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Degradation of Pinene by *Bacillus pallidus* BR425

Natarajan Savithiry[1], Douglas Gage[2], Weijie Fu[1] & Patrick Oriel[1,*]
[1] *Departments of Microbiology and* [2] *Biochemistry Michigan State University East Lansing, MI 48824, USA (* author for correspondence)*

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Abstract

An aerobic thermophile has been isolated from an α-pinene enrichment culture. The isolate, which was designated BR425, has been tentatively identified as *Bacillus pallidus* using 16S ribosomal RNA gene sequencing and organism morphology. Monophasic and biphasic incubations of BR425 cells with α-pinene, β-pinene, and limonene yielded a number of oxidized monoterpene metabolites with carveol as a common metabolite. A pinene degradation pathway with carveol and carvone as central metabolic intermediates is suggested.

Introduction

Pinenes, the major constituents of turpentine, are bicyclic monoterpenes which are produced in significant quantities by plants of the Pinaceae family. Because of their volatility, pinene emissions from conifer forests and during pulping operations constitute a major source of biogenic hydrocarbons (Lindskog & Potter 1995; Stromvall & Petersson 1993). The metabolism of pinenes by microorganisms has been little studied, as they have limited water solubility, and are membrane-destructive to procaryotic and eucaryotic microorganisms (Andrews et al.1980). In an early report in which a number of pinene metabolites were identified, catabolism of α-pinene by *Pseudomonas* strain PL was suggested to proceed by isomerization of the pinene to limonene with subsequent oxidation to perillic acid prior to ring cleavage and further catabolism utilizing a β-oxidation pathway (Shukla & Bhattacharyya 1968). An alternative pathway through limonene proposed by Gibbons & Pirt (1971) has been questioned (Trudgill 1990). A third pathway and some of the participating enzymes for *Nocardia* strain P18.3 (Griffiths et al. 1987) and *Pseudomonas fluorescens* NCIMB 11671 (Best et al. 1987) have been described, in which α-pinene is directly oxidized to pinene epoxide prior to ring cleavage. Recent evidence indicates the presence of an alternative pinene pathway in the pinene-epoxidizing *Pseudomonas fluorescens* strain NCIMB 11671, the metabolites for which have not yet been identified (Colcousi et al. 1996). Few of the enzymes participating in pinene catabolism or their encoding genes have so far been characterized.

Because of their important environmental roles including composting and waste treatment at elevated temperature, our laboratory has been engaged in exploring the metabolic diversity of aerobic thermophilic bacteria. In this report, we provide initial information on the degradation of α- and β- pinene by a newly isolated *Bacillus* thermophile, which has been tentatively identified as a strain of *B. pallidus*.

Materials and methods

Reagents and media

The (R)- enantiomer of α-pinene was selected for microbial degradation studies as this isomer predominates in North American pines. All monoterpenes were purchased from Aldrich Company (Milwaukee, Wisconsin), examined for purity using GC/MS (see below), and filter sterilized prior to use. M9 minimal salts medium (Maniatis et al. 1982) contained (per liter) Na_2 HPO_4, 6g; KH_2PO_4, 3g; NaCl, 0.5g; NH_4Cl 1g; pH 7.4. After autoclaving and cooling, 2

ml of 1M mgSO4 were added. LB medium has been described (Maniatis et al. 1982).

Organism isolation and growth

Pinene-degrading thermophiles were isolated from samples of dried wound exudate from a stand of white pine trees near Midland, Michigan. Enrichments were carried out in a 125 ml bottle containing 50 ml M9 minimal salts and 0.05 ml α-pinene, and were incubated at 60 °C in a gyrorotatory water bath-shaker. After 72 h incubation, samples of the enrichment culture were diluted and grown at 60 °C. on M9 salt plates in Petri dishes containing α-pinene in small glass tubes attached to the cover. Isolates were repeatedly transferred on these plates and retained as putative pinene utilizers. One isolate, designated BR425, exhibited good growth on α-pinene over a period of a few days, and was chosen for further study. For single liquid phase examination of growth and production of pinene metabolites, isolate BR425 was incubated in 100 ml serum bottles containing 40 ml M9 salts and pinene at the desired concentration and sealed with Teflon-coated stoppers and aluminum caps. Bottles were incubated in a gyrorotatory water bath shaker. Colony-forming units/ml (CFU) were enumerated by plating on LB agar at 60 °C.

Extraction and analysis of pinene biotransformation products

For analysis of pinene biotransformation products, liquid cultures of BR425 grown for the desired time were centrifuged at 12,000xg for 20 min. at 4 degrees C., acidified to pH 2.0 with HCl and extracted three times with equal volumes of ether. The ether fraction was evaporated to 25 ml and separated into neutral and acidic fractions by extraction (3X 0.6 volumes) with 5% NaOH. The ether fraction was concentrated with nitrogen, neutralized with 5% HCl, and analyzed by GC/MS. The NaOH fraction was acidified to pH 2.0 and reextracted (3x0.3volumes) with ether. Following concentration, the ether fraction was analyzed using a Hewlett-Packard HP5890 gas chromatograph with a MSD HP 5970 detector and a fused silica capillary column (0.25 mm I.D. X 30M DB-wax). Conditions used were: helium carrier gas, injection port and detector port at 240 °C., column temperature from 40–240 °C. at 7°/min with 2 min initial hold time.

Two-phase biotranformation studies

For two-phase biotranformation studies by suspensions of BR425 cells, 50 ml of BR425 cells grown in LB medium were washed with M9 medium and resuspended to 10^9 cells per ml in 0.05 M K_2HPO_4 buffer, pH 7.0 with 0.6 ml of pinene or limonene added. Following shaking in a gyrorotatory water bath at 50 °C., samples of the monoterpene phase were injected into the GC/MS for direct analysis.

16S Ribosomal RNA sequence analysis

Partial analysis of the BR425 16S ribosomal RNA gene sequence was obtained using chromosomal DNA prepared as described by Maniatis et al. 1982. PCR amplification and sequencing of the 16S ribosomal gene was carried out as described by Maltseva et al. 1996, using the Michigan State University Automated Sequencing Facility. Sequences were analyzed using the ribosomal database accessed using the GCG program (Genetics Computer Group, Madison, WI).

Assay of pinene oxide lyase

For assay of pinene oxide lyase, cells grown in LB broth into late exponential phase were disrupted using brief bursts of sonication at 4 °C. followed by removal of cell debris by centrifugation for 30 min. at 12,000xg. Enzyme assay utilized the spectrophotometric procedure of Griffiths et al.
1987.

Results

Characterization of isolate BR425

Isolate BR425 was plated from an α-pinene enrichment culture of white pine sap exudate and demonstrated good growth on M9 plates with α-pinene vapor, producing small creamy colonies in two or three days. Although the isolate exhibited a growth optimum at 55 °C. (data not shown) a lower growth temperature of 50 °C. was utilized for convenience. Microscopic examination indicated large rod-shaped bacteria with terminal endospores. Analysis of the 16S ribosomal gene sequence data approximately 350 nucleotides from each end yielded *Bacillus pallidus* (emb/z26930/BP16SRNA) as the best sequence match, with the only sequence differences being G for A at position 6 and T for C at position 573.

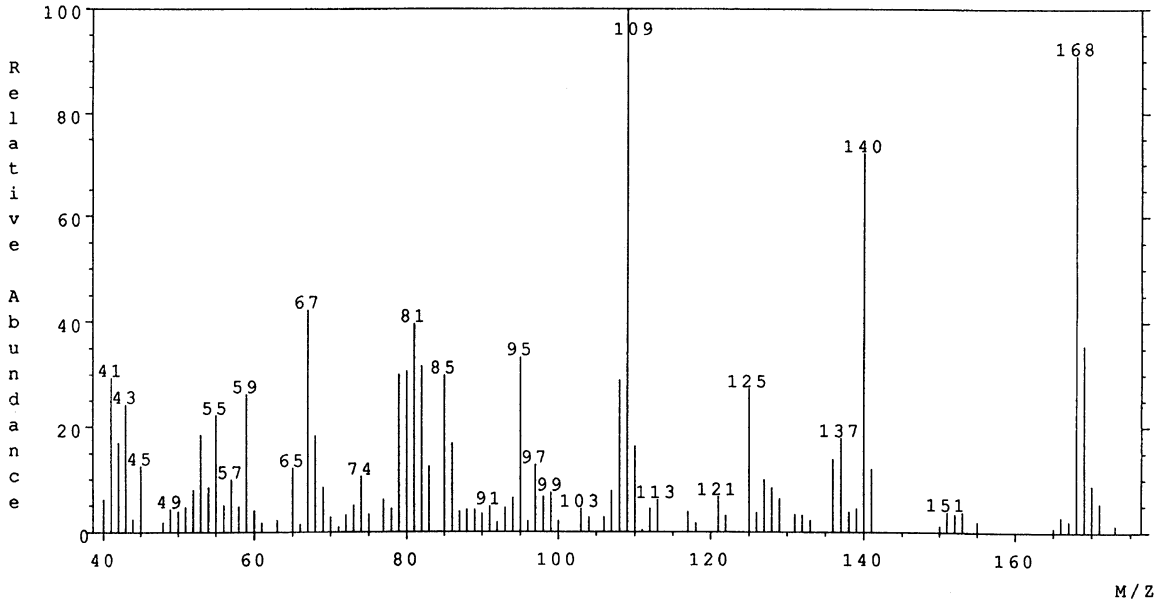

Figure 1. Fragmentation pattern of acid metabolite from BR425 growth on α-pinene or carvone.

Table 1. Metabolites formed during incubation of BR425 cells with 15mM α-pinene

Metabolites (μM/l)	0h	24h	48h	72h
β-pinene	0	0.08	0.22	0.15
limonene	0	0.59	1.32	0.44
pinocarveol	0	6.7	5.4	3.4
pinocarvone	0	5.8	6.3	5.5
myrtenol	0	3.8	3.4	2.1
myrtenal	0	3.1	3.1	2.9
carveol	0	29	42	9.7
carvone	0	8.5	18	5.4

Growth of BR425 on monoterpenes

Although growth of BR425 on α-pinene or limonene vapor on plates was readily observed, growth in liquid culture containing M9 salts and dissolved pinene or other monoterpenes was much more limited, although reproducible. Between three to fivefold increases in CFU were observed for α-pinene, β-pinene, limonene, and carveol over a 48 hour period using 5-15mM dissolved monoterpenes, whereas either no CFU increases or CFU decreases were observed for myrtenol, myrtenal, α-terpineol, and pinene epoxide (data not shown).

Metabolites produced during single-phase incubation with α-pinene

Ether extracts of BR425 culture supernatants incubated with 15mM α-pinene at a density of approximately 10^6 CFU/ml were examined at various times with the results shown in Table 1. Significant amounts of the neutral metabolites pinocarveol, pinocarvone, carveol and carvone were observed together with lesser amounts of myrtenol, myrtenal, limonene, and β-pinene in two independent experiments. None of these metabolites were observed in the absence of the thermophile. In addition to these neutral metabolites, an acid metabolite of molecular mass 168 was observed showing the fragmentation pattern shown in Figure 1. This acid metabolite was also observed during incubation with carvone in similar experiments, and was not identified in a library search. This fragmentation pattern is similar but not identical with acid metabolites of the same mass number previously reported during growth of *Pseudomonas* strains on pinene (Griffiths et al. 1987; Tudroszen et al. 1977).

Metabolites produced during two-phase incubations with α-pinene and limonene

In previous work with a limonene-degrading *E. coli* recombinant (Savithiry et al. 1997), incubations with neat limonene provided a convenient method for identification of neutral terpenoid metabolites, as the

Table 2. Metabolites formed during two-phase incubation of BR425 cells with monoterpenes

Metabolites (μM/l)	Organic phase		
	α-pinene	β-pinene	limonene
α-pinene	–	ND	ND
β-pinene	32	–	ND
limonene	10	8.1	–
pinocarveol	135	127	ND
pinocarvone	48	45	ND
myrtenol	154	113	ND
myrtenal	130	83	ND
α-terpineol	ND	ND	9.2
carveol	94	63	67
carvone	trace	trace	50

Average of two experiments in 24h incubation; ND: not detected

organic phase insures saturation of substrate in the aqueous phase, while facilitating removal and concentration of metabolites in the organic phase which can be utilized for direct identification using GC/MS eliminating the need for aqueous extraction. Experiments using incubations using BR425 cell suspensions with α-pinene, β-pinene, and limonene are shown in Table 2. For incubations with α-pinene and β-pinene, similar metabolites were identified to those obtained by ether extraction during single-phase incubation using dissolved α-pinene, except that metabolites were found in higher concentrations, and carvone was only observed in trace quantities. With limonene as substrate, carveol and carvone were found in significant quantitities, together with a small amount of α-terpineol.

Discussion

On the basis of morphology, growth characteristics, and partial 16S ribosomal RNA gene sequence, we have tentatively identified thermophilic isolate BR425 as a new strain of *Bacillus pallidus*, with confirmation awaiting more extensive examinations. *B. pallidus* was first described by Scholz et al. (1987) as a dominant member of yeast factory sewage and in municipal sludge, and a recent isolate has been found to degrade both aromatic and aliphatic nitriles (Cramp et al. 1997). While isolate BR425 could grow readily on plates supplied with pinene or limonene vapor, growth in liquid culture containing dissolved monoterpenes was limited. Similar problems with growth of

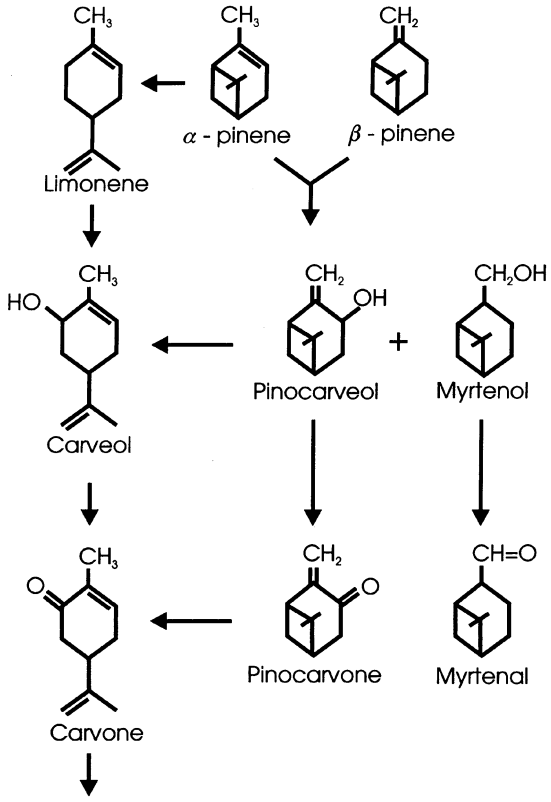

Figure 2. Possible biochemical conversion steps in the pinene catabolic pathway of *B. pallidus* BR425.

monoterpene-degrading isolates in anaerobic liquid culture have been reported (Harder & Probian 1995), and may result from monoterpene accumulation in and damage to cytoplasmic membranes (for review, see Sikkema et al. 1995).

In previous studies of monoterpene degradation, multiple oxidized metabolites have been observed, which were explained by the existence of multiple monoterpene pathways in the isolate (Shukla & Bhattacharyya 1968; Dhavlikar et al. 1966). For *B. pallidus* BR425, both the α-pinene C-10 oxidation product myrtenol and the C-3 oxidation product pinocarveol together with their further oxidation products myrtenal and pinocarvone were observed, suggesting either a monooxygenase capable of oxidation of either of these allylic carbons, or the existence of more than one pinene monooxygenase in the isolate. While information is not yet available to confirm one of these alternatives, we have recently discovered and cloned the gene for a novel limonene monooxygenase in a limonene-degrading *Bacillus stearothermophilus* strain BR388 which utilizes combined NADH and FAD to oxidize

limonene at both allylic carbons to produce a mixture of carveol and perillyl alcohol (Cheong TK and Oriel P, submitted). It will be of interest to determine whether BR425 possesses a similar monooxygenase capable of oxidation of bicyclic α-pinene at both allylic positions.

Thermophile isolate BR425 resembles the previously-described *Pseudomonas* PL in ability to degrade limonene as well as pinene, but differs in production of carveol and carvone from limonene rather than perillyl alcohol and perillyl aldehyde (Shukla & Bhattacharyya 1968). Inability to observe pinene epoxide as a metabolite or pinene epoxide lyase activity suggests that a pinene epoxidation pathway is not utilized. Based on the production of carveol and carvone from incubations with α-pinene, β-pinene, and limonene as well as production of the same acid metabolite during incubations with carvone and α-pinene, we suggest that carveol and carvone are central growth intermediates in BR425 pinene metabolism. Carveol and carvone could be produced from α- or β-pinene by either isomerization to limonene followed by oxidation to carveol and further oxidation to carvone, or by oxidation to pinocarveol or pinocarvone followed by isomerization to carveol or carvone, respectively. Since all these intermediates were observed, utilization of both routes shown in Figure 2 catalyzed by a limited number of isomerase, monooxygenase, and dehydrogenase enzymes with broad substrate range appears possible. Experiments are currently underway to test this possibility by characterization of the participating enzymes and encoding genes for these steps.

Acknowledgments

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