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ORIGINAL PAPER

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Biotransformation of *p*-xylene and 2,6-dimethylnaphthalene by xylene monooxygenase cloned from a *Sphingomonas* isolate

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Abstract *Sphingomonas* strain ASU1 was isolated from an industrial wastewater bioreactor and grew on 2,6-dimethylnaphthalene (2,6-DMN) as the sole carbon/energy source. The genes for a xylene monooxygenase were cloned from strain ASU1. Expression of the ASU1 xylene monooxygenase was compared to expression of the pWWO xylene monooxygenase in *Escherichia coli*. Both monooxygenases transformed *p*-xylene and 2,6-DMN by initially hydroxylating one methyl group. In addition, the ASU1 monooxygenase also hydroxylated the second methyl group on *p*-xylene and 2,6-DMN whereas the pWWO monooxygenase hydroxylated the second methyl group only on *p*-xylene. Endogenous *E. coli* enzymes contributed to further oxidation of the resulting aromatic alcohols to form aromatic carboxylates.

Introduction

The intermediates in bacterial oxidation of methylnaphthalenes to naphthoate and methylnaphthoates are similar to intermediates of the upper Tol pathway for metabolism of methylbenzenes (Fig. 1) (Miyachi et al. 1993; Grifoll et al. 1995; Dutta et al. 1998). The *xyl* genes on Tol plasmid pWWO are organized into two operons. The upper pathway operon encodes the enzymes for oxidation of *m*-xylene and *p*-xylene to form the corresponding methylbenzyl alcohol, methylbenzaldehyde, and finally methylbenzoate (Harayama et al. 1986, 1989). The lower pathway operon encodes enzymes that convert methylbenzoate into precursors of the Krebs cycle (Franklin et al. 1981; Harayama and Rekik 1990).

The genes for oxidation of methylnaphthalenes have not been identified. However, it is likely that homologs of the *xyl* genes and genes for naphthalene oxidation (*nah* genes) are involved in oxidation of methylnaphthalenes (Fig. 1). Xylene monooxygenase (encoded by *xyl*M and *xyl*A) initiates the Tol pathway by hydroxylating one methyl group on compounds such as *p*-xylene (Fig.1) (Assinder and Williams 1990). Accordingly, xylene monooxygenase or a similar monooxygenase may initiate metabolism of methylnaphthalenes. Since the bacteria in wastewater bioreactors are diverse (Bramucci and Nagarajan 2000), we used bioreactor sludge to isolate bacteria that degrade 2,6-dimethylnaphthalene (2,6-DMN). In the present report, we describe the activity of a xylene monooxygenase that was cloned from a *Sphingomonas* strain that grew with 2,6-DMN as the sole source of carbon and energy.

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Materials and methods

Media and culture conditions

S12 medium, S12 agar, M9 medium, LB medium and LB agar (Sambrook et al. 1989; Bramucci et al. 2002) were used for culturing bacteria. S12 medium and S12 agar were supplemented with 0.001% yeast extract. S12 agar was supplemented with aromatic compounds by placing a few flakes of solid compounds or 10 µl of liquid compounds on the interior of the Petri dish lid (Bramucci et al. 2002). All chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise indicated.

Isolation and characterization of strain ASU1

Activated sludge from an industrial wastewater bioreactor was inoculated into S12 medium with a few flakes of 2,6-DMN in a screw-cap Erlenmeyer flask. The enrichment culture was incubated at 28°C with shaking and was diluted 1:10 every 4–7 days. Bacteria that degraded 2,6-DMN were isolated after spreading samples of the enrichment culture onto S12 agar with 2,6-DMN at 28°C. Strain ASU1 was tested for growth on aromatic substrates by streaking onto S12 agar. Growth was evaluated after 3–5 days at 30°C.

Strain ASU1 16S rRNA genes were amplified by the polymerase chain reaction (PCR) using primers JCR14 (5′-ACGGGCGGTGTGTAC-3′) and JCR15 (5′-GCCAGCAGCCGCGGTA-3′) and sequenced (Bramucci et al. 2002). ASU1 DNA was prepared by suspending several colonies in 100 µl of water. The suspension was frozen at –20°C, thawed, heated to 90°C for 10 min and centrifuged.

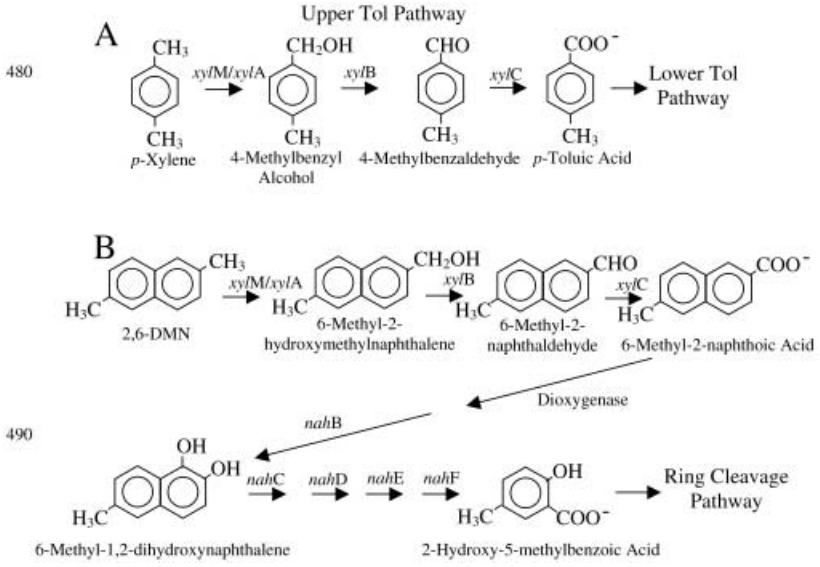

Fig. 1A, B Bacterial oxidation of methybenzenes and methylnaphthalenes. **A** Tol pathway. **B** Methylnaphthalene pathway with suggested roles for *xyl* genes and *nah* genes

For analysis of 2,6-DMN degradation, strain ASU1 was grown in S12 medium with a few flakes of 2,6-DMN for 5 days at 30°C. Cells were collected by centrifugation, washed with S12 medium and resuspended in 600 ml of S12 medium. Half of the suspension was supplemented with 2,6-DMN. The other culture was a no-carbon control. Samples (75 ml) were extracted with 50 ml of ethyl acetate and analyzed by GC/MS.

Cloning and sequencing of xylene monooxygenase genes

A cosmid library was constructed by ligating a partial *Sau*3A (Promega, Madison, Wis.) digest of ASU1 DNA to SuperCos 1 vector DNA (Stratagene, La Jolla, Calif.). Individual recombinant *Escherichia coli* colonies were incubated in microtiter wells containing 200 µl of LB broth with 50 µg/ml ampicillin at 37°C for 16 h on a shaking platform. Pools of cultures were tested for the *xyl*A gene by PCR using a commercial kit (Perkin Elmer, Norwalk, Conn.). The PCR primers (xylAF1: 5′-CCGCACGATTGCAAGGT-3′; xylAR1: 5′-GGTGGGCCACACAGATA-3′) were based on the plasmid pNL1 *xyl*A sequence (GenBank accession no. AF079317). PCR was performed for 1 min at 94°C and then cycled 40 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Positive pools were deconvoluted by retesting each individual culture that formed the pool.

The sequence of a cosmid with *xyl*A was determined from a library of overlapping fragments of cosmid DNA that had been cloned into the *Sma*I site of plasmid pUC18 (Amersham Pharmacia, Piscataway, N.J.). Cosmid DNA was fragmented by partial digestion with *Hae*III or by forcing the DNA through a nebulizer (IPI Medical Products, Chicago, Ill.) with filtered air (22 psi for 30 s). DNA fragments of 2–4 kb in size were excised from a 0.8% low-melting-point agarose gel and purified using a Gene Clean Kit (Qbiogene, Carlsbad, Calif.). Nebulized DNA was repaired in a polishing reaction [4 µl 10× polynucleotide kinase buffer (Promega), 1 µl 10 mM ATP, 1 µl T4 DNA Polymerase (6 U/µl; Promega), 1 µl polynucleotide kinase (6 U/µl; Promega), 30 µl nebulized DNA, 1.6 µl dNTPs (stock solution containing 2.5 nM of each dNTP), 1.4 µl water] that was incubated at 37°C for 1 h. Clones containing fragmented DNA were inoculated into each well of a 96 square-well plate (Beckman Coulter, Fullerton, Calif.) containing 1 ml of LB with 50 µg/ml ampicillin, 0.2% glucose and 20 mM Tris-HCl, pH 7.5. The plates were incubated at 37°C for 16 h on a shaking platform. Plasmid DNA was prepared using the Qiaprep 96 Turbo Miniprep Kit (Qiagen, Valencia, Calif.). The plasmids were sequenced on an automated ABI sequencer (Applied Biosystems, Foster City, Calif.) using pUC18 universal and reverse primers. The sequences were assembled using Sequencher 3.0 (Gene Codes, Ann Arbor, Mich.).

The *xyl*M and *xyl*A genes were amplified by PCR and cloned into pCR2.1-TOPO (TOPO TA Cloning Kit; Invitrogen, Carlsbad, Calif.). Primers ASU1MAF1 (5′-TAACTAAGGAGAAATCATATGGACGGACTGCG-3′) and ASU1MAR1 (5′-GGATCCCGGGTCTTTTTTTACGTGCGATTGCTGCG-3′) were used to amplify the ASU1 genes. Primers WWOF1 (5′-TAAGTAGGTGGATATATGGACAC-3′) and WWOR2 (5′-GGATC-CCTAGACTATGCATCGAACCAC-3′) were used to amplify the pWWO genes contained in the American Type Culture Collection (ATCC) strain 33015. The samples were incubated for 1 min at 94°C and then cycled 40 times at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. After the last cycle, the samples were incubated at 72°C for 10 min.

Transformation of *p*-xylene and 2,6-DMN by *E. coli*

For transformation of *p*-xylene, cells were grown for 24 h at 37°C with shaking in 50 ml of LB in a 500 ml screw-cap Erlenmeyer flask. The cells were collected by centrifugation and resuspended in 50 ml of M9 medium with 20 µg/ml tryptophan, 0.4% glycerol, 0.4% casamino acids, and 50 µg/ml ampicillin. *p*-Xylene (100 µl) was supplied to the culture by evaporation from a sterile 13×100 mm glass tube reservoir inside the culture flask. In addition, 10 µl of *p*-xylene was added directly to the culture medium at the beginning of the experiment. After 5.5 h, 1 ml of 20% glycerol was added to the medium. The culture was incubated at 37°C with shaking.

For transformation of 2,6-DMN, cells were grown for 24 h at 37°C with shaking in 50 ml of M9 medium containing 20 µg/ml tryptophan, 0.4% glycerol, 0.4% casamino acids, and 50 µg/ml ampicillin in a 500 ml screw-cap Erlenmeyer flask. The cells were collected by centrifugation and resuspended in 50 ml of fresh M9 medium supplemented as above. The culture was additionally supplemented with 25 mg 2,6-DMN in 10 ml perfluoro compound FC-75 (ACROS) and was incubated at 37°C with shaking.

Analytical methods

For reverse phase HPLC analysis, 1.5 ml samples were collected and filter-sterilized using 0.2 µm Acrodisc GHP membrane filters (Gelman). Analyses were performed on a Hewlett Packard 1100

with a photo diode array UV-visible detector set at 230 nm (primary wavelength), 254 nm (secondary wavelength) and 450 nm as background reference. For analysis of *p*-xylene transformation products, the column was a Hewlett Packard Zorbax SB-C8 (4.6 mm ×25 cm). The column temperature was 50°C. The mobile phase was acetonitrile (solvent S1) and 0.02 mM ammonium acetate (solvent S2). The gradient was: (1) 0–25 min S1 increased from 10% to 30%, (2) S1 increased to 95% over the next 9 min, (3) S1 remained at 95% for the next 4 min, and (4) S1 decreased to 10% in 4 min. The flow rate for the mobile phase was 1.0 ml/min. For analysis of 2,6-DMN transformation products, the column was a Hewlett Packard Zorbax SB-C18 (4.6×12.5 mm, 5 µm). The gradient was: (1) 0–3 min 10% S-1, (2) S1 increased to 100% in 33 min, and (3) S1 decreased to 10% in 3 min. The flow rate for the mobile phase was 0.9 ml/min.

For GC/MS analysis, samples were acidified to pH 2 with concentrated HCl and extracted in an equal volume of ethyl acetate. Anhydrous sodium sulfate or magnesium sulfate was use to remove residual water, and the samples were dried with a gentle flow of nitrogen. The samples were resuspended in 1 ml bis(trimethylsilyl)trifluroacetamide silylation reagent (Supelco, Bellefonte, Pa.) and derivatised at 80°C for *p*-xylene products and at 90°C for 2,6-DMN products.

For *p*-xylene products, mass spectra were acquired in the negative ion mode on a Agilient 5973 Network MSD (single stage quadrupole) with a Hewlett-Packard 6890A GC. The samples were run on an HP-5MS 0.5% phenylmethylsiloxane column with 1 µl splitless injection and 13 min delay time before activating the mass spectrometer. The GC column temperature conditions were (1) 50°C for 1 min, (2) the temperature was increased at 10°C/min to 250°C and (3) held at 250°C for 5 min.

For 2,6-DMN products, mass spectra were acquired in the negative ion mode on a Finnigan SSQ 7000 (single stage quadrupole) or Hewlett-Packard 5970 MSD (single stage quadrupole) with a Hewlett-Packard 5980 II GC. The samples were run on an MDN-5S column with 1 µl splitless injection and 13 min delay time before activating the mass spectrometer. The GC column temperature conditions were (1) 50°C for 5 min, (2) the temperature was increased at 10°C/min to 300°C and (3) held at 300°C for 5 min.

Results

Isolation and characterization of *Sphingomonas* strain ASU1

Strain ASU1 was isolated from an enrichment culture in which 2,6-DMN was the sole source of carbon and energy. Strain ASU1 grew on toluene, *m*-xylene, *p*-xylene, naphthalene, 1-methylnaphthalene, 2-methylnaphthalene and various isomers of dimethylnaphthalene as sole carbon source but was unable to grow on benzene. Microscopic examination indicated that strain ASU1 was a Gram-negative rod. The ASU1 16S rRNA gene sequence had the highest homology (99.6% identity) to the 16S rRNA gene sequence of *Sphingomonas* strain MBIC3020 (GenBank accession no. AB025279.1).

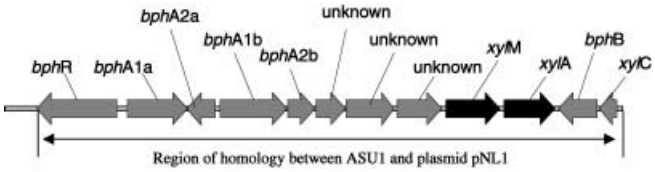

Fig. 2 Physical map of the 12.5 kb sequence derived from strain ASU1 that includes the genes for *xyl*M and *xyl*A

Two major products were detected by GC/MS in cultures of strain ASU1 after 5 days of incubation when 2,6-DMN was the sole source of carbon and energy. The first product was 6-methyl-2-naphthoic acid and the second product was 2-hydroxy-5-methylbenzoic acid (Table 1). After an additional 4 days of incubation, 6-hydroxymethyl-2-naphthoic acid and 2,6-naphthalenedicarboxylic acid were also detected (Table 1). These results were consistent with observations for other bacteria that grow on 2,6-DMN (Dutta et al. 1998).

Identification and cloning of genes for xylene monooxygenase from strain ASU1

The ability of strain ASU1 to grow on methylbenzenes, naphthalene and methylnaphthalenes is similar to *Novosphingobium aromaticivorans* strain F199 (ATCC strain 700278) that grows on naphthalene and all isomers of xylene (Fredrickson et al. 1991). Strain F199 contains plasmid pNL1, which has several *xyl* gene and *nah* gene homologs (Romine et al. 1999). PCR experiments with primers based on the pNL1 homologs for salicylaldehyde dehydrogenase (*nah*E), xylene monooxygenase (*xyl*M and *xyl*A), benzyl alcohol dehydrogenase (*xyl*B) and benzaldehyde dehydrogenase (*xyl*C) indicated that strain ASU1 contained these genes (data not shown). A cosmid library of strain ASU1 DNA was screened for *xyl*A. A partial sequence from a positive cosmid was 12.5 kb in length and contained adjacent open reading frames for *xyl*M and *xyl*A (Fig. 2). Positions 632–12,591 of the 12.5 kb sequence displayed 92% base sequence

Table 1 GC/MS data for products formed by *Sphingomonas* strain ASU1 during growth on 2,6-dimethylnaphthalene (2,6-DMN). *RT* Retention time

Product	GC RT	m/z of major ion peaks	Suggested structure[a]
1	21.60	296(M^+ TMS)(1), 281(100), 223(20), 149(30), 73(75)	2-Hydroxy-5-methyl-benzoic acid TMS ester
2	25.56	258(M^+ TMS)(514), 243(100), 199(80), 169(62.8), 141 (45.7), 122(17.1), 115(34.2) 73(29)	6-Methyl-2-naphthoic acid TMS ester
3	27.43	346(M^+ TMS)(3.6), 331(100), 257(7.2), 73(30.9)	6-Hydroxymethyl-2-naphthoic acid TMS ester
4	29.70	360(M^+ TMS)(30), 345(100), 301(40), 271(38), 228(32), 184(30), 154(60), 126(60.5), 73(30)	2,6-Naphthalenedicarboxylic acid TMS ester

[a] Based on comparison with authentic standards

Table 2 GC/MS data for products formed by *Escherichia coli* strain TOP10(pMGB123) during growth on *p*-xylene or 2,6-DMN

Carbon Source	Product	GC RT	m/z of major ion peaks	Suggested structure[a]
p-Xylene	1	15.50	194(M^+ TMS)(15.6), 179(100), 149(31.25), 105(96.8), 73(18.75)	4-Methylbenzyl alcohol TMS ester
	2	17.02	208(M^+ TMS) (10), 193 (100), 149(60), 119(80), 91(40)	*p*-Toluic acid TMS ester
	3	21.28	281(M^+ TMS)(81.8), 265(10), 251(10), 207(100), 193(20), 163(18), 135 (25), 73(95.45)	4-Hydroxymethylbenzoic acid TMS ester
	4	19.90	282(M^+ TMS)(18.75), 267(37.5), 237(15.6), 193(56.25), 179(78.1), 147(28.1),103(62.5), 73(100)	1,4-Benzenedimethanol TMS ester
2,6-DMN	1	24.4	244(M^+ TMS)(50), 229(30), 155(100), 73(20)	6-Methyl-2-hydroxymethyl-naphthalene TMS ester
	2	25.7	258(M^+ TMS)(50), 243(100), 199(90), 169 (80), 141(60), 122(35), 115(50), 73(10)	6-Methyl-2-naphthoic acid TMS ester
	3	29.9	360(M^+ TMS)(30), 345(100), 301(40), 271(40), 228(35), 18428), 154(60), 126(65), 73(31)	2,6-Naphthalenedicarboxylic acid TMS ester

[a] Based on comparison with authentic standards

Table 3 Products formed by *E. coli* strains TOP10(pMGB123) and TOP10(pMGB125) during growth on *p*-xylene. *MBA* Methyl benzyl alcohol, *BDM* benzenedimethanol, *HMBA* hydroxymethylbenzoic acid

	Product (μg/ml)				
Strain	Time (h)	4-MBA	*p*-Toluate	1,4-BDM	4-HMBA
TOP10(pMGB123)	0	ND[a]	ND	ND	ND
	6	783.7	23.1	ND	ND
	24	764.0	27.8	ND	ND
	48	351.1	30.5	5.4	0.3
TOP10(pMGB125)	0	ND	ND	ND	ND
	6	301.8	4.8	ND	ND
	24	475.7	12.2	ND	ND
	48	271.2	23.7	ND	ND

[a] Not detected

identity with plasmid pNL1 between positions 133,938 and 145,937. The *xyl*M and *xyl*A homologs from ASU1 had 97% and 98% base sequence identity, respectively, with the corresponding pNL1 homologs.

Expression of the ASU1 xylene monooxygenase in *E. coli*

A 2.3 kb PCR product that contained the *xyl*M and *xyl*A genes of strain ASU1 was cloned into plasmid pCR 2.1-TOPO, creating plasmid pMGB123. pMGB123 caused *E. coli* to produce indigo on LB agar, indicating that a functional xylene monooxygenase was expressed from the cloned genes (Keil et al. 1987). The *xyl*M and *xyl*A genes for the well-characterized Tol plasmid pWWO xylene monooxygenase were similarly cloned and expressed from plasmid pMGB125 in *E. coli* for comparison.

Several products were detected in cultures of strain TOP10(pMGB123) that expressed the ASU1 xylene monooxygenase during incubation with *p*-xylene. The HPLC retention times (RT) and mass spectra (Table 2) of the products corresponded to authentic standards of 4-methylbenzyl alcohol (RT =22.4 min), *p*-toluic acid (RT =27.1 min), 1,4-benzenedimethanol (RT =7.5 min) and 4-hydroxymethylbenzoic acid (RT =9.9 min). The 4-methylbenzyl alcohol and *p*-toluic acid appeared first and were followed by 1,4-benzenedimethanol and 4-hydroxymethylbenzoic acid (Table 3). 4-Methylbenzyl alcohol and *p*-toluic acid were the only compounds detected by HPLC under the same conditions for strain TOP10(pMGB125) that expressed the pWWO xylene monooxygenase (Table 3). However, small amounts of 1,4-benzenedimethanol and 4-hydroxymethylbenzoic acid were detected by GC/MS in concentrated samples of culture supernatant (data not shown). None of these products were detected in cultures of cells with cloned monooxygenase genes that lacked *p*-xylene, nor were they detected in cultures of strain TOP10(pCR 2.1-TOPO) that contained *p*-xylene (data not shown). In separate experiments, 10.1 μg/ml *p*-toluic acid was detected by HPLC after 24 h in cultures of strain TOP10 (pCR 2.1-TOPO) that initially had 400 μg/ml 4-methylbenzyl alcohol. Taken together, these results indicated that both xylene monooxygenases hydroxylated *p*-xylene to produce predominately 4-methylbenzyl alcohol. In addition, the ASU1 monooxygenase hydroxylated the second methyl groups on 4-methylbenyl alcohol and possibly *p*-toluic acid more readily than the pWWO xylene monooxygenase. However, endogenous *E. coli* enzymes contributed to transformation of 4-methylbenzyl alcohol to *p*-toluic acid.

Incubation with 2,6-DMN resulted in detection of two products by HPLC in cultures of strain TOP10-(pMGB123). After 48 h, 4.17 μg/ml of 6-methyl-2-

hydroxymethylnaphthalene (RT =21.70 min) and 0.33 µg/ml 6-methyl-2-naphthoic acid (RT =13.91 min) were present in the culture. The identities of these products were confirmed by GC/MS (Table 2). After 24 h of additional incubation, 2,6-naphthalenedicarboxylic acid was detected by GS/MS (Table 2). Similarly, 17.3 µg/ml of 6-methyl-2-hydroxymethylnaphthalene and 0.57 µg/ml 6-methyl-2-naphthoic acid were present in a TOP10 (pMGB125) culture after 48 h. None of these products were detected in cultures of TOP10(pCR 2.1-TOPO) that contained 2,6-DMN, or in cultures of strain TOP10 (pMGB123) or TOP10(pMGB125) that lacked 2,6-DMN (data not shown). These results indicated that both xylene monooxygenases hydroxylated one methyl group on 2,6-DMN to form 6-methyl-2-hydroxymethylnaphthalene. In addition, the ASU1 xylene monooxygenase hydroxylated the second methyl group at a low level to produce 2,6-naphthalenedicarboxylic acid.

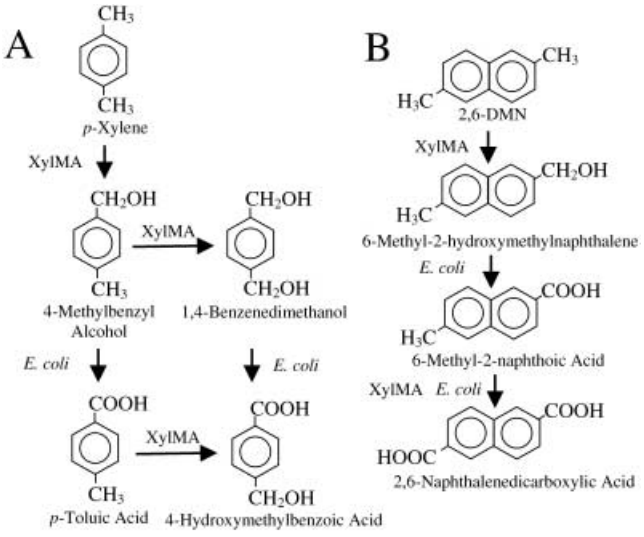

Fig. 3A, B Oxidation products of *p*-xylene and 2,6-dimethylnaphthalene (2,6-DMN) detected in cultures of recombinant *Escherichia coli* expressing cloned ASU1 xylene monooxygenase or cloned pWWO xylene monooxygenase. *XylMA* indicates a xylene monooxygenase encoded by *xyl*M and *xyl*A. **A** Products of *p*-xylene oxidation. **B** Products of 2,6-DMN oxidation

Discussion

Since the microbial communities in wastewater bioreactors are complex (Bramucci and Nagarajan 2000), we used activated sludge to isolate strain ASU1. The ASU1 DNA with *xyl*M and *xyl*A was highly homologous to plasmid pNL1. Plasmid pNL1 is contained in *N. aromaticivorans* strain F199, which was isolated from deep subsurface sediments (Fredrickson et al. 1991). We did not determine if strain ASU1 contained additional DNA that was homologous to pNL1. Nevertheless, sequencing the 12.5 kb fragment of DNA containing ASU1 *xyl*M and *xyl*A demonstrated that this DNA is present in wastewater bioreactors and that genes associated with pNL1 may be distributed across diverse environments.

ASU1 grew on 2,6-DMN by oxidizing one methyl group to form 6-methyl-2-naphthoic acid. This compound was then most likely converted to 6-methyl-1,2-dihydroxynaphthalene by a dioxygenase, with further metabolism proceeding as for naphthalene oxidation (Fig. 1) (Eaton and Chapman 1992). Our results indicated that the ASU1 *xyl*M and *xyl*A genes encoded a xylene monooxygenase that converted 2,6-DMN to 6-methyl-2-hydroxymethylnaphthalene in *E. coli*. This observation is consistent with this enzyme initiating oxidation of 2,6-DMN in strain ASU1. The variety of methylated aromatics that are substrates for ASU1 growth and the ability of the ASU1 xylene monooxygenase to hydroxylate *p*-xylene and 2,6-DMN suggests that ASU1 may use a single monooxygenase to attack several different methylated aromatics. Genetic analysis of strain ASU1 will be necessary to confirm that xylene monooxygenase has a role in metabolism of 2,6-DMN, *p*-xylene and other methylated aromatics.

The Tol plasmid pWWO xylene monooxygenase has been cloned and expressed in *E. coli* (Harayama et al. 1986; Wubbolts et al. 1994; Bühler et al. 2000, 2002). The pWWO xylene monooxygenase oxidizes one methyl group on a variety of substituted toluenes to form the corresponding benzyl alcohols in *E. coli*. The pWWO xylene monooxygenase has a relaxed substrate specificity and oxidizes benzyl alcohol and benzaldehyde to form benzoic acid in *E. coli* (Harayama et al. 1986; Bühler et al. 2000, 2002). We could not determine if either the ASU1 xylene monooxygenase or the pWWO xylene monooxygenase oxidized aromatic alcohols and aldehydes because, in our hands, *E. coli* converted 4-methylbenzyl alcohol to *p*-toluic acid. Nevertheless, the ASU1 xylene monooxygenase was clearly required for hydroxylation of methyl groups on aromatics in *E. coli* (Fig. 3). 4-methylbenzyl alcohol was the predominate product formed from *p*-xylene by both monooxygenases. However, 1,4-benzenedimethanol and 4-hydroxymethylbenzoic acid were also detected, particularly in cultures containing the ASU1 xylene monooxygenase. Hence, the ASU1 xylene monooxygenase could hydroxylate both methyl groups of *p*-xylene.

The activity of the pWWO xylene monooxygenase has been tested with a variety of monocyclic aromatic substrates (Harayama et al. 1986; Wubbolts et al. 1994; Bühler et al. 2000, 2002). However, the activity of the pWWO xylene monooxygenase with methylnaphthalenes has not been described previously. Both xylene monooxygenases hydroxylated a single methyl group on 2,6-DMN to produce 6-methyl-2-hydroxymethylnaphthalene and 6-methyl-2-naphthoic acid (Fig. 3). In addition, the ASU1 xylene monooxygenase oxidized the second methyl group resulting in formation of 2,6-naphthalenedicarboxylic acid.

Pandoraea strain IR3 oxidizes both methyl groups on *p*-xylene to produce terephthalic acid (Bramucci et al. 2002). However, it is not known if a single monooxygenase is responsible for oxidation of both methyl groups. Although the pWWO xylene monooxygenase has been

extensively characterized, we are not aware of any previous report indicating that this monooxygenase or any other similar monooxygenase oxidizes more than one methyl group on any aromatic substrate. Partial oxidation of multiple methyl groups on aromatic rings is difficult to achieve by chemical catalysis. However, our results suggest that the xylene monooxygenases encoded by pWWO and strain ASU1 could be used in bioprocesses such as transformation of *p*-xylene to 1,4-benzenedimethanol or 4-hydroxymethylbenzoic acid.

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