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Isolation and Characterization of Isopimaric Acid-Degrading Bacteria from a Sequencing Batch Reactor

ANN E. J. WILSON,[1] EDWARD R. B. MOORE,[2] AND WILLIAM W. MOHN[1]*

Department of Microbiology and Immunology and Pulp and Paper Center, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada,[1] and Department of Microbiology, GBF-National Research Center for Biotechnology, Brunswick, Germany[2]

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We isolated two aerobic, gram-negative bacteria which grew on the diterpene resin acid isopimaric acid (IpA) as the sole carbon source and electron donor. The source of the isolates was a sequencing batch reactor treating a high-strength process stream from a paper mill. The isolates, IpA-1 and IpA-2, also grew on pimaric and dehydroabietic acids, and IpA-1 grew on abietic acid. Both strains used fatty acids, but neither strain used camphor, sitosterol, or betulin. Strain IpA-1 grew anaerobically with nitrate as an electron acceptor. Strains IpA-1 and IpA-2 had growth yields of 0.19 and 0.23 g of protein per g of IpA, respectively. During growth, both strains transformed IpA carbon to approximately equal amounts of biomass, carbon dioxide, and dissolved organic carbon. In both strains, growth on IpA induced an enzymatic system which caused cell suspensions to transform all four of the above resin acids. Cell suspensions of IpA-1 and IpA-2 removed IpA at rates of 0.56 and 0.13 μmol mg of protein^{-1} h^{-1}, respectively. Cultures and cell suspensions of both strains failed to completely consume pimaric acid and yielded small amounts of an apparent metabolite from this acid. Cultures and cell suspensions of both strains yielded large amounts of three apparent metabolites from dehydroabietic acid. Analysis of 16S rDNA sequences indicated that the isolates are distinct members of the genus *Pseudomonas* sensu stricto.

Resin acids, including isopimaric acid (IpA), are tricyclic diterpenes produced in many tree species, particularly conifers. All resin acids may be classified into two families, pimaranes and abietanes, based on the C-13 substituent (Fig. 1). In general, resin acids are a highly toxic component of pulp mill effluents (27, 29, 33). More specifically, IpA is the most toxic resin acid in mechanical and kraft pulp mill effluents (17, 18) and may be more recalcitrant than other resin acids in both treatment systems and natural waters (10). In addition, resin acids are a component of pitch (3), which interferes with the papermaking process. This interference is increasingly problematic as pulp and paper mills attempt to recirculate water to minimize wastewater discharge.

Although resin acids affect the productivity and environmental impact of pulp and paper mills, information about their biodegradation is presently very limited. Some bacteria can grow on abietanes, but none have been shown to grow on pimaranes. Over 20 years ago, six isolates, presumably members of the *Bacteria* domain, were reported to grow on abietic acid (AbA), dehydroabietic acid (DhA), or their derivatives (2, 5, 6, 19, 28). Recently, two bacterial isolates were reported to grow on the abietanes AbA and DhA but not to grow on the pimaranes pimaric acid (PiA) and IpA (21). Five presumably bacterial isolates were reported to grow on DhA (1). These five isolates removed from medium the abietanes AbA, DhA, 7-oxo-DhA, and 12-chloro-DhA but not the pimaranes PiA, IpA, and sandaracopimaric acid. *Bacillus psychrophilus* was reported to transform DhA without using it as a growth substrate (4).

Some fungi can transform resin acids including abietanes and pimaranes, but none have been shown to degrade resin acids. *Mortierella isabellina* can hydroxylate DhA, AbA, and IpA at several positions (14–16), forming products with reduced toxicity (30). *Ophiostoma piliferum* is sold as a commercial product for removing extractives, including resin acids, from wood prior to pulping (3). Four fungi, including *O. piliferum*, reduced the resin acid content of wood chips (34). The mechanism of removal was not determined and may have been nondegradative; for example, hydroxylation, polymerization, or covalent binding to the wood may have occurred. The pimaranes tested were reduced to a lesser extent than were most of the abietanes, and only two of the four fungi reduced IpA.

No organism capable of growth on pimaranes or of pimarane degradation has been reported. However, aerobic biological treatment systems can effectively remove resin acids, including pimaranes. One example is a sequencing batch reactor (SBR) used for this study, which efficiently removed several resin and fatty acids from a high-strength process stream from a paper mill (11). Although treatment systems can remove these contaminants, the systems are still prone to periodic failure. These failures may occur in part because system designers and operators do not know the basic parameters for optimal resin acid biodegradation. Understanding the physiology, ecology, and biochemistry of pimarane-degrading microorganisms would provide insight into the necessary procedures for optimization and stabilization of treatment systems. This is the first report of pimarane-degrading microorganisms. We characterize two isolates (strains IpA-1 and IpA-2), including their phylogeny, their specificities for resin acids and other compounds, their carbon mass balances during growth on IpA, and the inducibility of their IpA biodegradation.

MATERIALS AND METHODS

Cultures and media. The mineral medium for enrichment and subsequent experiments was previously described (21), except that the enrichment medium

* Corresponding author. Mailing address: Department of Microbiology and Immunology, University of British Columbia, 300—6174 University Blvd., Vancouver, B.C., Canada, V6T 1Z3. Phone: (604) 822-4285. Fax: (604) 822-6041. Electronic mail address: wmohn@unixg.ubc.ca.

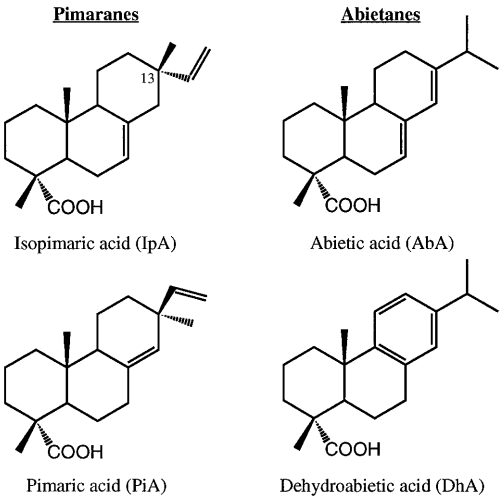

FIG. 1. Resin acids used in this study. For IpA, C-13 is indicated.

contained (per liter) 0.18 g of $MgCl_2$ and 0.022 g of $CaCl_2 \cdot H_2O$. A liquid enrichment culture contained 100 μM IpA as the sole organic substrate and was incubated stationary at 20 to 25°C. After three consecutive passages on liquid medium, the culture was streaked on solid medium containing 100 μM IpA to obtain isolated colonies. Solid media contained 1.5% Select Agar (BBL, Cockeysville, Md.). Stock cultures were maintained on tryptic soy broth (Difco, Detroit, Mich.) with 1.0 g of pyruvate per liter added. Resin acids (Helix Biotechnologies, Richmond, B.C. Canada) were of high purity, except PiA, which is significantly contaminated by another resin acid, sandaracopimaric acid. The purities reported (Table 1) are those certified by the manufacturer. Resin acids, benzoate, and phenol were dissolved in NaOH. Betulin, camphor, hexadecane, and sitosterol were dissolved in acetone. Resin acids and other poorly water-soluble substrates were present in the cultures as insoluble suspensions. Most substrates were added to autoclaved culture medium from filter-sterilized stock solutions in water or the above solvents. Arabinose, carboxymethyl cellulose, galactose, mannose, and xylan were added from autoclaved stock solutions. Linoleic acid, a liquid, was filter sterilized and added directly. Palmitic acid crystals were added directly without sterilization (growth never occurred in uninoculated control cultures). The use of substrates was tested in stationary (except as noted) 2.5-ml cultures incubated at 20 to 25°C. Anaerobic use of substrates was tested in 10-ml cultures incubated on a tube roller in 26-ml tubes having a headspace of N_2 and sealed with serum bottle stoppers. Anaerobic cultures had 0.90 g of glucose per liter and, when indicated, 1.70 g of sodium nitrate per liter. The presence or absence of growth on resin acids and other poorly water-soluble substrates (which interfere with the measurement of optical density) was evaluated by microscopy. Growth on other substrates was quantified by measuring the optical density at 610 nm. Oxidation of a battery of substrates was tested with GN microplates (Biolog, Hayward, Calif.).

Time courses. Growth curves and IpA removal curves were determined with individual 1-ml cultures for each protein or IpA sample. Entire cultures were used for the analysis because of the difficulty in obtaining representative subsamples of insoluble IpA. IpA was extracted (see below) from the cultures in the culture tubes, which had Teflon-lined screw-caps. Each culture received a 1% inoculum from a late-log-phase culture and was incubated on a tube roller at 30°C.

Carbon mass balance determination. The fate of carbon during growth on 0.060 g of IpA per liter was determined with 10-ml cultures incubated in 26-ml tubes sealed with serum bottle stoppers. The cultures were incubated to early stationary phase (IpA-1 for 14 h and IpA-2 for 24 h) on a tube roller at 30°C. One set of replicate cultures was used for CO_2 analysis. 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 analysis. Another set of replicate cultures was used for protein and dissolved organic carbon (DOC) analysis. 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 of carbon by multiplying by 1.82 (ratio of dry weight to amount of protein) and by 0.60 (ratio of amount of carbon to dry weight). The supernatants were acidified as above to remove CO_2 and assayed for DOC. Values for net carbon as CO_2, biomass, and DOC were determined as the values for cultures grown on IpA minus the corresponding values for inoculated control cultures with no IpA as substrate.

Cell suspensions. Cell suspensions were prepared as previously described (21). The cells were grown to late log phase on either 0.12 g of IpA per liter or 0.3 g of glucose per liter. The cells were washed and suspended in culture medium which did not contain trace elements, vitamins, or $FeSO_4$. Where indicated, 50 μg of chloramphenicol per ml was added. Cell suspensions had an optical density at 610 nm of 0.37 to 0.77, which corresponds to 82 to 480 μg of protein per ml. Killed cell suspensions were boiled for 10 min. Aliquots of 1 ml were incubated in screw-cap tubes on a tube roller at 30°C. Entire tubes were used for each resin acid sample.

Analytical methods. Protein was quantified by the bicinchoninic acid method (31). For the protein assay, the cells were lysed by being vortexed in 1% sodium dodecyl sulfate at room temperature or by being incubated at 95°C with 1 M NaOH. Resin acids, with 12,14-dichlorodehydroabietic acid as an internal standard, were quantified by extraction with ethyl acetate, derivitization with diazomethane, and analysis by gas chromatography as previously described (21). CO_2 was quantified by gas chromatography by directly injecting 100 μl of headspace sample on a Shimadzu GC-8A gas chromatograph equipped with a Haysep DB column (3.05-m by 3.2-mm [diameter] packed column) and a thermal conductivity detector. For the CO_2 analysis, the injector and detector temperatures were set at 140°C, the column temperature was set at 100°C, and the carrier gas was He at 30 ml/min. DOC was quantified with an automatic Shimadzu TOC-500 total organic carbon analyzer equipped with an autoinjector.

Determination and analysis of 16S rRNA gene sequences. Nucleic acids were isolated from late-exponential-phase cultures by the procedure of Giovannoni (8). Nearly complete 16S rRNA genes were amplified by PCR (23) with a forward primer hybridizing at positions 8 to 27 and a reverse primer hybridizing at the complement of positions 1541 to 1525 (*Escherichia coli* 16S rRNA gene sequence numbering). PCR was carried out with a GeneAmp 9600 (The Perkin-Elmer Corp., Norwalk, Conn.) and conditions as reported previously (13). PCR-derived DNA was purified with Centricon-100 microconcentrators (Amicon GmbH, Witten, Germany) and sequenced directly with an Applied Biosystems 373A DNA Sequencer as specified by the manufacturer (Perkin-Elmer, Applied Biosystems GmbH, Weiterstadt, Germany) for *Taq* cycle sequencing with fluorescent-dye-labeled dideoxynucleotides (13). Sequence data were aligned with reference rRNA (and rRNA gene) sequences (20, 32), using the evolutionarily conserved primary sequence and secondary structure as references (9, 35). Evolutionary distances (12) were calculated from complete sequence pair similarities by using only unambiguously determined nucleotide positions.

TABLE 1. Substrate use by IpA degraders

Substrate	Concn (g/liter)	Substrate use[a] by:	
		IpA-1	IpA-2
Wood sugars			
L-Arabinose	1.0	(G)	G
Carboxymethyl cellulose	1.0	NG	NG
Cellobiose		NO	NO
D-Galactose	1.0	NG	G
D-Glucose	1.0	G	G
Glucuronate		NO	O
D-Mannose	1.0	NG	G
D-Xylose	1.0	NG	(G)
Resin acids			
AbA (96% pure)[b]	0.06	(G)	NG
DhA (99% pure)[b]	0.06	(G)	(G)
IpA (99% pure)[b]	0.06	G	G
PiA (88% pure)[b]	0.06	G	G
Fatty acids			
Linoleic	1.0	G	G
Palmitic	1.0	G	G
Other substrates			
Acetate	1.0	G	G
Benzoate[b]	0.24	G	G
Betulin[b]	0.05	NG	NG
Camphor[b]	0.05	NG	NG
Ethanol	0.20	G	G
Hexadecane[b]	0.50	G	NG
Glycerol	1.0	G	G
Methanol	0.10	NG	NG
Phenol[b]	0.01	NG	G
Pyruvate	1.0	(G)	G
Sitosterol[b]	0.05	NG	NG

[a] G, supports growth as sole organic substrate; (G), supports slow growth; NG, does not support growth; O, oxidized in Biolog assay; NO, not oxidized.
[b] Cultures were incubated on a tube roller.

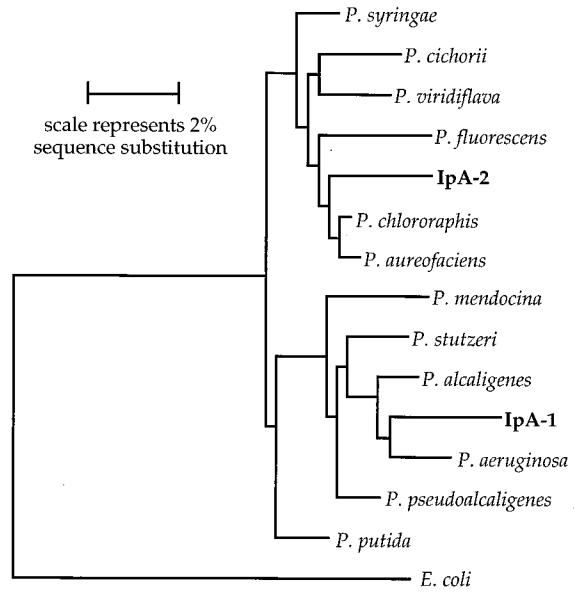

FIG. 2. Unrooted dendrogram showing the estimated phylogenetic relationships of strains IpA-1 and IpA-2 with 12 *Pseudomonas* species. The dendrogram was generated from comparisons of nearly complete 16S rRNA gene sequences by using a weighted, least-squares distance method (24).

Nucleotide sequence accession numbers. The 16S rRNA gene (rDNA) sequences determined for IpA-1 and IpA-2 have been deposited in the EMBL Nucleotide Database under accession numbers X96787 and X96788, respectively.

RESULTS AND DISCUSSION

Isolation and characterization. By using IpA as the sole organic carbon source and electron donor, six strains of gram-negative aerobic bacteria were isolated. The source of the isolates was a laboratory-scale SBR treating pulp mill wastewater (11) from which DhA-degrading bacteria were previously isolated (21). By using the criteria of cell morphology, colony morphology, substrate range, and temperature range, two different strain types emerged. These types are represented by strains IpA-1 (five isolates) and IpA-2 (one isolate). These two isolates are short, motile, rods. When grown on IpA, strain IpA-1 was 1.5 to 2.0 μm by 1.0 μm and strain IpA-2 was 2.0 to 3.0 μm by 1.5 μm. Both isolates formed clumps of cells during the early log phase of growth on IpA. When grown on IpA, colonies of IpA-1 were clear, translucent, smooth, asymmetrical, and flat. Colonies of IpA-2 were pale yellow, opaque, smooth, circular, and convex. Both isolates were positive for oxidase and catalase.

The substrate and temperature ranges were similar for IpA-1 and IpA-2. Both strains grew on most resin and fatty acids tested, which represent the major resin and fatty acids in the SBR influent (Table 1). IpA-2 grew on a slightly wider variety of other substrates, particularly wood sugars, which were probably present in the SBR influent. Neither strain grew on camphor (a monoterpene), sitosterol (a steroid), or betulin (a triterpene), which all commonly occur in wood. IpA-1 grew at 21, 30, and 37°C but not 40°C, whereas IpA-2 grew at 21 and 30°C but not 37°C. Both strains grew aerobically on glucose, but neither grew anaerobically (fermentatively) on glucose. IpA-1 grew anaerobically on glucose plus nitrate, but IpA-2 did not. Strains IpA-1 and IpA-2 share with many other resin acid degraders the ability to use fatty acids and the inability to use many sugars, aromatic compounds, and terpenes other than resin acids (21, 22). Strains IpA-1 and IpA-2 use a broader range of resin acids than the previously reported resin acid-degrading isolates and are the first microorganisms reported to grow on pimaranes.

Phylogeny. Cluster analysis of the 16S rDNA sequences of IpA-1 and IpA-2 (Fig. 2) indicated that IpA-1 and IpA-2 clearly group with species of *Pseudomonas* sensu stricto. This genus belongs to the gamma subclass of the *Proteobacteria* (36) and to rRNA group I (7, 26). IpA-1 and IpA-2 had 90.5 to 97.6% sequence similarity to 27 reference species of *Pseudomonas*. IpA-1 had the greatest sequence similarity (97.0%) to *Pseudomonas alcaligenes*, and IpA-2 had the greatest sequence similarity (97.6%) to *Pseudomonas chlororaphis*. These sequence similarities are lower than those typically found between members of the same species. The two isolates were not more closely related to one another than they were to reference species of *Pseudomonas*. The above physiological characteristics of both isolates are consistent with those of the genus *Pseudomonas* (25). The phylogenetic analysis shows that IpA-1 and IpA-2 probably each represent a new species within *Pseudomonas*. It would be of interest to know how these isolates are phylogenetically related to other resin acid degraders and how widely distributed among groups of microorganisms is the ability to use resin acids.

Growth on resin acids. Both IpA-1 and IpA-2 grew on IpA as a sole organic substrate (Fig. 3). Both strains grew faster and to higher density on pimaranes than on abietanes (Table 1). The strains differed in the use of AbA, which was used by only IpA-1. Growth on each resin acid was confirmed by at least three serial passages on mineral medium with a particular resin acid as the sole organic substrate. The resin acids were completely removed in these cultures, with the exception of PiA, which was not completely degraded by cultures after long (14-day) incubations. Time course analyses showed that both strains have similar growth and IpA removal rates (Fig. 3). The growth of IpA-1 and IpA-2 on IpA was not exponential and occurred at maximum rates of 3 and 4 μg of protein ml^{-1} h^{-1}, respectively. The growth rates may have been limited by the insolubility of IpA. Strain IpA-1 had a consistently shorter lag period after inoculation than did IpA-2.

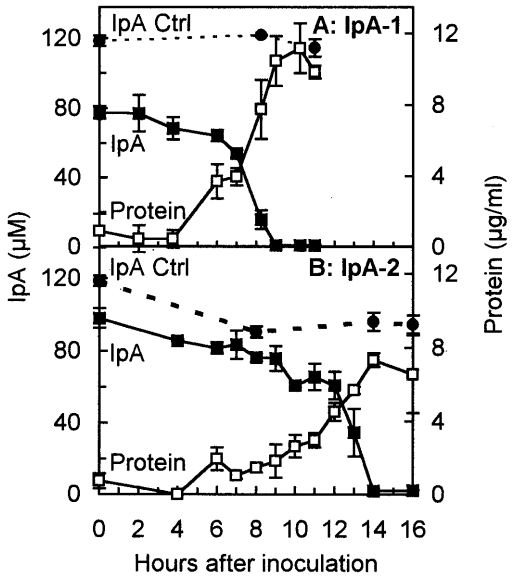

FIG. 3. Growth on and removal of IpA by strains IpA-1 (A) and IpA-2 (B); Ctrl, uninoculated cultures; bars indicate standard deviation (n = 3).

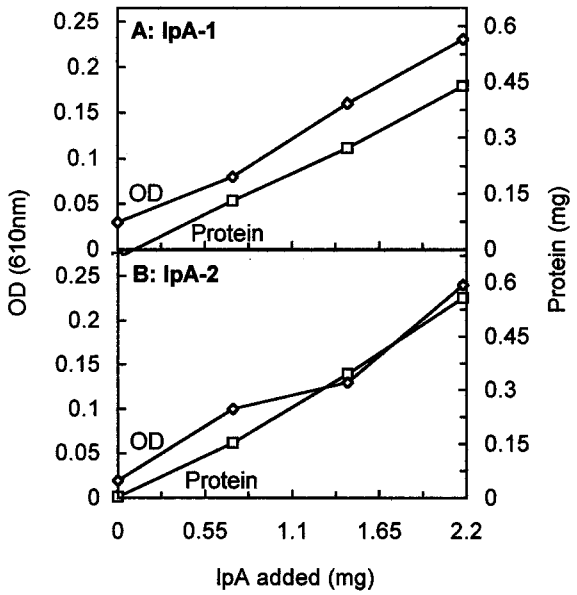

FIG. 4. Growth yields of IpA-1 (A) and IpA-2 (B) on IpA (n = 2). Cultures of 12 ml were incubated to early stationary phase at 30°C on a tube roller. OD, optical density.

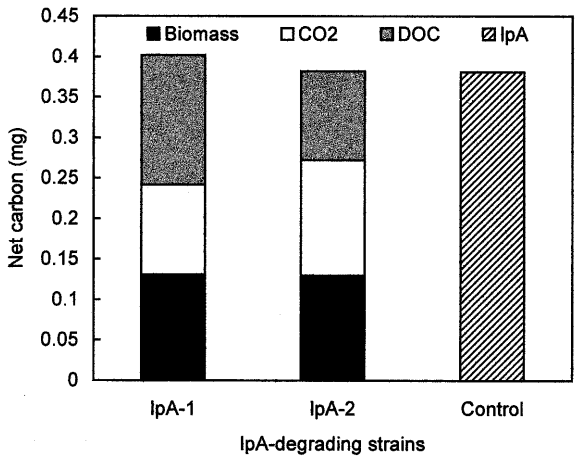

FIG. 5. Carbon mass balance for IpA-1 and IpA-2 grown on IpA in 10-ml cultures (n = 3). Control, uninoculated medium.

The two strains reported here were isolated from the same SBR as were the previously described abietane degraders (21). The differing specificities for use of resin acids by the two groups are complementary, with one group using only abietanes and the other group more efficiently using pimaranes. These specificities suggest a need for complex communities to accomplish resin acid removal in treatment systems. Changes of species and sources of trees used for pulping result in changes of resin acid composition in process waters. Such changes probably cause shifts in populations of resin acid degraders in treatment systems for those waters. These shifts may be accompanied by a temporary failure of a system to remove particular resin acids. It may be possible to manipulate microbial populations to avoid such failures. It would be useful to monitor populations of resin acid degraders in treatment systems experiencing normal physicochemical fluctuations.

Cultures of both IpA-1 and IpA-2 showed a linear relationship between cell yield (as protein or optical density) and IpA provided (Fig. 4). This indicates that IpA was the limiting growth factor in the cultures and that it was not toxic at the highest concentration used (0.18 g/liter). The growth yields of strains IpA-1 and IpA-2 were 0.19 and 0.23 g of protein per g of IpA, respectively. Assuming that the cells are 55% protein, these growth yields correspond to 0.35 and 0.42 g of cell dry weight per g of IpA.

Because the growth yields of IpA-1 and IpA-2 on IpA were lower than expected, a carbon mass balance was determined. When grown on IpA, both strains converted approximately one-third of the carbon to biomass, one-third to CO_2 and one-third to DOC (Fig. 5). The DOC did not include IpA or other compounds detectable by the gas chromatographic analysis for IpA. These data do not conclusively indicate complete mineralization of IpA. Two possible explanations for the unidentified DOC follow. First, IpA might be incompletely mineralized and the DOC might contain the degradation product(s). Second, IpA might be completely mineralized and the DOC might contain material synthesized by the isolates, such as exopolymer, biosurfactant, or autolyzed cells.

Cell suspensions. Both strains have inducible, heat-labile IpA removal activity. Cell suspensions of IpA-1 and IpA-2 completely removed IpA in 4 h (Fig. 6). The initial IpA removal rates for both strains were linear. These removal rates, in the presence of chloramphenicol, were 0.56 and 0.13 μmol mg of protein^{-1} h^{-1} for IpA-1 and IpA-2, respectively. Resin acid removal was not induced in glucose-grown cells (Table 2). In the absence of the protein synthesis inhibitor chloramphenicol, IpA induced its own removal by glucose-grown IpA-1 cells, but the other resin acids did not induce their own removal. In the absence of chloramphenicol, all four resin acids induced their own removal by glucose-grown IpA-2 cells. With more time, the three resin acids other than IpA presumably could induce their own degradation by IpA-1, because this isolate does grow on any of the four resin acids as a sole organic substrate (Table 1). Both IpA-1 and IpA-2 may be particularly adapted to the use of IpA, because induction of

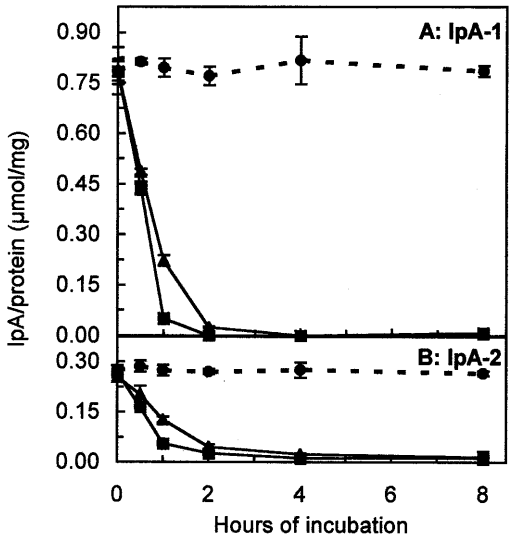

FIG. 6. Removal of IpA by cell suspensions of IpA-1 (A) and IpA-2 (B). Symbols: ■, without chloramphenicol; ▲, with chloramphenicol; ●, boiled controls. Bars indicate standard deviation (n = 3). The cells used were grown on IpA.

TABLE 2. Removal of four resin acids by cell suspensions of strains IpA-1 and IpA-2[a]

Strain	Growth substrate	Treatment	% removal of resin acid (mean ± SD, $n = 3$)			
			IpA	PiA	DhA	AbA
IpA-1	IpA	+ Chloramphenicol	100 ± 0.0	87 ± 2.7	100 ± 0.0	100 ± 0.0
		− Chloramphenicol	100 ± 0.0	91 ± 1.4	100 ± 0.0	100 ± 0.0
		Killed control	15 ± 12.1	18 ± 6.4	−9 ± 10.3	8 ± 6.7
	Glucose	+ Chloramphenicol	−1 ± 8.2	−26 ± 15.6	−3 ± 8.1	2 ± 1.4
		− Chloramphenicol	100 ± 0.0	8 ± 29.4	6 ± 5.3	13 ± 2.6
		Killed control	12 ± 13.3	9 ± 19.3	−48 ± 15.2	15 ± 6.1
IpA-2	IpA	+ Chloramphenicol	100 ± 0.0	77 ± 3.4	100 ± 0.0	100 ± 0.0
		− Chloramphenicol	100 ± 0.0	93 ± 2.0	100 ± 0.0	100 ± 0.0
		Killed control	−21 ± 47.2	14 ± 24.6	−5 ± 5.0	1 ± 3.1
	Glucose	+ Chloramphenicol	11 ± 8.1	7 ± 1.9	−1 ± 0.9	−5 ± 42.9
		− Chloramphenicol	100 ± 0.0	74 ± 2.4	80 ± 17.4	80 ± 0.5
		Killed control	2 ± 5.9	7 ± 10.4	13 ± 10.9	0 ± 7.0

[a] Suspensions of cells grown on IpA were incubated for 8 h, and suspensions of cells grown on glucose were incubated for 10 h. The initial resin acid concentration was 200 μM.

IpA removal appeared to be faster than induction of the removal of the other resin acids.

The above results are consistent with substrate-induced resin acid-degrading enzyme systems in IpA-1 and IpA-2. The abietane degraders previously reported (1, 21) also have inducible enzyme systems responsible for resin acid removal. It appears that IpA-1 and IpA-2 each have a single enzyme system capable of removal of all resin acids tested, since IpA induced the removal of all these resin acids (Table 2). Similarly, DhA induced removal of both DhA and AbA by two abietane degraders (21). This does not rule out the existence of other resin acid-transforming systems induced by other substrates. The rates of IpA removal found in this study are more than 10 times lower than the rates of DhA removal previously determined in studies of similar cell suspensions (21). Interestingly, the induced enzyme system of IpA-2 removed AbA even though IpA-2 will not grow on AbA. It appears that IpA-2 can cometabolize AbA in the presence of another resin acid substrate.

Cell suspensions and cultures of IpA-1 and IpA-2 yielded apparent metabolites from DhA, including two transiently produced compounds and relatively large amounts of three compounds which were consistently produced and were stable. Thus, DhA does not appear to be completely mineralized by the two strains. The DhA products may result from diversion of the metabolic pathway which supports growth. Impurities in the DhA (99% pure) are insufficient to account for the observed growth on DhA. Cell suspensions and cultures yielded a relatively small amount of one apparent PiA metabolite, which was consistently produced and was stable. Like the cultures (see above), cell suspensions of IpA-1 and IpA-2 failed to completely remove PiA. No IpA or AbA metabolites were detected. The patterns of apparent DhA and PiA metabolites were identical for both IpA-1 and IpA-2, suggesting that the two strains have very similar enzyme systems catalyzing resin acid transformations. Characterization of the biochemical pathway(s) of resin acid degradation would help us to better understand the specificities for resin acids and to determine whether IpA is completely mineralized.

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