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ORIGINAL PAPER

Dehydration of the off-flavor chemical 2-methylisoborneol by the R-limonene-degrading bacteria *Pseudomonas* sp. strain 19-rlim and *Sphingomonas* sp. strain BIR2-rlima

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Abstract The terpene 2-methylisoborneol (MIB), a major cause of off-flavor in farm-raised catfish and drinking water, is transformed by various different terpene-degrading bacteria. Two of these, the R-limonene-degrading strains *Pseudomonas* sp. 19-rlim and *Sphingomonas* sp. BIR2-rlima, dehydrated MIB with the formation of odorless metabolites 2-methylenebornane and 4-methylcamphene. These metabolites which have a structural resemblance to camphor, could be further transformed by camphor-degrading bacteria to more oxidized products. The bacterial dehydrations demonstrated here may have application in removing MIB where it is a problem.

Keywords 2-Methylisoborneol · Dehydration · R-limonene · *Pseudomonas* · *Sphingomonas* · Biotransformation

Introduction

2-Methylisoborneol (MIB), a product of actinomycetes and cyanobacteria, is occasionally responsible for a camphoraceous earthy-musty off-flavor and smell in farm-raised catfish or drinking water. This off-flavor reduces the marketability of catfish, resulting in losses to farmers of millions of dollars annually (Engle et al. 1995). Since it is difficult to control the growth of MIB-producing cyanobacteria in catfish ponds, a practical means to alleviate the off-flavor problem might be to introduce MIB-degrading bacteria into those ponds where they could remove the off-flavor chemical as it is produced. To this end, bacteria able to grow with various terpenes and coincidentally to transform MIB have been isolated and characterized.

In a previous report (Eaton and Sandusky 2009) several camphor-degrading bacteria represented by *Pseudomonas putida* G1, *Rhodococcus ruber* T1 (NCIMB 9784), and *Rhodococcus wratislaviensis* DLC-cam demonstrated three distinct patterns of oxidation of MIB. Together, they were able to hydroxylate MIB at all three secondary carbons. In this study, a separate group of terpene-degrading bacteria that catalyze the dehydration of MIB were isolated and identified. This biotransformation by two representative strains will be demonstrated along with some further transformations of the dehydration products.

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Materials and methods

Chemicals and media

MIB was obtained from Dalton Pharma Services, Toronto, ON, Canada; R-(+)-limonene and

D-camphor were from Aldrich Chemical Company, Milwaukee, WI. All chemicals were of the highest purity available. The minimal medium was R medium (Eaton 2001), solidified when needed with 1.6% Nobel Agar (Difco, Detroit, MI).

Acid dehydration of MIB was carried out by dissolving 10 mg MIB in 1 ml methylene chloride. HCl (40 µl of 5 N) was added and the mixture was incubated at room temperature for several hours. Progress of the reaction was monitored by GC-MS analysis of samples diluted in methylene chloride. When the reaction appeared complete, 40 µl of 5 N NaOH was added. The solution was dried over sodium sulfate, methylene chloride was removed with nitrogen gas at 40°C and the products were re-dissolved in ethanol. The concentrations of dehydration products and residual MIB were not determined.

Bacterial strains

Bacteria were isolated from environmental sources by enrichment culture using R-limonene, as sole carbon and energy source. For taxonomy, 16S ribosomal RNA genes were amplified by PCR and sequenced as previously described (Eaton and Sandusky 2009). DNA sequence searches of the GenBank database were carried out using BLASTN (Altschul et al. 1990). Camphor-degrading strains *Pseudomonas putida* G1 (ATCC 17453), *Pseudomonas* sp. SWS3-camc, *Rhodococcus wratislaviensis* DLC-cam, and *Rhodococcus ruber* T1 (NCIMB 9784) were described previously (Eaton and Sandusky 2009).

Screening

For the initial screening of MIB biotransformations, bacteria were inoculated into 2 ml minimal medium supplemented with 0.1% succinate and 0.025% yeast extract to which about 1 mg solid or 1 µl liquid terpene was added as inducer in 20 ml glass scintillation vials with PTFE-lined caps. After overnight growth, about 0.5 mg MIB was added to each vial and the incubations continued for 3 days. The cultures were extracted with 2 ml methylene chloride which was dried over sodium sulfate and analyzed by GC-MS.

Time series transformations

Minimal medium (100 ml) containing 0.1% succinate and 0.025% yeast extract in as many 500 ml bottles as required was inoculated with a 10 ml overnight culture grown in the same medium. To each bottle was added either 25–50 µl R-limonene (neat), 67 µl ethanol containing 50 mg D-camphor, or no inducer and the cultures were incubated at 30°C for 16 h with shaking. The cultures were pooled, harvested by centrifugation at 6000 rpm for 15 min in a FiberLite F-14 rotor, washed with the same medium without inducer, and resuspended in a total of 25–33 ml of the same medium without inducer. Two milliliters of culture were added to each of 12–15 new vials to which either 2 µl MIB in ethanol or the dehydration products 2-methylenebornane + 4-methylcamphene in ethanol was subsequently added. Vials were incubated at 30°C with shaking at 250 rpm. At intervals, individual vials were removed and extracted with 2 ml methylene chloride which was subsequently dried over sodium sulfate. One microliter was analyzed by GC-MS. Culture densities at the beginning of time series incubations were recorded at 600 nm in 1 cm cuvettes with a Perkin-Elmer Lambda 35 spectrophotometer.

Analysis of metabolites

GC-MS analyses were carried out using an Agilent 6890 gas chromatograph in the splitless mode with an HP5-ms column (30 m × 0.25 mm i.d., 0.25 µm film thickness) coupled to an Agilent 5973 mass selective detector. Helium (1 ml min^{-1}) was the carrier gas, the inlet was set at 250°C, and the oven was programmed as follows: 50°C for 2 min then increasing 20°C per minute to 250°C. The mass spectrometer operated in the electron ionization mode at 70 eV and a source temperature of 230°C. Mass spectra were acquired over a 50–300 amu range at 2.94 scans/s.

Results and discussion

Pseudomonas sp. strain 19-rlim (16S rRNA gene GenBank accession #EF091149) was obtained from soil taken from beneath a katsura tree in Dow Gardens, Midland MI, while *Sphingomonas* sp. strain

BIR2-rlima (16S rRNA gene GenBank accession #EF153191) came from soil from beneath a dead river birch on Octavia Street, New Orleans, LA. Both were isolated by enrichment culture using R-limonene as sole carbon and energy source in minimal medium. When screened for transformation of MIB, both strains accumulated putative dehydration products (identified by comparison of mass spectra to the NIST database accompanying the Agilent 5973N GC-MS). Several other strains isolated by selective enrichment on other terpenes including α-pinene, β-pinene, limonene, and γ-terpinene incubated with MIB yielded the same products (unpublished results). Although extensive effort was made at the same time to isolate bacteria able to grow with MIB as sole carbon and energy source, none was isolated.

Dehydration of MIB by *Pseudomonas* sp. strain 19-rlim

When R-limonene grown-*Pseudomonas* sp. strain 19-rlim was incubated with MIB (Fig. 1), it removed 95% of MIB in 8 h with the accumulation of a compound eluting at 5.54 min (peak area = 45% of MIB peak area at start) as well as a minor compound eluting at 5.22 min (5% of MIB peak area at start). The mass spectra (Fig. 2) of these compounds are identical to those previously described for 2-methylenebornane (5.54 min) and 4-methylcamphene (5.22 min) (Schumann and Pendelton 1997). Strain 19-rlim grown in the absence of the inducer, R-limonene, did not transform MIB (data not shown).

Dehydration of MIB by *Sphingomonas* sp. strain BIR2-rlima

The second strain, R-limonene-grown *Sphingomonas* sp. strain BIR2-rlima also rapidly removed MIB but differed from Pseudomonas sp. strain 19rlim in that the compound eluting at 5.22 min was the major accumulating product with very little of the 5.54 min-eluting product (Fig. 3a). The mass spectra of these products (not shown) were identical to those of 2-methylenebornane and 4-methylcamphene produced by strain 19rlim. There were also several very minor peaks that increased in size over time eluting between 8.4 and 8.7 min.

Oxidation of MIB by *Sphingomonas* sp. strain BIR2-rlima

When *Sphingomonas* sp. strain BIR2-rlima was grown in the absence of inducer (Fig. 3b) it did not accumulate the dehydration products of R-limonene-induced cells. Instead it converted MIB to an array of oxidation products eluting at 8.40, 8.60, 8.63, and

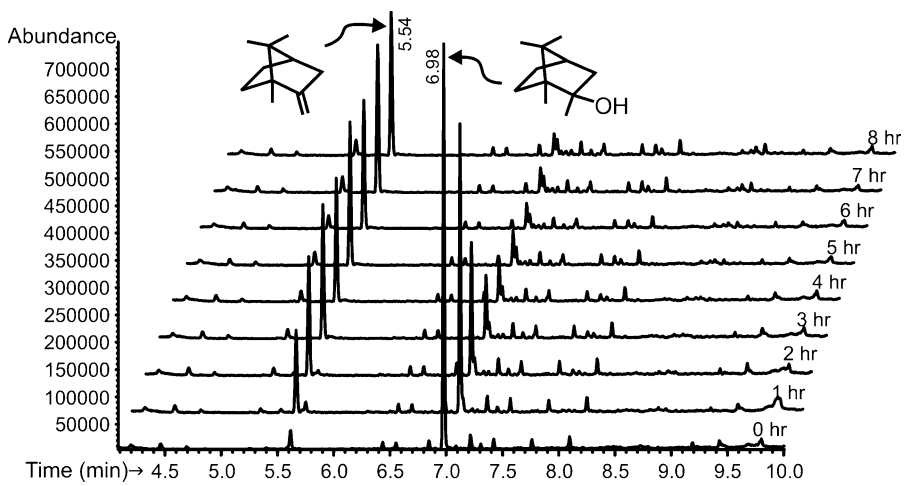

Fig. 1 Incubation of limonene-induced *Pseudomonas* sp. 19-rlim with MIB. The time series was carried out as described in Materials and Methods. Strain 19-rlim was resuspended in minimal medium to $OD_{600} = 15.9$ and distributed in 2 ml portions into scintillation vials to which 2 µl MIB (1/100 w/v in ethanol, about 120 nmol) was then added. Samples were taken at the beginning of the incubation and at 1 h intervals, extracted with methylene chloride and analyzed by GC-MS. The small peak eluting at 5.6 min at 0 h is residual R-limonene

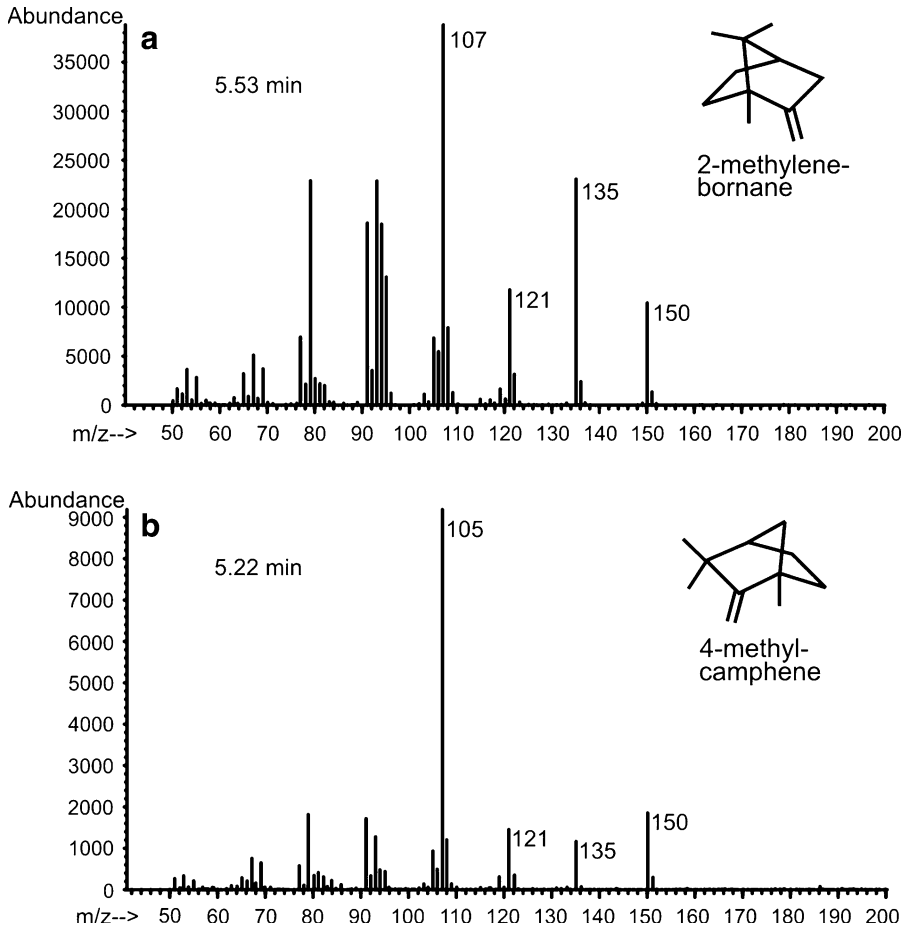

Fig. 2 Mass spectra of the two major products formed from MIB by *Pseudomonas* 19-rlim. **a** 2-methylenebornane, (**b**) 4-methylcamphene

8.67 min (the minor products seen in the incubation with R-limonene-induced cells). When strain BIR2-rlima was grown with D-camphor as inducer (data not shown), it also gave the same products but in a shorter time (4.5 h with twice the MIB concentration and a starting culture $OD_{600} = 10.4$). The mass spectra of these compounds are identical to those of compounds previously shown to be formed from MIB by various camphor-degrading strains (Eaton and Sandusky 2009): 6-hydroxy-MIB (52%, 8.60 min, *m/z* = 169 (100), 151 (28), 133 (6), 125 (20), 123 (37), 111 (37), 109 (86)), 5-hydroxy-MIB (35%, 8.63 min, *m/z* = 184 (3), 169 (3), 166 (20), 151 (73), 148 (4), 139 (2), 137 (3), 133 (9), 123 (47), 109 (100), 105 (9), 95 (37), 93 (48)), and 3-hydroxy-MIB (13%, 8.67 min, *m/z* = 184 (1), 169 (2), 166 (13), 151 (31), 148 (6), 139 (9), 133 (7), 123 (84), 111 (18), 108 (33), 95 (80), 85 (36), 81 (27), 74 (100)) (Eaton and Sandusky 2009).

The bacterial dehydration of MIB has been demonstrated twice in previous research although the transformations were far from complete. In both cases, the yield of metabolites was small and could easily have been the result of minor acid-catalyzed dehydration that occasionally occurs during isolation or analysis of MIB (Eaton, unpublished observation). Incubation of sludge from a gravel filter with MIB yielded seven metabolites that were tentatively identified (from GC-MS analysis) and included the two dehydration products, 2-methylenebornane and 2-methyl-2-bornene (Sumitomo 1992). Using the backwash water from a biological filter as a source, two strains, a *Pseudomonas* sp. and an *Enterobacter* sp., were isolated by enrichment with MIB (Tanaka

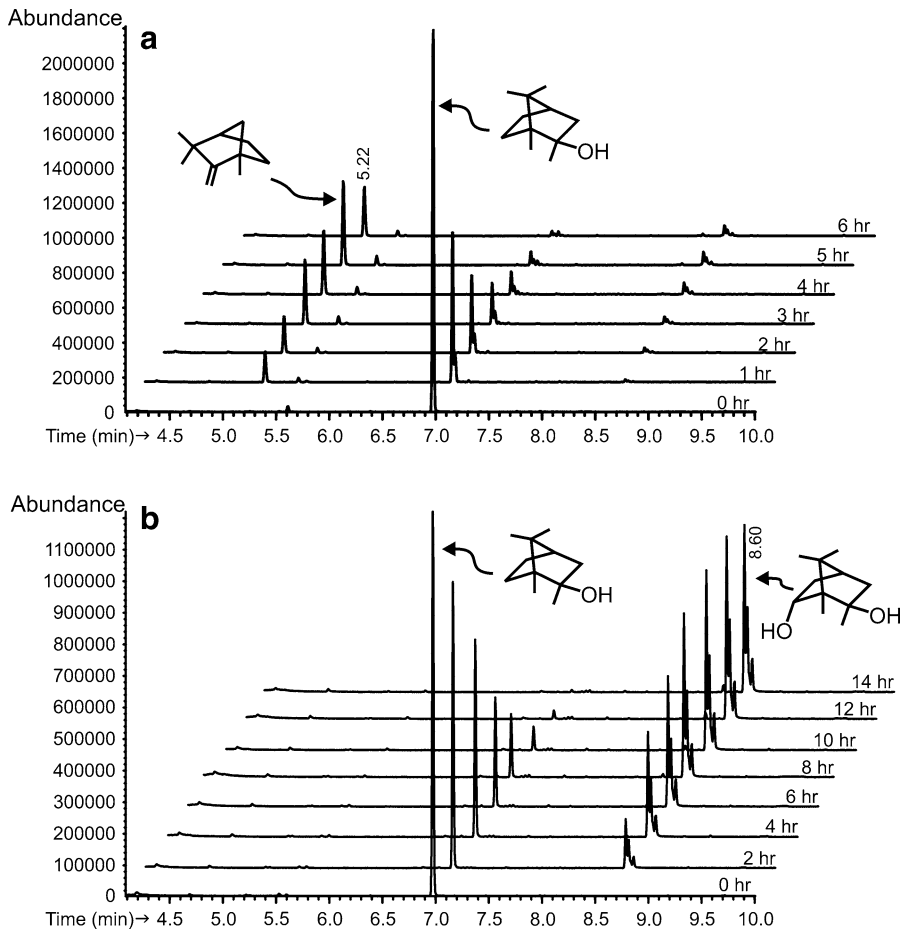

Fig. 3 Incubation of *Sphingomonas* sp. strain BIR2-rlima with MIB. The time series was carried out as described in Materials and Methods. **a** R-limonene-induced strain BIR2-rlima was resuspended in minimal medium to $OD_{600} = 6.35$ and distributed in 2 ml portions into scintillation vials to which 2 µl MIB (1/25 w/v in ethanol, about 480 nmol) was added. Samples were taken at the beginning of the incubation and at 1 h intervals, extracted with methylene chloride and analyzed by GC-MS. **b** Uninduced strain BIR2-rlima was resuspended in minimal medium to $OD_{600} = 9.0$ and distributed into scintillation vials to which 2 µl MIB (1/50 w/v in ethanol, about 240 nmol) was added. Samples were taken at the beginning of the incubation and at 2 h intervals, extracted with methylene chloride and analyzed by GC-MS

et al. 1996). The latter strain converted a small portion of the MIB to chemicals identified as 2-methylenebornane and a rearranged product, 2-methylcamphene. It is likely that these minor products are the same as those identified here (see below).

Acid-catalyzed dehydration and a proposed mechanism

Acid-catalyzed dehydration of MIB was first studied by Martin et al. (1988a, b) who misidentified the products as 2-methyl-2-bornene and 2-methylenebornane. Their published mass spectra of these products are included in the NIST database of mass spectra that accompanies the Agilent 5973 GC-MS used here. Their product identifications were later corrected by Schumann and Pendelton (1997) who generated different mixtures of MIB dehydration products by three methods: treatment with thionyl chloride in pyridine, treatment with sulfuric acid, and synthesis and elimination of a methyl xanthate. The dehydration products were identified by GC-MS and NMR spectroscopy and shown for the sulfuric acid treatment to be approximately equal amounts of 2-methylenebornane

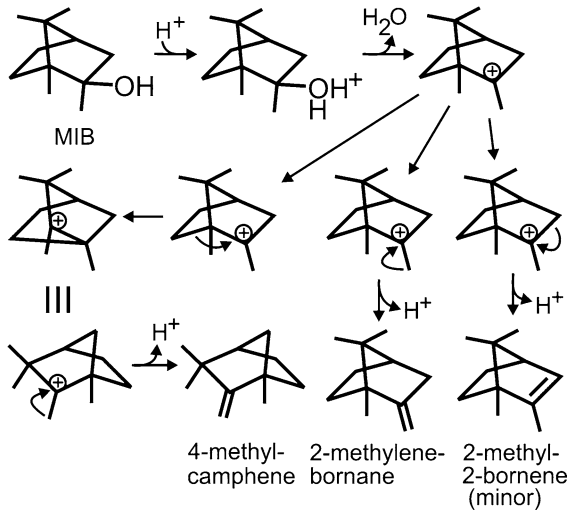

Fig. 4 Proposed reactions following acid treatment of MIB. Protonation of MIB leads to elimination of water and formation of the carbocation at the upper right. This can undergo at least three rearrangements; that leading to 2-methyl-2-bornene occurs much less frequently than the other two

(previously identified by Martin et al. as 2-methyl-2-bornene) and 1-methylcamphene (previously identified as 2-methylenebornane; its name is changed here to 4-methylcamphene, according to the IUPAC system, IUPAC 1979) along with 3% 2-methyl-2-bornene (Fig. 4). Rather than yielding 2-methyl-2-bornene as a major product, proposed by Martin et al., the carbocation intermediate underwent the 1,2-alkyl group migration known as the Wagner-Meerwein rearrangement (March 1992, Birladeanu 2000) to yield 4-methylcamphene as shown in Fig. 4. In accord with the results of Schumann and Pendelton (1997), the acid-catalyzed dehydrations carried out here to produce the substrates for the experiments described below also produced approximately equal amounts of 2-methylenebornane and 4-methylcamphene.

Because of the identities of the products accumulated by *Pseudomonas* sp. strain 19-rlim and *Sphingomonas* sp. strain BIR2-rlima, it is reasonable to propose that the biological dehydration mechanism may be identical: following an enzyme-catalyzed protonation of the hydroxyl group and subsequent dehydration, spontaneous carbocation rearrangements lead to the observed products.

Biotransformations of dehydration products

R-limonene-induced *Pseudomonas* sp. 19-rlim and R-limonene-induced *Sphingomonas* sp. BIR2-rlima converted MIB to two major products, 2-methylenebornane and 4-methylcamphene, although in differing ratios (Figs. 1, 3a). It was unclear whether the difference between these two strains occurred because the major accumulating chemical was also the major product formed or because other products were formed and then further degraded. To investigate this, a mixture of 2-methylenebornane and 4-methylcamphene, prepared by acid-catalyzed dehydration of MIB (as in Fig. 4), was incubated with strains 19-rlim and BIR2-rlima as well as with a few camphor-degrading bacteria which had been previously screened for their ability to act on these products.

Pseudomonas sp. strain 19-rlim, grown in the presence of R-limonene or without added inducer did not change either compound (data not shown). This suggests that 2-methylenebornane is the major MIB dehydration product in strain 19-rlim and that little 4-methylcamphene is formed by that strain.

Sphingomonas sp. strain BIR2-rlima, grown with either R-limonene or camphor, removed both dehydration products; 2-methylenebornane (5.53 min) was removed more rapidly than 4-methylcamphene (5.22 min) (Fig. 5a). This supports (but does not prove) the possibility that both dehydration products may be formed by this strain from MIB with 2-methylenebornane degraded as rapidly as it is formed, leaving 4-methylcamphene. The products formed by strain BIR2-rlima have molecular weights of 180 and 182, suggesting the addition of two oxygen atoms ($C_{11}H_{16}O_2$) and ($C_{11}H_{18}O_2$), and 196, suggesting three added oxygen atoms ($C_{11}H_{16}O_3$).

The camphor-degrading bacteria *Pseudomonas putida* G1, *Sphingomonas wratislaviensis* DLC-cam (not shown), and *Pseudomonas* sp. SWS3-camc also accumulated products of molecular weights 180, 182, and 196 when incubated with 2-methylenebornane and 4-methylcamphene. They are represented here by *Pseudomonas* sp. strain SWS3-camc which appeared to degrade only 2-methylenebornane (Fig. 5b).

Strains BIR2-rlima and SWS3-camc have some products in common; thus, metabolites eluting at

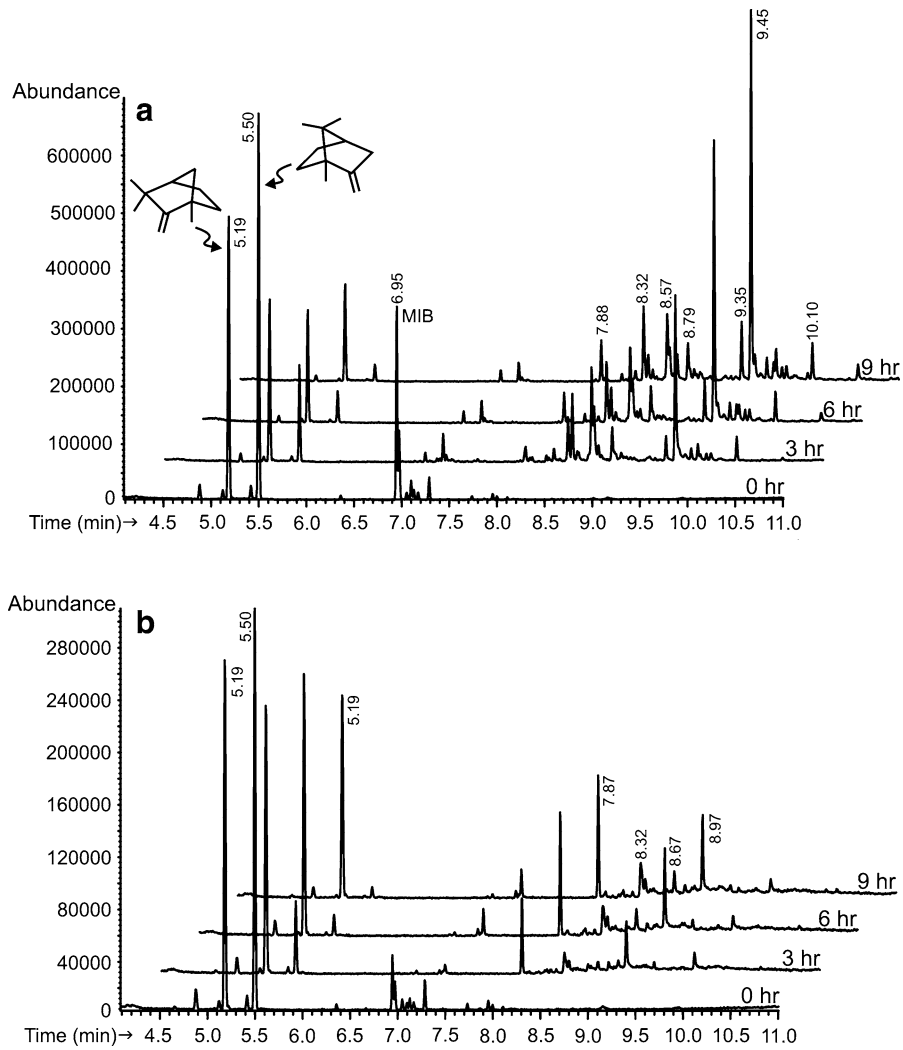

Fig. 5 Incubation of camphor-induced bacteria with 2-methylenebornane and 4-methylcamphene. **a** *Sphingomonas* sp. BIR2-rlima was resuspended in minimal medium to $OD_{600} = 10$ and distributed into scintillation vials to which 2 µl 2-methylenebornane + 4-methylcamphene in ethanol was added. Samples were taken at the beginning of the incubation and at 3 h intervals, extracted with methylene chloride and analyzed by GC-MS. (Note the contaminating MIB peak at 6.95 min in the starting sample.) **b** *Pseudomonas* sp. SWS3-camc was resuspended in minimal medium to $OD_{600} = 12.8$ and distributed into scintillation vials to which 2 µl 2-methylenebornane + 4-methylcamphene in ethanol was added. Samples were taken at the beginning of the incubation and at 3 h intervals, extracted with methylene chloride and analyzed by GC-MS

8.67–8.68 min ($M^+ = 196$) have similar mass spectra (not shown) as do the metabolites eluting at 7.87–7.88 min ($M^+ = 180$). Strain BIR2-rlima produced more metabolites than SWS3-camc probably due to the facts that (a) a significant concentration of MIB (25% of total) was present at the start of the incubation, so that a product of MIB oxidation, 6-hydroxy-MIB (8.57 min, $M^+ = 182$), was prominent and (b) unlike SWS3-camc, strain BIR2-rlima also acted on 4-methylcamphene, yielding a different group of products than the transformation of 2-methylenebornane. None of these products has been identified, although based on previous studies of MIB transformation (Eaton and Sandusky 2009) and the resemblance of the substrates to camphor, they are likely to be analogous to camphor-pathway intermediates and acted on by enzymes of the camphor catabolic pathway (Fig. 6), including

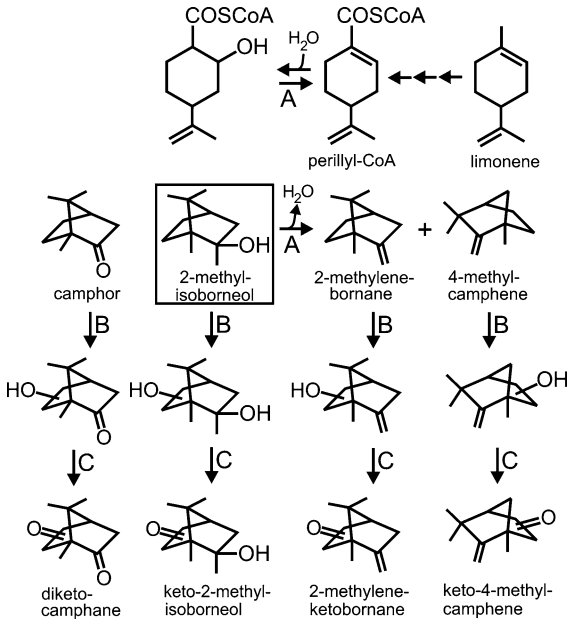

Fig. 6 Proposed transformations of MIB by R-limonene and camphor-degrading bacteria. R-limonene is metabolized by a multistep pathway through perillyl-CoA which is converted to 2-hydroxy-4-isopropenylcyclohexane carboxyl-CoA by a reversible hydratase (enzyme A) (Duetz et al. 2003). Camphor metabolism is initiated by cytochrome P450 camphor hydroxylase (enzyme B) followed by hydroxycamphor dehydrogenase (enzyme C). These enzymes act on MIB and/or dehydration products as indicated

hydroxyketones, diketones, and ketolactones (Gunsalus and Marshall 1971).

There are a few hydratase/dehydratase enzymes involved in R-limonene catabolism that might also catalyze the dehydration of MIB. One of these is the enzyme that converts perillyl-CoA to 2-hydroxy-4-isopropenylcyclohexane carboxyl-CoA (Fig. 6).

Application

Although Martin et al. (1988a, b) claimed that the MIB dehydration products may be at least partially responsible for musty odor in catfish, a subsequent study (Mills et al. 1993) clearly demonstrated that those compounds do not have a musty taste or odor. Other researchers (Finato et al. 1992, Napolitano et al. 1996) have synthesized various cyclic and non-cyclic tertiary alcohols and shown them to have earthy or earthy/camphor odors resembling that of MIB. Together, these data suggest that the tertiary hydroxyl is important for the earthy taste and odor and that by removing it, one can eliminate earthy off-flavor.

R-Limonene-induced *Pseudomonas* sp. strain 19-rlim and *Sphingomonas* sp. strain BIR2-rlima dehydrate MIB to 2-methylenebornane and 4-methylcamphene. These or other bacteria performing these enzyme-catalyzed dehydrations may be useful for eliminating MIB from catfish ponds and, thus, MIB-caused off-flavor from catfish. For this to occur, some strain modifications will be needed. Most significantly, the need for an inducer should be eliminated by either isolation of constitutive mutants or cloning and constitutive expression of the dehydrating enzymes in a new host strain.

Both benthic and planktonic cyanobacteria are responsible for producing MIB. Depending on the organism and environmental conditions, the relative intracellular and extracellular concentrations of MIB can vary widely (Jüttner and Watson 2007). These variables are likely to affect the bioavailability of MIB to degrading bacteria. MIB produced and retained within cyanobacteria subsequently consumed by catfish would not be degradable by the bacteria described here.

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