pulp and paper mill effluents as well as in the sediments of water bodies receiving such effluents (11, 12). The reduced use of Cl_2 has reduced the production of chlorinated DhAs, but they are still commonly found in effluents (11, 12). The accumulation of resin acids in sediments suggests that they are environmentally persistent, but conclusive evidence for persistence in various aquatic environments is lacking. Not surprisingly, Cl-DhA appears to be more persistent than DhA and other resin acids in pulp mill biological treatment systems (6, 17).

Resin acids, including DhA, are the major contributors to toxicity of pulp and paper mill effluents to fish (18, 24). Chlorinated DhAs are more toxic to fish than DhA (19, 22, 27). Resin acids are hydrophobic and have been shown to bioaccumulate in fish (21). By analogy to other pollutants, one might expect the chlorinated analogs of DhA to have greater potential than DhA for bioaccumulation, but there is no published evidence for this hypothesis. For the reasons described above, there is cause for concern about the environmental effects of chlorinated DhAs, and there is a need for better understanding of the fate of these compounds in the environment and in biological treatment systems for pulp and paper mill effluents.

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Currently, very little is known about biodegradation of chlorinated DhAs. Bicho et al. (2) described five bacterial isolates which grow on DhA and are able to remove from their medium 12-Cl-DhA but do not appreciably remove 14-Cl-DhA nor diCl-DhA. Kutney et al. (8–10) found that the fungus *Mortierella isabellina* can nondegradatively transform the three chlorinated DhAs mentioned above to hydroxylated and carbonylated derivatives which are not further metabolized by the fungus but which are less toxic to fish. The fungus primarily hydroxylated C-2, C-15, and C-16 of chlorinated DhAs. No microorganisms have previously been reported to grow on chlorinated DhAs, and biodegradation of these compounds has not been conclusively demonstrated previously.

Recently, our research group has isolated and characterized at least 12 distinct bacterial strains which grow on various resin acids (15, 16, 26). In this study, we examined the ability of these 12 strains to metabolize chlorinated DhAs. All of the *Proteobacteria* able to grow on DhA could also use a mixture of 12-Cl-DhA and 14-Cl-DhA (Cl-DhA) as a sole organic substrate, but none grew on diCl-DhA. Growth on Cl-DhA of two of the isolates, *Sphingomonas* sp. strain DhA-33 and *Ralstonia* sp. strain BKME-6, was examined in detail. Growth yields of both strains on Cl-DhA were low, and removal of Cl-DhA was incomplete, particularly removal by BKME-6. DhA-33 accumulated a metabolite, tentatively identified as 3-oxo-14-chlorodehydroabietin (Fig. 1). Both strains transformed a significant fraction of Cl-DhA carbon to unidentified dissolved organic carbon (DOC). In natural environments and in biological treatment systems, bacterial degradation of chlorinated DhAs can be expected, particularly where DhA is also present as a substrate. However, residual chlorinated DhAs, as well as identified and unidentified metabolites, may persist in such systems. These persistent compounds have the potential of negative environmental effects.

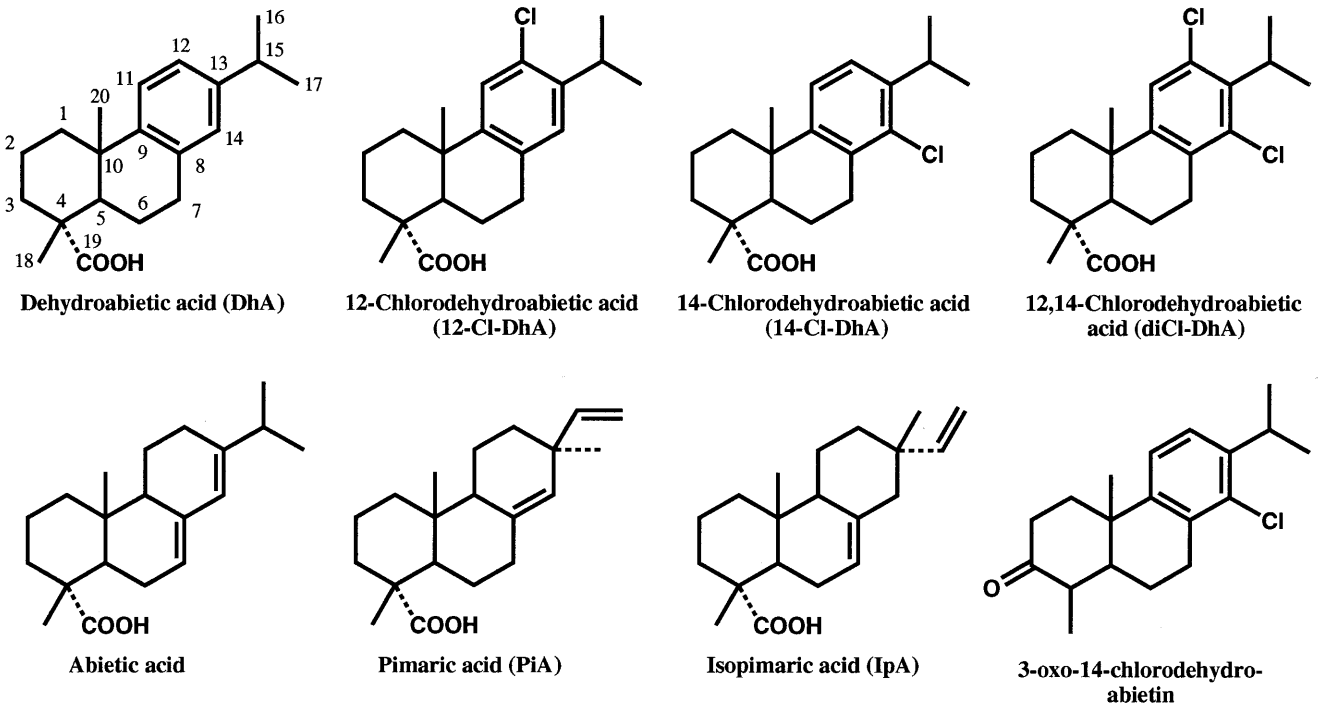

FIG. 1. Chemical structures discussed in this paper. Numbering of the carbon atoms is shown for DhA.

MATERIALS AND METHODS

Cultures and media. The bacterial strains used are from three sources, (i) a laboratory-scale bioreactor treating mechanical newsprint whitewater (15, 26), (ii) soil from a temperate forest (16), and (iii) an aerobic lagoon treating kraft pulp mill effluent (1). The third group comprises two strains, BKME-6 and BKME-9, which were a gift from P. Bicho. The mineral medium for cultures was described previously (15). DhA (99%), Cl-DhA (85 to 95%; 1:1 mixture of 12-Cl-DhA and 14-Cl-DhA), and diCl-DhA (95 to 99%) were from Helix Biotechnologies, Richmond, British Columbia, Canada. Organic substrates were added to sterile mineral medium from filter-sterilized stock solutions dissolved in water (pyruvate and glucose), in aqueous NaOH (DhA and Cl-DhA), or in methanol (diCl-DhA). Resin acids were present in the cultures as insoluble suspensions. Cultures were incubated at 30°C in tubes on a tube roller or in flasks on a shaker. Growth curves and Cl-DhA removal curves were determined as described previously (26), by use of individual 1-ml cultures for each protein or Cl-DhA sample.

Carbon mass balance determination. The fate of carbon during growth on Cl-DhA was determined by using 5-ml cultures incubated in 26-ml tubes sealed with serum bottle stoppers. The cultures were incubated to the early stationary phase on a tube roller at 30°C. Strain DhA-33 was grown for 70 h on 400 μM Cl-DhA, and strain BKME-6 was grown for 72 h on 800 μM Cl-DhA. One set of triplicate cultures was used to quantify residual Cl-DhA and production of a metabolite. Another set of triplicate cultures was used to quantify CO_2 production. After incubation, these replicates were acidified with 4 M HCl to pH 2.0 to drive CO_2 into the headspace of the tubes for its analysis. A third set of triplicate cultures was used to quantify protein and DOC production. After incubation, these replicates were centrifuged for 20 min at 16,000 × *g* to pellet the cells. The cell pellets were assayed for protein, and this value was converted to biomass carbon by multiplying by 1.82 (ratio of dry weight/protein) and multiplying by 0.60 (ratio of carbon/dry weight). The supernatants were acidified as described above to remove CO_2 and assayed for DOC and dissolved Cl-DhA, the latter being subtracted from the net DOC values. Values for net increase in carbon as a metabolite, CO_2, biomass, and DOC were determined as values for cultures grown on Cl-DhA less the corresponding values for inoculated control cultures having no Cl-DhA as substrate. Values for net decrease in carbon as Cl-DhA were determined as values for uninoculated control cultures having Cl-DhA as substrate less the corresponding values for cultures grown on Cl-DhA.

Cell suspensions. Cell suspensions were prepared as described previously (15). Strain DhA-33 was grown to the late log phase on 1.0 g of glucose per liter, and strain BKME-6 was grown on 1.0 g of pyruvate per liter. Cells were washed and suspended in culture medium which did not contain trace elements, vitamins, or $FeSO_4$. Where indicated, 50 μg of chloramphenicol or 30 μg of tetracycline per ml was added. Magnesium was omitted from the medium when tetracycline was added. Cell suspensions had an optical density at 610 nm of approximately 0.9, which corresponds to approximately 100 μg of protein per ml. Killed cell suspensions were boiled for 10 min. Cell suspension aliquots of 1 ml were incubated in screw-cap tubes on a tube roller at 30°C. Entire tubes were used for each analytical sample.

Analytical methods. Protein was quantified by the bicinchoninic acid method (20). For the protein assay, cells were lysed by adding 1% sodium dodecyl sulfate or by adding 1 M NaOH and incubating at 95°C. CO_2 was quantified by gas chromatography as described previously (26). DOC was quantified with an automatic Shimadzu model TOC-500 total organic carbon analyzer. Resin acids were quantified by extraction with ethyl acetate, derivatization with diazomethane, and gas chromatography as described previously (15), except that isopimaric acid (IpA) was used as the internal standard. Some gas chromatography samples were alternatively derivatized with bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich Canada, Mississauga, Ontario) to detect potential metabolites with hydroxyl substituents. Mass spectra were determined with a Varian model 3400 gas chromatograph connected to a Varian Saturn model 4D ion trap mass spectrometer. The column and chromatography conditions were essentially those described previously (15). A metabolite of Cl-DhA was extracted from a 2-day-old DhA-33 culture with ethyl acetate. The extract was evaporated to dryness and redissolved in methanol. The metabolite was purified by high-pressure liquid chromatography with a Hewlett-Packard 1050 system equipped with a 4-mm by 125-mm HP octyldecyl silane-hypersil (C_{18}) column (Hewlett-Packard) and a UV-visible detector. The eluent was acetonitrile–aqueous 0.1% acetic acid (55:45) at a flow rate of 2.0 ml/min. Samples of 100 μl were injected, and effluent fractions containing the metabolite were collected. The ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectrum of the purified metabolite was determined by the University of British Columbia Regional Chemistry High-Resolution NMR Analytical Services and Research Support Facility.

RESULTS

Strains using Cl-DhA. Of 12 strains able to grow on resin acids, 7 were able to grow on Cl-DhA (Table 1). The criterion for growth on Cl-DhA was three successive passages on mineral medium with Cl-DhA as the sole organic substrate. The strains using Cl-DhA were diverse members of the class *Proteobacteria* (alpha, beta, and gamma subclasses) which grow well on the abietanes, DhA and abietic acid (AbA), but are unable to use the pimaranes, pimaric acid (PiA) and IpA. For each of these strains, the growth rate and cell yield on Cl-DhA

TABLE 1. Use of resin acids by 12 distinct bacterial strains[a]

Strain	Phylogeny	Growth on[b]:					% Removal of:	
		DhA	AbA	PiA	IpA	Cl-DhA	12-Cl-DhA	14-Cl-DhA
DhA-33	*Sphingomonas*	+	+	−	−	+	96	87
DhA-35	*Zoogloea*	+	+	−	−	(+)	88	48
BKME-6	*Ralstonia*	+	+	−	−	(+)	48	23
DhA-53	*Burkholderia*	+	+	−	−	(+)	68	8
DhA-54	*Burkholderia*	+	+	−	−	(+)	68	7
IpA-51	*Burkholderia*	−	−	−	+	−	74	10
DhA-51	*Pseudomonas*	+	+	−	−	(+)	0	0
BKME-9	*Pseudomonas*	+	+	−	−	(+)	76	13
IpA-1	*Pseudomonas*	(+)	(+)	+	+	−	0	0
IpA-2	*Pseudomonas*	(+)	−	+	+	−	0	0
DhA-55	Gram positive	+	+	+	+	−	0	0
IpA-13	Gram positive	+	+	+	+	−	0	0

[a] Data from references 15 and 26 and from this study.
[b] +, growth; (+), poor growth; −, no growth.

were less than that on DhA. Removal of Cl-DhA was seldom complete. Most strains removed much more 12-Cl-DhA than 14-Cl-DhA. One exception was strain DhA-33, which grew on Cl-DhA better than the other strains and which almost completely removed both Cl-DhA isomers. The Cl-DhA removal data reported (Table 1) were determined after the third passage on mineral medium with Cl-DhA and were consistent with results after the second passage. *Sphingomonas* sp. strain DhA-33 and *Ralstonia* sp. strain BKME-6 were selected for a more-detailed examination.

Growth of strains DhA-33 and BKME-6 on Cl-DhA. Strain DhA-33 grew on 400 μM Cl-DhA (200 μM each isomer) with a doubling time of 7.0 h (Fig. 2A). Both 12-Cl-DhA and 14-Cl-DhA were removed simultaneously by DhA-33 to residual levels of approximately 25 μM. After a 7-day incubation of DhA-33 cultures, the residual levels of 12-Cl-DhA and 14-Cl-DhA were 7 and 11 μM, respectively. Strain BKME-6 had a biphasic growth curve (Fig. 2B). During the first phase, BKME-6 had a doubling time of 3.1 h and 12-Cl-DhA was reduced to a concentration of approximately 100 μM. Because of the low rate of 14-Cl-DhA removal, it is impossible to conclude when this removal began, either simultaneously with 12-Cl-DhA removal or after 12-Cl-DhA removal slowed. During the second phase, growth of BKME-6 slowed dramatically to a doubling time of 56 h and both Cl-DhA isomers were slowly removed. After a 7-day incubation of BKME-6 cultures, the residual levels of 12-Cl-DhA and 14-Cl-DhA were 49 and 134 μM, respectively. Growth curves for each strain were consistent in at least three independent experiments. Time courses for Cl-DhA removal were determined in one experiment. The growth yields on Cl-DhA were similar for DhA-33 and BKME-6, the values being 0.13 and 0.15 g of protein/g of Cl-DhA removed, respectively (Fig. 3). The linear growth yield plots indicate that Cl-DhA was the growth-limiting factor in those cultures. The highest Cl-DhA concentration tested, 3,200 μM, completely inhibited growth of DhA-33 but did not inhibit growth or reduce the growth yield of BKME-6. In cultures of both strains with higher initial Cl-DhA concentrations, residual concentrations of Cl-DhA were higher (data not shown). The percent residual Cl-DhA remained approximately constant, with one exception. The percent residual 12-Cl-DhA in

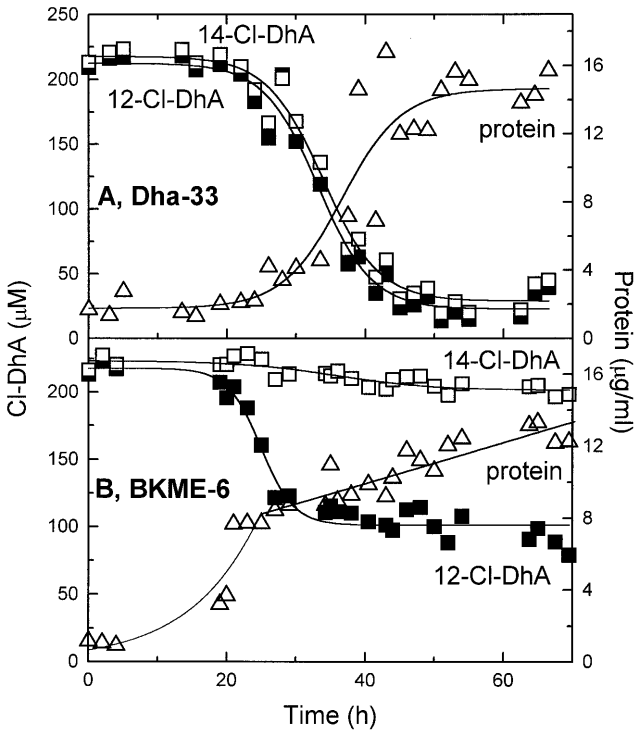

FIG. 2. Growth of strains DhA-33 (A) and BKME-6 (B) on Cl-DhA and removal of each Cl-DhA isomer.

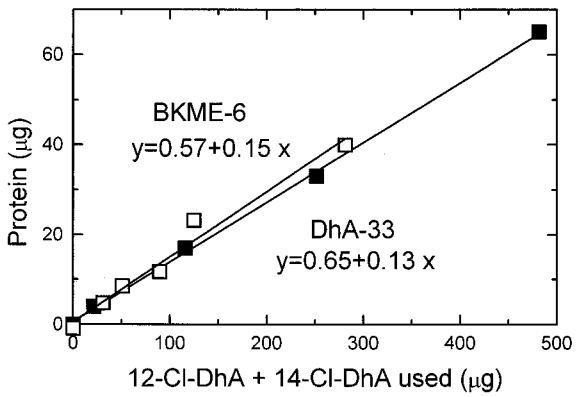

FIG. 3. Growth yields of strains DhA-33 and BKME-6 on Cl-DhA.

BKME-6 cultures rose as initial Cl-DhA concentrations were increased, reaching 55% residual 12-Cl-DhA when 3,200 μM Cl-DhA was initially present.

A metabolite. A probable metabolite always accumulated during growth of DhA-33 on Cl-DhA and was detected by gas chromatography. No other Cl-DhA metabolites of either strain were detected in growing cultures when gas chromatography samples were derivatized with either diazomethane or bis(trimethylsilyl)trifluoroacetamide (the latter method allowing detection of alcohols which might be missed by the former method). Based on all available evidence, we conclude that the Cl-DhA metabolite of DhA-33 was most likely 3-oxo-14-chlorodehydroabietin (Fig. 1), which resulted from addition of a ketone group to and removal of the carboxyl group from 14-Cl-DhA. The metabolite chromatographed without derivatization, and neither derivatizing agent changed its retention time, indicating that it lacked carboxyl or hydroxyl substituents to react with those agents. The infrared absorption spectrum had a prominent carbonyl peak at 1,706 cm^{-1} but lacked O—H stretch near 3,000 cm^{-1}, suggesting a ketone substituent. The mass spectrum had a molecular ion at *m/z* 304 (7.7%), which corresponding to the molecular weight of the proposed structure. Other major ion fragments were *m/z* 289 (100%), corresponding to loss of a methyl group, and *m/z* 247 (53%), corresponding to loss of an isopropyl group. The major mass spectrum peaks were accompanied by M+2 peaks, and for the two largest peaks, *m/z* 289 and *m/z* 247, the M/M+2 ratios were 100:33 and 100:34, respectively, indicating a single chlorine substituent. The ^{1}H-NMR spectrum of the metabolite included resonances of adjacent aromatic protons on C-11 (7.11 ppm, doublet, J = 8.3 Hz) and C-12 (7.21 ppm, doublet, J = 8.3 Hz). The presence of these hydrogen substituents indicates that the chlorine substituent is at C-14. Relative to the ^{1}H-NMR spectrum of 14-Cl-DhA, that of the metabolite shows a change in the resonance of the methyl protons on C-18 from a singlet at 1.25 ppm to a doublet at 1.11 ppm (J = 6.57 Hz), suggesting decarboxylation of 14-Cl-DhA resulting in a new hydrogen substituent at C-4. The assignment of the ketone to C-3 is not certain; however, it seems likely since decarboxylation reactions usually involve a nearby electron-withdrawing group (see Discussion).

Carbon mass balance. A carbon mass balance was determined for late-growth-phase cultures of strain DhA-33 on 400 μM Cl-DhA and strain BKME-6 on 800 μM Cl-DhA (Fig. 4). Approximately equal amounts of carbon from Cl-DhA were incorporated into biomass and converted to CO_2 by both strains, with the percent conversions being higher for DhA-33. The metabolite of DhA-33 was quantified based on the assumption that it had a similar response factor for the flame ionization detector of the gas chromatograph as did Cl-DhA (no authentic standard was available). Both strains produced a substantial amount of DOC which could not be identified. In agreement with previous experiments (Table 1 and Fig. 2B), large amounts of both Cl-DhA isomers remained in BKME-6 cultures. The overall carbon recoveries were 71 and 92% for DhA-33 and BKME-6, respectively. If the response factor for the metabolite of DhA-33 described above is actually lower than estimated, the amount of metabolite produced would have been underestimated, which may account for the low carbon recovery of 71%.

Removal of Cl-DhA by cell suspensions. Removal of Cl-DhA was inducible in both strains (Fig. 5A and 6A). Chloramphenicol prevented induction in DhA-33, and tetracycline prevented induction in BKME-6. The latter strain was resistant to chloramphenicol. Boiling cells of either strain completely prevented Cl-DhA removal (data not shown). In cell suspensions

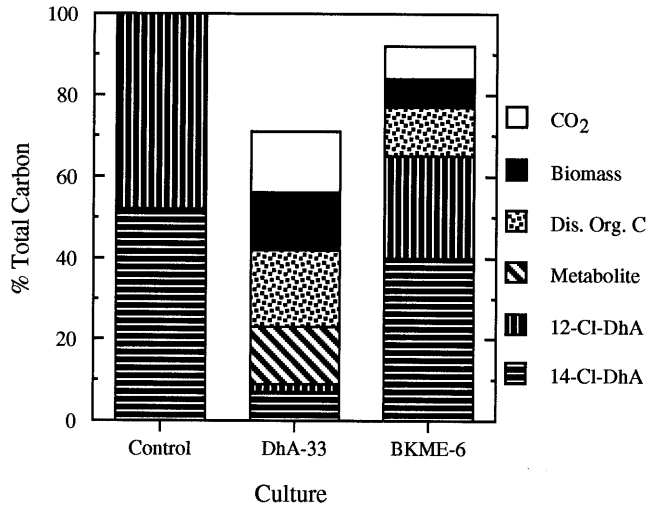

FIG. 4. Carbon mass balance for strains DhA-33 and BKME-6 grown on Cl-DhA, with cultures in the late growth phase.

of both strains, 12-Cl-DhA removal appeared to commence more than 1 h before 14-Cl-DhA removal, although this trend was less clear for strain DhA-33. Substantial residual concentrations of both Cl-DhA isomers were left by both strains in cell suspensions, as in cultures (Fig. 2).

Cell suspensions of strain DhA-33 removed 12- and 14-Cl-DhA at similar rates (Fig. 5A), i.e., 0.23 and 0.24 μmol mg of

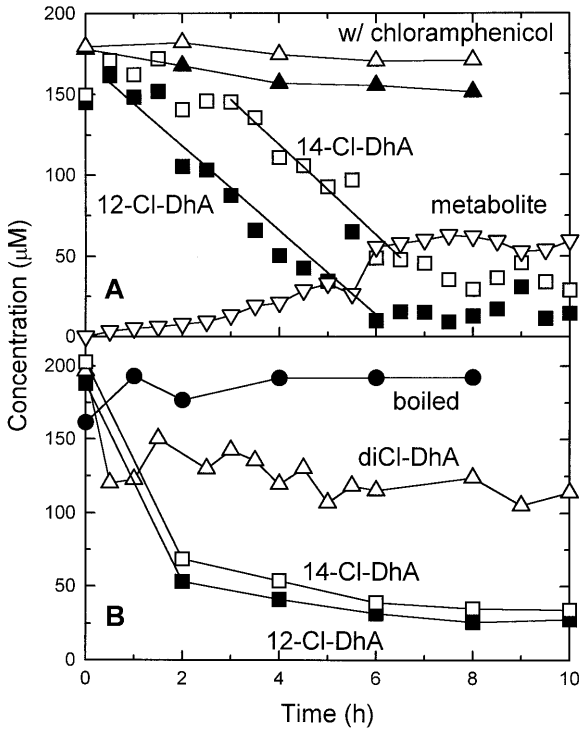

FIG. 5. Removal of Cl-DhA or diCl-DhA by DhA-33 cell suspensions grown on glucose (A) or on glucose with Cl-DhA added 4 h prior to harvesting cells (B). The metabolite is probably 3-oxo-14-chlorodehydroabietic. Unlabeled symbols in panel A: ▲, 12-Cl-DhA; △, 14-Cl-DhA.

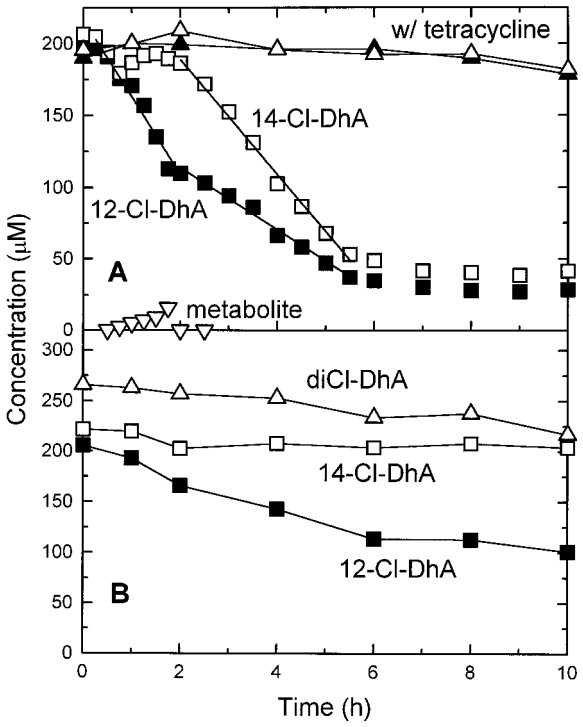

FIG. 6. Removal of Cl-DhA or diCl-DhA by BKME-6 cell suspensions grown on pyruvate (A) or on pyruvate with Cl-DhA added 10 h prior to harvesting cells (B). The metabolite is different from that in Fig. 5A. Unlabeled symbols in panel A: ▲, 12-Cl-DhA; △, 14-Cl-DhA.

protein^{-1} h^{-1}, respectively. As did cultures of DhA-33, cell suspensions of DhA-33 accumulated the metabolite described above. Cell suspensions of DhA-33 removed diCl-DhA when previously induced by growth in the presence of Cl-DhA (Fig. 5B) but failed to remove diCl-DhA when not preinduced (data not shown). The residual concentration of diCl-DhA in induced cell suspensions was more than 100 μM. Probable metabolites of diCl-DhA were detected. The results from cell suspensions of DhA-33 were essentially the same in two independent experiments.

Cell suspensions of strain BKME-6 removed 12-Cl-DhA at two rates (Fig. 6A). The initial removal rate of 12-Cl-DhA was 0.28 μmol mg of protein^{-1} h^{-1}; then, when 14-Cl-DhA removal began, the removal rate of 12-Cl-DhA decreased to 0.11 μmol mg of protein^{-1} h^{-1}. BKME-6 removed 14-Cl-DhA at a rate of 0.21 μmol mg of protein^{-1} h^{-1}. A probable 12-Cl-DhA metabolite accumulated until 14-Cl-DhA removal began and then disappeared. Identification of the metabolite was not practical because its concentration was very low. This pattern of removal rates and metabolite accumulation by BKME-6 was observed in three independent experiments. Cell suspensions of BKME-6 failed to remove diCl-DhA either when previously induced by growth in the presence of Cl-DhA (Fig. 6B) or when not preinduced (data not shown). Surprisingly, BKME-6 cells that were previously induced by growth in the presence of Cl-DhA, washed, concentrated, and suspended with Cl-DhA left a residual 12-Cl-DhA concentration of 100 μM and almost completely failed to remove 14-Cl-DhA (Fig. 6B). This result was observed in two independent experiments.

DISCUSSION

In previous work (15, 16, 26), our research group has identified three groups of bacterial isolates based on specificity for use of resin acids as growth substrates (Fig. 1 and Table 1). Those groups are (i) diverse *Proteobacteria* which use only abietanes, such as DhA and AbA, (ii) *Pseudomonas* spp. which use pimaranes, such as PiA and IpA, and grow poorly, if at all, on abietanes, and (iii) gram-positive bacteria which use abietanes and pimaranes. In the present study, we found that all the members of the first group, and no members of the other two groups, can grow on Cl-DhA. Growth of *Ralstonia* sp. strain BKME-6 and *Pseudomonas* sp. strain BKME-9 on Cl-DhA is consistent with the report of Bicho et al. (2) indicating that these two strains can remove 12-Cl-DhA from their medium. In this study, the percent removal of 14-Cl-DhA by both strains was higher and the percent removal of 12-Cl-DhA by BKME-9 was higher than that in the previous study. These differences are probably due to differences in culture conditions in the two studies.

Correlation of the ability of members of *Proteobacteria* to use Cl-DhA with their ability to use DhA (Table 1) suggests that Cl-DhA is degraded by an enzyme system which evolved by selection for the ability to use the naturally occurring substrate DhA. This hypothesis is consistent with the relative inefficiency of the enzyme system in degrading Cl-DhA, as evidenced by the high residual levels of substrate and the slow growth on Cl-DhA (Table 1 and Fig. 2). The nine strains previously found to grow well on DhA, including DhA-33, generally reduce DhA to undetectable levels (14, 15, 26). In strain DhA-33, the enzyme system degrading Cl-DhA also appears to degrade diCl-DhA, since Cl-DhA induces diCl-DhA removal (Fig. 5B).

The Cl-DhA metabolite produced by strain DhA-33, tentatively identified as 3-oxo-14-chlorodehydroabietin (Fig. 1), may result from hydroxylation of C-3 of 14-Cl-DhA, followed by formation of a ketone by a dehydrogenase. Subsequent decarboxylation of C-4 could be enzymatic or spontaneous. Decarboxylation reactions commonly occur when β-carbonyl groups are present (25). The ketone is believed to facilitate decarboxylation by stabilizing the carbanionic transition state. The unchlorinated analog of the metabolite described above, 3-oxodehydroabietin, was previously identified as a trace metabolite during growth of *Flavobacterium resinovorum* on DhA (5). Subsequent studies reported in 1973 concluded that this metabolite was likely an intermediate in the DhA biodegradation pathway of *Flavobacterium resinovorum* (3) but not likely in the pathway used by a *Pseudomonas* sp. and an *Alcaligenes* sp. (4). These proposed DhA biodegradation pathways have not been confirmed by the use of newer methodology. The present study does not provide evidence indicating whether 3-oxo-14-chlorodehydroabietin is a degradation intermediate or the product of a side reaction. *Pseudomonas* sp. strain BKME-9 cultures did not accumulate 3-oxo-14-chlorodehydroabietin during growth on Cl-DhA, and current studies of DhA degradation by this strain (13) suggest that 3-oxo-dehydroabietin is not an intermediate of DhA degradation by BKME-9.

The biphasic growth curve of strain BKME-6 (Fig. 2B) suggests that this strain uses both Cl-DhA isomers as growth substrates. If the growth yield of BKME-6 on 12-Cl-DhA is constant, then removal of 12-Cl-DhA during the second growth phase does not appear sufficient to support growth during that phase. However, 14-Cl-DhA appears to be used very slowly by BKME-6. It is less clear whether DhA-33 uses both Cl-DhA isomers. Since all 14-Cl-DhA consumed by

DhA-33 does not appear to be transformed to 3-oxo-14-chlorodehydroabietin, some 14-Cl-DhA may support growth.

Relatively little of the Cl-DhA removed by DhA-33 and BKME-6 was mineralized (Fig. 4). DhA-33 and BKME-6 converted 32 and 43%, respectively, of the carbon removed (not total carbon) to biomass plus CO_2, respectively. In the case of DhA-33, an estimated 15% of carbon removed was recovered as a metabolite. For DhA-33 and BKME-6, substantial amounts of the carbon removed were recovered in the form of unidentified DOC, i.e., 20 and 35%, respectively. The fact that the DOC was not detected by gas chromatography suggests that the DOC was either too polar to be extracted by ethyl acetate or had too high a boiling point for chromatography. This DOC may represent metabolites of Cl-DhA or carbon incorporated into biomass and subsequently turned over by secretion or cell lysis. Similar partial transformation of IpA to DOC was previously observed (26), while DhA appeared to be almost completely mineralized (15).

All of the strains using Cl-DhA left relatively high residual concentrations of both isomers (Table 1 and Fig. 2). This phenomenon could be due to low affinity for Cl-DhA or to production of a metabolite which competitively inhibits the degradative enzyme system. The latter possibility seems more likely, particularly in the case of BKME-6. Competitive inhibition by a metabolite could explain the higher residual Cl-DhA concentrations which occurred with higher initial Cl-DhA concentrations for both strains (growth yield experiment). Such inhibition could also explain the reduced ability of BKME-6 cells to remove Cl-DhA when they were previously grown in the presence of Cl-DhA (Fig. 6B). This observation suggests that an inhibitory factor is retained by the washed, suspended cells and has a greater effect on 14-Cl-DhA degradation than on 12-Cl-DhA degradation. When cultures are transferred, the concentration of such an inhibitory factor would be greatly reduced relative to the concentration of Cl-DhA, perhaps allowing resumed degradation activity. If any metabolite(s) of either strain limited removal of Cl-DhA, it occurred without a toxic effect, since the growth yield of both strains was constant over the range of initial Cl-DhA concentrations tested (Fig. 3).

This study indicates the potential for biodegradation of Cl-DhA but suggests that these compounds are more recalcitrant than DhA. In accordance with this study, Cl-DhA and diCl-DhA have been shown to be relatively recalcitrant in biological treatment systems (17) and to accumulate in aquatic sediments (11, 12). The extent of Cl-DhA biodegradation remains to be determined in biological treatment systems where low Cl-DhA concentrations, alternative removal mechanisms, and complex chemical and biological factors make such determinations difficult. If the organisms examined in this study are representative of chlorinated DhA-degrading organisms, one would expect biological treatment systems to most effectively degrade low concentrations of Cl-DhA, and one might doubt whether diCl-DhA can be effectively biodegraded. Peak levels have been estimated to be 0.6 ppm of Cl-DhA and 1.1 ppm of diCl-DhA in pulp and paper mill effluents (17). Such levels would rise if technology allowing mills to reuse process waters was developed. At the lowest concentration tested in this study, strain DhA-33 reduced 64 μM (21 ppm) Cl-DhA to below 1 μM (0.3 ppm). Cl-DhA removal rates for activated sludge and aerated lagoon biomass were estimated to range from 1.3 to 9.4 μg mg of biomass^{-1} day^{-1} (17), which is well below the maximum Cl-DhA removal rate (for both isomers simultaneously) observed in this study, i.e., 157 μg (0.47 μmol) mg of protein^{-1} h^{-1}.

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REFERENCES

1. **Bicho, P., V. Martin, and J. N. Saddler.** 1994. Enrichment and characterization of resin acid degrading bacteria from bleached kraft effluent, p. 379–383. *In* 1994 International Environmental Conference TAPPI Proceedings. TAPPI Press, Atlanta, Ga.
2. **Bicho, P. A., V. Martin, and J. N. Saddler.** 1995. Growth, induction, and substrate specificity of dehydroabietic acid-degrading bacteria isolated from a kraft mill effluent enrichment. Appl. Environ. Microbiol. **61:**3245–3250.
3. **Biellmann, J. F., G. Branlant, M. Gero-Robert, and M. Poiret.** 1973. Dégradation bactérienne de l'acide déhydroabiétique par *Flavobacterium resinovorum*. Tetrahedron **29:**1227–1236.
4. **Biellmann, J. F., G. Branlant, M. Gero-Robert, and M. Poiret.** 1973. Dégradation bactérienne de l'acide déhydroabiétique par un *Pseudomonas* et une *Alcaligenes*. Tetrahedron **29:**1237–1241.
5. **Biellmann, J. F., R. Wennig, P. Daste, and M. Raynaud.** 1968. Microbial degradation of diterpenic acids. Chem. Commun. **1968:**168–169.
6. **Easty, D. B., L. G. Borchardt, and B. A. Wabers.** 1978. Wood-derived toxic compounds removal from mill effluents by waste treatment processes. TAPPI J. **61:**57–59.
7. **Furman, G. S., and D. B. Easty.** 1983. Chlorination of dehydroabietic acid under pulp bleaching conditions. TAPPI J. **67:**89.
8. **Kutney, J. P., E. Dimitriadis, G. M. Hewitt, P. J. Salisbury, M. Singh, J. A. Servizi, D. W. Martens, and R. W. Gordon.** 1982. Studies related to biological detoxification of kraft pulp mill effluent. IV. The biodegradation of 14-chlorodehydroabietic acid with *Mortierella isabellina*. Helv. Chim. Acta **65:**1343–1350.
9. **Kutney, J. P., E. Dimitriadis, G. M. Hewitt, P. J. Salisbury, M. Singh, J. A. Servizi, D. W. Martens, and R. W. Gordon.** 1983. Studies related to biological detoxification of kraft pulp mill effluent. VI. The biodegradation of 12,14-chlorodehydroabietic acid with *Mortierella isabellina*. Helv. Chim. Acta **66:**921–928.
10. **Kutney, J. P., G. M. Hewitt, P. J. Salisbury, M. Singh, J. A. Servizi, D. W. Martens, and R. W. Gordon.** 1983. Studies related to biological detoxification of kraft pulp mill effluent. VII. The biotransformation of 12-chlorodehydroabietic acid with *Mortierella isabellina*. Helv. Chim. Acta **66:**2191–2197.
11. **Lee, H.-B., and T. E. Peart.** 1991. Determination of resin and fatty acids in sediments near pulp mill locations. J. Chromatogr. **547:**315–323.
12. **Lee, H.-B., and T. E. Peart.** 1992. Supercritical carbon dioxide extraction of resin and fatty acids from sediments at pulp mill sites. J. Chromatogr. **594:**309–315.
13. **Martin, V., J. N. Saddler, and W. W. Mohn.** 1996. Mutational analysis of the dehydroabietic acid biodegradation pathway of *Pseudomonas* sp. BKME-9 isolated from bleached kraft mill effluent, abstr. K-109, p. 553. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
14. **Mohn, W. W.** Unpublished data.
15. **Mohn, W. W.** 1995. Bacteria obtained from a sequencing batch reactor that are capable of growth on dehydroabietic acid. Appl. Environ. Microbiol. **61:**2145–2150.
16. **Mohn, W. W., A. E. Wilson, P. Bicho, and E. Moore.** 1996. Phylogeny and physiology of resin acid-degrading bacteria, abstr. K-107, p. 553. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
17. **Mueller, J. C., J. M. Leach, and C. C. Walden.** 1977. Detoxification of bleached kraft mill effluents—a manageable problem. TAPPI J. **60:**136–137.
18. **Priha, M. H., and E. T. Talka.** 1986. Biological activity of bleached kraft mill effluent (BKME) fractions and process streams. Pulp Pap. Can. **87:**143–147.
19. **Servizi, J. A., D. W. Martens, R. W. Gordon, J. P. Kutney, M. Singh, E. Dimitriadis, G. M. Hewitt, P. J. Salisbury, and L. S. L. Choi.** 1986. Microbiological detoxification of resin acids. Water Pollut. Res. J. Can. **21:**119–129.
20. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150:**76–85.
21. **Stuthridge, T. R., K. Johnsen, H. Hoel, and J. Tana.** 1995. Bioaccumulation of resin acids in trout exposed to thermomechanical pulping effluents and their receiving waters, p. 533–538. *In* 49th Appita Annual General Confer-

ence Proceedings. Appita, Carlton, Australia.
22. **Sunito, L. R., W. Y. Shiu, and D. Mackay.** 1988. A review of the nature and properties of chemicals present in pulp mill effluents. Chemosphere **17:**1249–1290.
23. **Thakore, A. N., and A. C. Oehlschlager.** 1977. Structures of toxic constituents in kraft mill caustic extraction effluents from ^{13}C and ^{1}H nuclear magnetic resonance. Can. J. Chem. **55:**3298–3303.
24. **Walden, C. C., and T. E. Howard.** 1981. Toxicity of pulp and paper mill effluent—a review. Pulp Pap. Can. **82:**T143.
25. **Walsh, C.** 1979. Enzymatic reaction mechanisms. W. H. Freeman & Co., San Francisco, Calif.
26. **Wilson, A. E. J., E. R. B. Moore, and W. W. Mohn.** 1996. Isolation and characterization of isopimaric acid-degrading bacteria from a sequencing batch reactor. Appl. Environ. Microbiol. **62:**3146–3151.
27. **Zanella, E.** 1983. Effect of pH on acute toxicity of dehydroabietic acid and chlorinated dehydroabietic acid to fish and *Daphnia*. Bull. Environ. Contam. Toxicol. **30:**133–140.