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Identification and characterization of the AgmR regulator of *Pseudomonas putida*: role in alcohol utilization

Received: 10 September 2001 / Revised: 7 November 2001 / Accepted: 16 November 2001 / Published online: 16 January 2002
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Abstract Two-phase partitioning bioreactors (TPPBs) comprise an aqueous phase containing all non-carbon nutrients necessary for microbial growth and a solvent phase containing high concentrations of inhibitory or toxic substrates that partition at sub-inhibitory levels to the aqueous phase in response to cellular demand. This work aimed at eliminating the growth of *Pseudomonas putida* ATCC 11172 on medium-chain-length (C₈–C₁₂) aliphatic alcohols, hence enabling their use as xenobiotic delivery solvents within two-phase partitioning bioreactors. Experiments resulted in the isolation of a mini-Tn5 mutant unable to utilize these alcohols. The mutation, which also eliminated growth on glycerol and ethanol, was identified to be within a homologue of the *P. aeruginosa agmR* gene, which encodes a response regulator. Enzyme analysis of the *agmR*::Tn5Km mutant cell extracts revealed a 10-fold decrease in pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase activity. A knockout in a gene (*exaA*) encoding a PQQ-linked alcohol dehydrogenase slowed but did not eliminate growth on medium-chain-length alcohols or ethanol, suggesting metabolic redundancy within *P. putida* ATCC 11172. Analysis of *P. putida* KT2440 genome sequence data indicated the presence of two PQQ-linked alcohol dehydrogenase-encoding genes. The successful elimination of alcohol utilization in the *agmR* mutant indicates control by AgmR on multiple pathways and presents a useful strain for biotechnological applications requiring alcohol non-utilizing microbial catalysts.

Introduction

The greatest challenge in biodegradative approaches to pollutant remediation is appropriate delivery of xenobiotic(s) to the bacterial catalyst(s) to prevent substrate limitation or toxicity to the cells (Alexander 1994). A strategy developed to address this issue is two-phase partitioning bioreactor (TPPB) technology which involves the introduction of an immiscible, biocompatible, but non-metabolized organic solvent into the reactor to partition the pollutant and enable delivery of a sub-inhibitory concentration of xenobiotic to the bacterial cells in the aqueous phase (Collins and Daugulis 1996). Through careful solvent selection and the use of a TPPB configuration, it is possible to introduce and treat high concentrations of xenobiotic in the reactor at a rate determined by the metabolic demand of the microbial population (Daugulis 2001).

Pseudomonas putida ATCC 11172 is one of several micro-organisms specifically shown to be an effective xenobiotic degrader within a TPPB system (Collins and Daugulis 1997a, b). However, the broad biodegradative capabilities of this organism also include some potentially useful delivery solvents. To circumvent the organism's extensive degradative capabilities, TPPB systems have been forced to employ complex and often expensive organics, or compounds with sub-optimal process characteristics (e.g., low partition coefficient) as the delivery solvents. As such, solvent bioavailability has been a key limiting factor in the operation and commercial application of TPPB technology.

The accessibility, biocompatibility, generally low cost, and partitioning nature of medium-chain-length alcohols (MCLAs) make them desirable options as delivery organics within TPPB systems. Biochemical analysis has revealed that the degradation of MCLAs is within the metabolic range of *P. putida* ATCC 11172. The goal of this work therefore was to use genetic engineering to eliminate the ability of this *P. putida* strain to degrade MCLAs, hence enabling their use as delivery solvents in a TPPB system.

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Table 1 Bacterial strains and plasmids. ATCC American Type Culture Collection

Strain or plasmid	Description	Source
<i>Pseudomonas putida</i> ATCC 11172	Wild type	ATCC
<i>P. putida</i> AVP2	11172 derivative, <i>agmR</i> ::mini Tn5	This study
<i>P. aeruginosa</i> ATCC 17933	PAO with high quinoprotein ethanol dehydrogenase activity	ATCC; Rupp and Gorisch (1988)
<i>Escherichia coli</i> DH5 α	F ⁻ ϕ 80d <i>lacZ</i> , Δ M15 Δ (<i>lacZYA</i> ⁻ <i>argF</i>) U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (<i>r_K⁻</i> , <i>m_K⁺</i>), <i>supE44</i> , λ ⁻ , <i>thi-1</i> , <i>gyr96</i> , <i>relA1</i>	Low (1968); Hanahan (1983); Meselson and Yuan (1986)
<i>E. coli</i> S17-1	<i>thi</i> , <i>pro</i> , <i>hsdR</i> (<i>rm</i> ⁺), <i>recA</i> (derived from <i>E. coli</i> 294); carries plasmid RP4 derivative (<i>amp</i> ; <i>ter</i> ::Mu; <i>km</i> ::Tn7) integrated into chromosome Tc ^s ; Km ^s ; Ap ^r ; Tp ^r ; tra ⁺	Simon et al. (1983)
pCR 2.1 TOPO	F1; ColE1; <i>lacZ</i> α , Ap ^r ; Km ^r	Invitrogen (Carlsbad, Calif.)
pRK415	Tc ^r , <i>incP1</i> <i>mob</i> , <i>lacZ'</i> , cloning vector	Keen et al. (1988)
pUTKm	Ap ^r ; Tn5-based delivery plasmid with Km ^r	de Lorenzo et al. (1990); Herrero et al. (1990)
pAV2	pCR 2.1 TOPO harboring PCR amplified Tn insertion region	This study
pAV5	pCR 2.1 TOPO harboring insert with 11172 <i>agmR</i> amplified by PCR	This study
pAV8	pRK415 harboring insert with 11172 <i>agmR</i> amplified by PCR	This study

Metabolism of aliphatic alcohols occurs through two oxidative steps, requiring alcohol and aldehyde dehydrogenases (ADHs), with the resulting acids being further metabolized via the β -oxidative pathway and the tricarboxylic acid cycle. Bacteria are known to produce a number of ADHs with a variety of substrate specificities and, often, different physiological roles (Reid and Fewson 1994). In pseudomonads, the occurrence of both constitutive and inducible dehydrogenases has been observed and both NAD(P)-dependent and NAD-independent ADHs have been identified. The latter frequently contain pyrroloquinoline quinone (PQQ) as the prosthetic group (Toyama et al. 1995). In this work, mutation in the *AgmR* regulator of *P. putida* ATCC 11172 was shown to eliminate the organism's ability to grow on a variety of alcohols, a number of which possess desirable characteristics for delivery solvents within TPPB systems.

Materials and methods

Bacterial strains, plasmids, and cultivation

Bacterial strains and vectors used in this study are described in Table 1. Mineral salts medium (MSM) contained (per liter): 6 g K₂HPO₄, 4 g KH₂PO₄, 2 g (NH₄)₂SO₄, 0.66 g MgSO₄, 0.25 g CaCl₂, 5 ml trace element solution [prepared in distilled water and containing (per liter): 0.3 g H₃BO₃, 0.089 g ZnSO₄·7H₂O, 0.024 g Ni₂SO₄, 0.018 g (NH₄)₆Mo₇O₂₄·4H₂O, 0.003 g CuSO₄·5H₂O, 0.05 g MnSO₄, 0.19 g CoCl₂, 0.0006% FeCl₃ (v/v)] and was supplemented with either 11 g sodium pyruvic acid l⁻¹ (Sigma-Aldrich Chemical Co., Oakville, Ontario) or other carbon sources, as indicated in the text. To test bioavailability, MSM was supplemented with either: 0.25% (v/v) ethanol; 0.2% (v/v) glycerol; or 10% (v/v) MCLAs (Sigma-Aldrich). For cultivation on solid medium [MSM plus 1.5% (w/v) agar; Difco], 50–100 μ l of immiscible carbon substrates were applied to the lid of Petri plates. The medium contained no other carbon source. *P. putida* strains were cultivated at 30 °C, while *P. aeruginosa* and *Escherichia coli* strains were incubated at 37 °C. When required, antibiotic (Sigma-

Aldrich) supplementation of media was as follows: 50 μ g ampicillin ml⁻¹ (*E. coli*), 50 μ g kanamycin (Km) ml⁻¹ (*E. coli*), 150 μ g Km ml⁻¹ (*Pseudomonas*), 10 μ g tetracycline (Tc), ml⁻¹ (*E. coli*), 50 μ g Tc ml⁻¹ (*Pseudomonas*). Stock cultures were stored in Luria Bertani (LB; Difco laboratories, Detroit, Mich.) broth, supplemented with dimethylsulfoxide (7.5% v/v) at -70 °C.

Transposon mutagenesis with antibiotic enrichment

Transposon mutants were generated by delivery of mini-Tn5Km::pUT into recipient *P. putida* ATCC 11172 by conjugation with *E. coli* S17-1 (de Lorenzo et al. 1990; Berg and Howe 1989). Aliquots (100 μ l) of donor and recipient were mixed, spotted onto LB agar plates, and incubated overnight at 30 °C. After incubation, the cells were recovered from the plates with MSM. The cells were washed twice and then plated onto MSM plates which had pyruvate as the carbon source and contained Km, to select for *P. putida* cells that had acquired the transposon. Following incubation, colonies were washed from these selection plates, transferred to MSM with decanol as the sole carbon source, and grown for approximately 4 h. Ceftazidime (250 μ g ml⁻¹; GlaxoSmithKline, Mississauga, Ontario) was then added and incubation was continued for another 3–4 h. The cells were then washed and used to inoculate MSM supplemented with pyruvate and Km, followed by incubation overnight. This antibiotic enrichment was repeated twice. Cells were finally plated onto LB plates and screened for growth on MSM with decanol as carbon source. Potential mutants were further screened in liquid culture for growth on various MCLAs.

DNA procedures

Chromosomal DNA was isolated by the technique of Ausubel et al. (1995). Basic molecular biological techniques were carried out, including plasmid isolation, restriction digestion, agarose gel electrophoresis, and cloning, using standard procedures (Sambrook et al. 1989). Direct isolation of specific DNA bands from agarose gels was performed by excision of appropriate gel fragments and DNA isolation, using either Qiaquick (Qiagen, Mississauga, Ontario) or Prep-a-Gene (BioRad, Hercules, Calif.) extraction kits.

To complement the mutation in the *agmR* gene, a copy of the gene PCR-amplified from wild-type *P. putida* was cloned into the *EcoRI* site of the broad-host-range vector pRK415 (Keen et al.

1988), yielding pAV8. pAV8 and pRK415 (control) were transformed into competent AVP2 and transformants were selected on 50 Tc plates. Transformants were then screened for their ability to grow on MCLAs.

Oligonucleotide probes for DNA hybridizations were designed, based on consensus sequences for *alkB* and *alkJ*, synthesized by Cortec DNA Laboratory Services (St. Lawrence College, Kingston, Ontario), and were labeled with γ -³²P-ATP, using T4 polynucleotide kinase and following the manufacturer's instructions (Life Technologies, Gaithersburg, Mo.). Chromosomal DNA (1.5 μ g) was digested with various restriction endonucleases and the DNA fragments were resolved on 0.8% (w/v) agarose gels. Prior to hybridization, the gels were dried onto Gelbond film (FMC Bioproducts, Rockland, Me.), as described by Naczynski and Kropinski (1993). Denaturation and subsequent hybridizations were performed as described by Sambrook et al. (1989). The Gelbond/DNA was then exposed to Kodak Scientific imaging film (X-OMAT AR).

PCR procedures

Inverse PCR was used to identify the site of transposon insertion (Moreau et al. 1997). Chromosomal DNA was digested with *Pst*I (Life Technologies), the restriction endonuclease was then thermally inactivated (80 °C, 20 min), and aliquots were incubated with T4 DNA ligase (Life Technologies) overnight at 16 °C. The resulting self-ligated DNA was precipitated with ethanol and resuspended in sterile, distilled water to give a final concentration of 10 ng μ l⁻¹. This was used as the template in PCR with primers based on the mini-Tn5 sequence.

Traditional PCR reactions were used for screening and amplification of entire genes. PCR reactions (100 μ l) contained either 2 μ l self-ligated chromosomal DNA or 30 ng native chromosomal DNA, 50 pmol of each primer, 0.2 mM concentrations of each deoxynucleotide triphosphate, 1 unit Taq DNA polymerase (Life Technologies), 1.5 mM MgCl₂, and 5 μ l DMSO. A thermocycler was programmed as follows: 2 min warm-up/hold at 94 °C, followed by 30 cycles of: 1 min at 94 °C, 1 min at annealing temperature (56 °C for inverse PCR), and 2 min at 72 °C, followed by a 10 min hold at 72 °C. PCR products were cloned into a pCR 2.1 TOPO vector, following the manufacturer's protocol (Invitrogen, Carlsbad, Calif.).

Sequence assembly and analysis

DNA sequencing was carried out by Cortec, using the fluorescent dye-dideoxy chain-termination method (Sanger et al. 1982). Sequences were stripped of poor-quality and vector sequences; and were then aligned and assembled, using Seqman software (DNA-Star, Madison, Wis.). ORFs were identified and analyzed, using Editseq (DNASTar) and ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). Sequences were scanned for homologues, using the BLAST suite of programs (Altschul et al. 1990, 1997). Protein masses and isoelectric points were determined online, using ProtParam tools (<http://www.expasy.ch/tools/protparam.html>). Similar sequences and homologues were compared using GeneStream's Align program (<http://www2.igh.cnrs.fr/bin/align-guess.cgi>). Proteins were examined for conserved motifs using Pfam (<http://pfam.wustl.edu/hmmsearch.shtml>), Prosite (<http://www.expasy.ch/tools/scnpsite.html>) and Smart (<http://smart.embl-heidelberg.de/index.shtml>). The sequences described in this work have been deposited with GenBank, under accession numbers AF315634 (*agmR*) and AY048591 (*exaA*).

Cell extracts and enzyme assays

Cultures grown overnight in pyruvate MSM were used to inoculate 0.5 l of fresh pyruvate MSM containing an appropriate inducing carbon source (either ethanol or decanol, 0.25% v/v). Uninduced extracts were prepared from cultures grown on pyruvate on-

ly. Following a 24-h growth period, cells were harvested for preparation of cell-free extracts. Cells were first washed several times with 30 ml of ice-cold phosphate buffered saline and then resuspended in 3 ml of ice-cold 50 mM Tris (pH 8.0) containing 3 mM CaCl₂ and 10 μ g deoxyribonuclease I (Boehringer Mannheim, LaVal, Canada). Cells were lysed using a French-Press (American Instrumentation Co, Silver Springs, Md.) at 55–70 MPa. The broken cell suspension was centrifuged at 6,000 g for 15 min to remove cell debris and unbroken cells; and the supernatant was used as the cell-free extract for NAD-dependent alcohol dehydrogenase assays. For PQQ assays, the extracts were centrifuged at 60,000 g for 1 h to remove membrane components; and the supernatant was retained for enzyme assay.

NAD-dependent dehydrogenase activity was measured as the increase in optical density at 340 nm (OD₃₄₀) caused by the reduction of NAD⁺. Standard assay reactions were performed at room temperature (23–25 °C) in a total volume of 1 ml, composed of 800 μ l of 0.032 M sodium pyrophosphate buffer (pH 9.0), 50 μ l of 0.15 mM NAD⁺, 100 μ l of 1 mM KCN, and 25 μ l of cell-free extract. The cuvette was blanked and the reaction initiated with 25 μ l of 0.05 M substrate. PQQ-linked alcohol dehydrogenase activity was measured spectrophotometrically by following the reduction of the artificial electron acceptor dichloroindophenol (DCIP) at 600 nm. Reaction cuvettes contained 650 μ l of 100 mM Tris HCl (pH 9.0) buffer, supplemented with 50 μ l of 5 mM ethylamine as an activator, 50 μ l of 0.1 mM KCN, and 25 μ l of cell-free extract, and reactions were initiated with a premix of DCIP, phenazine methosulfate, and substrate (0.2 mM, 1.0 mM for the dyes respectively, with 0.5 mM ethanol/glycerol, or 100 mM MCLA). A unit of enzyme was defined as a Δ OD min⁻¹ of 0.01; and activity was normalized with respect to the protein concentration of the cell-free extract. Protein content was determined using a modified Bradford assay (Bradford 1976), with bovine serum albumin as a standard.

Results

Growth analysis of *P. putida* ATCC 11172

Analysis of the substrate utilization pattern of *P. putida* ATCC 11172 revealed growth on C₂, C₈–C₁₂, and C₁₆ primary alcohols (but not on alkanes) as sole carbon sources, suggesting that this bacterium does not harbor the OCT plasmid. This was further indicated by the lack of hybridization with an *alkB* (alkane hydroxylase) probe and the absence of mercury resistance (an additional phenotype attributed to genetic elements on the OCT plasmid (Harder and Kunz 1986). Southern hybridization and PCR amplification efforts using primers to the *P. oleovorans alkJ* (alcohol dehydrogenase) sequence also failed to detect a homologue (data not shown). The apparent lack of an OCT plasmid and the absence of a chromosomal homologue for *alkJ* suggest the occurrence of an alternative pathway for MCLA utilization in this strain of *P. putida* (Chen et al. 1995).

Isolation of a *P. putida* ATCC 11172 alcohol non-utilizing mutant and identification of the affected gene

Transposon mutagenesis using the mini-Tn5::pUT delivery system (Berg and Howe 1989) was used to generate *P. putida* ATCC 11172 mutants no longer capable of

growing on decanol as sole carbon source. Over 10,000 mini-Tn5 mutants were screened and three exhibiting loss of growth on decanol were isolated (AVP1–AVP3). We focused on AVP2, which exhibited the greatest stability in maintenance of the mutant phenotype. Further growth analysis revealed a loss of growth on other MCLAs and on ethanol and glycerol.

The site of insertion of Tn5 was defined by inverse PCR and sequencing, which revealed that the transposon was inserted within an ORF with an overall nucleotide identity of 86% to *agmR* from *P. aeruginosa* (GenBank accession no. M60805). To obtain the sequence of the entire ORF, primers based on the *P. aeruginosa agmR* sequence were designed and used to amplify the corresponding segment from *P. putida* ATCC 11172 (AF315634).

The product of *P. putida agmR* is a polypeptide of 221 amino acids, with strong homology to the response regulators of two-component regulatory systems. The protein has a predicted mass of 24.4 kDa and an isoelectric point (pI) of 5.9. Pfam and Smart analysis identified the presence of two modules, a N-terminus receiving domain (34% homology to Pfam 00072), and a C-terminal helix-turn-helix motif (41% homology to Smart 00421). The C-terminal output domain of AgmR classifies in the LuxR subfamily and is believed to be involved in DNA-binding. That the mutation in *agmR* was solely responsible for the observed phenotype was verified by cloning the ORF into pRK415 (yielding pAV8) and introduction into AVP2. Transformants harboring pAV8 exhibited a restored ability to grow on alcohols. Acquisition of pRK415 did not affect growth (i.e., cells still exhibited the alcohol non-utilizing phenotype).

ADH activity

An analysis of ADH activity in the wild type and mutant was conducted in an effort to relate the mutation to a specific enzyme activity. Enzyme assays revealed the presence of very low-level, constitutive, NAD-dependent ethanol dehydrogenase activity in both wild-type and mutant extracts. No activity was measured using glycerol or decanol as substrates, indicating this ADH activity is not responsible for the growth of *P. putida* on decanol.

The PQQ assay used in this work was based on an assay developed to measure PQQ-dependent ADH activity identified in *P. aeruginosa* ATCC 17933 (Rupp and Gorisch 1988) and this strain was used as a control in establishing a functioning assay system in our laboratory. PQQ-linked ADH activity in both *P. aeruginosa* ATCC 17933 and *P. putida* ATCC 11172 was inducible and observed only in cell extracts prepared from cultures grown in the presence of ethanol. Significant differences in activity levels between the wild-type and mutant strains were observed with both ethanol and decanol as substrates, suggesting that this enzyme activity is implicated in the decanol- and ethanol-oxidizing capability of

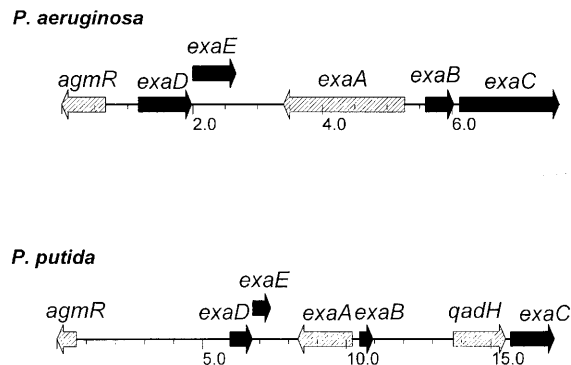


Fig. 1 The *agmR-exa* gene cluster of *Pseudomonas aeruginosa* (constructed from sequence data; GenBank accession no. AJ009858) and the *agmR-exa* cluster of *P. putida* (constructed from KT2440 sequence data at <http://www.tigr.org>). Genes encode: *exaD* sensor kinase, *exaE* response regulator, *exaA* quinoprotein ethanol dehydrogenase, *exaB* cytochrome c precursor, *qadH* uncharacterized pyrroloquinoline quinone (PQQ)-linked alcohol dehydrogenase detected from *P. putida* KT2440 genome sequence data, *exaC* aldehyde dehydrogenase. Scale markings represent DNA length in kilobases

Table 2 Pyrroloquinoline quinone-linked enzyme activity of *P. putida* strains. Enzyme units were measured as described in Materials and methods. Induction in all cases was with ethanol. Uninduced data give the average of two trials; induced data give the average of three trials; endogenous data are from induced cells with no substrate

Substrate	Strain 11172	Strain AVP2
	(enzyme units mg ⁻¹ protein)	
Uninduced (ethanol)	3.1	4.6
Induced		
Ethanol	90.7	9.8
Decanol	91.1	7.6
Endogenous	5.9	5.7

P. putida ATCC 11172 (Table 2). Efforts to assess activity from cells grown in the presence of decanol were unsuccessful, probably due to the inability to completely remove the organic during the preparation of extracts, resulting in enzyme saturation by residual substrate. Glycerol was not a substrate for the enzyme(s) under the assay conditions used.

Sequence of a quinoprotein ethanol dehydrogenase homologue in *P. putida* and identification of an *exa* gene cluster

In *P. aeruginosa* ATCC 17933, a quinoprotein ethanol dehydrogenase (QEDH) encoded by the 1,872 base pair *exaA* gene has been fully characterized. The *exaA* gene itself is part of a gene cluster encoding a PQQ-linked alcohol-utilization system (Fig. 1; Schobert and Görisch 1999). Using primers based on the *P. aeruginosa exaA* gene, a homologue was PCR-amplified from *P. putida*

Fig. 2 Clustal W alignment of ExaA amino acid sequences from *P. putida* ATCC 11172 (11172; this work, AY048591), KT2440 genome sequence (KT2440; <http://www.tigr.org>), *P. aeruginosa* PA01 (PA01; AJ009858), and the additional quinoprotein alcohol dehydrogenase (*QadH*) identified in the *P. putida* KT2440 sequence. Consensus amino acids of the PQQ beta-propeller repeat (Pfam 00564) occurring in enzymes with PQQ as cofactor are indicated by arrows and identical amino acids are highlighted

11172	MTIRSLPALSPALSVRVLVLLAGGLALGNVATAATAAPAAPAGKSVTWEDIANDHLTTKDV	60
KT2440	MTIRSLPALSPALSVRVLVLLMAGSLALGNVATAASTPAAPAGKSVTWEDIANDHLTTQDV	60
PA01	MTTRTSPAPAGLLRPS-LHCLAFVALG-----SAGAALAKDVTWEDIANDDKTTGDV	52
QadH	MTRSFRRLPLFAVSLVLSAMLLAG-----AAHAAVSNEEILQDPKPNPQQI	44
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11172	LQYGMGTNAQRWSPLAQVNDQNVFKLTPAWSYSGFDEKQKRGQESQLSSSDGVVYVVTGSYS	120
KT2440	LQYGMGTNAQRWSPLAQVNDKNVFKLTPAWSYSGFDEKQKRGQESQAIIVSDGVVYVVTGSYS	120
PA01	LQYGMGTHAQRWSPLKQVNADNVFKLTPAWSYSGFDEKQKRGQESQAIIVSDGVVYVVTASYS	112
QadH	VTNGLGVQGRYSPLDLLNVNPKLRPVWAFSGFGEKQKRGQQAQPLIKDGMVYLTGSYS	104
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11172	RVFALDAKTGKRLWYINHRLPDNIRPCCDVVNRGAAIFGDKIYFGTLDARLIALDKHTGK	180
KT2440	RVFALDAKTGKRLWYINHRLPDNIRPCCDVVNRGAAIYGDKIYFGTLDARVIALDKRTGK	180
PA01	RFALDAKTGKRLWYINHRLPDDIRPCCDVVNRGAAIYGDKVFFGTLDASVVALNKNKTGK	172
QadH	RVFAVDARTGKRLWYDARLPDDIRPCCDVINRGVALYGNLFFGTLDARLVALNKNKTGK	164
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11172	VVWNKKFGDHSAGYTMGTAPVLIKDKTSGKVLIIHGSSGDFEFGVVGQLFARDPDTGEEVW	240
KT2440	VVWNKKFGDHSAGYTMGTAPVLIKDKTSGKVLIIHGSSGDFEFGVVGQLFARDPDTGEEVW	240
PA01	VVWNKKFADHGAGYTMGTAPVIVKDKTSGKVLIIHGSSGDFEFGVVGRLFARDPDTGEEIW	232
QadH	VVWSKKVADHKEGYSISAAPMIVNGK-----LITGVAGGEFGVVGKIQAYNPENGELLW	218
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11172	MRPFVEGHMGRNLNGKDSPTPTGDVKAPSWPDDPTTETGKVEAWSHGGGAPWQSASFDPETN	300
KT2440	MRPFVEGHMGRNLNGKDSPTPTGDVKAPSWPDDPTTETGKVEAWSHGGGAPWQSASFDPETN	300
PA01	MRPFVEGHMGRNLNGKDSPTPTGDVKAPSWPDDRNSPTGKVESWSHGGGAPWQSASFDAETN	292
QadH	MRPTVEGHMGVYKDGKAIENGISG-----GEAGKTWPGLWKTGGAPWLGYYDPETN	273
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11172	TIIIVGAGNPGPWNTWARTSKDGNPHDFDSLTYSGQVGVDPSTGEVVKWFYQHTPNDAWDFS	360
KT2440	TIIIVGAGNPGPWNTWARTSKDGNPHDFDSLTYSGQVGVDPSTGEVVKWFYQHTPNDAWDFS	360
PA01	TIIIVGAGNPGPWNTWARTAKGGNPHDYDSLTYSGQVGVDPSSGVEVVKWFYQHTPNDAWDFS	352
QadH	LILFGTGNPAPWNSHLRPG-----DNLVSSRLALNDDGTGKWHFQSTPHDGDWDFD	325
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11172	GNNELVLFYDKDKNGNVKATAHADRNNGFFVVDNRNNGKLNQAFPFVDNITWASHIDLKT	420
KT2440	GNNELVLFYDKDKNGNVKATAHADRNNGFFVVDNRNNGKLNQAFPFVDNITWASHIDLKT	420
PA01	GNNELVLFYKAKDGKIVKATAHADRNNGFFVVDNRNNGKLNQAFPFVDNITWASHIDLKT	412
QadH	GVNELISFNKYD-GGKEVKAAATADRNNGFFVVDNRNNGKLNQAFPFVDNITWATGLD-KD	383
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11172	GRPVENXGQRPAPKPLPGETKGPVEVSPFPLGGKNWNPMAYSQDTGLFYIPGNQWKEEYW	480
KT2440	GRPVENXGQRPAPKPLPGETKGPVEVSPFPLGGKNWNPMAYSQDTGLFYIPGNQWKEEYW	480
PA01	GRPVREGQRPPLPEPGQKHGKAVEVSPFPLGGKNWNPMAYSQDTGLFYIPANHWKEDYW	472
QadH	GRPIYNDASRPGAP-GSEAKGSSVFVAPFLGAKNWNMPMAYNKDTGLFYIPSNWGMADIW	442
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11172	TEEVNYKKGSAIYLGMGFRIKRMYYDDHVGTLRAMDPTTGKLVWEHKEHLPLWAGVLTATKGN	540
KT2440	TEEVNYKKGSAIYLGMGFRIKRMYYDDHVGTLRAMDPTTGKLVWEHKEHLPLWAGVLTATKGN	540
PA01	TEEVSYTKGSAIYLGMGFRIKRMYYDDHVGTLRAMDPTTGKLVWEHKEHLPLWAGVLTATAGN	532
QadH	NEGIAKKGAAFLGAGFTIKPLNEDYIGVLRADIPVSGKEVVRHKNYAPLWGGVLTATKGN	502
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11172	LVFTGTGDFGFFKAFDAKTGKELWKFQTSVSPITWEQDGEQYIGVTVGYGGAVPLWG	600
KT2440	LVFTGTGDFGFFKAFDAKTGKELWKFQTSVSPITWEQDGEQYIGVTVGYGGAVPLWG	600
PA01	LVFTGTGDFGFFKAFDAKTGKELWKFQTSVSPITWEQDGEQYIGVTVGYGGAVPLWG	592
QadH	LVFTGTPEGFLOAFNAKTGDKVWFQTSVSPITWEQDGEQYIGVTVGYGGAVPLWG	562
	***** * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *	
		* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
11172	GDMAELTKPVAQGGSFVWFKIPSWDNKTAQR--	631
KT2440	GDMAELTKPVAQGGSFVWFKIPSWDNKTAQR--	631
PA01	GDMADLTPVAQGGSFVWFKIPSWDNRTASR--	623
QadH	GEVAKRVKDFNQGGMLWTFKLPKQLQQTASVKP	595
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ATCC 11172 (AY048591). The *exaA* gene product from *P. putida* ATCC 11172 shared 86% amino acid identity with ExaA of *P. aeruginosa* (AJ009858), and 96% identity with the ExaA of *P. putida* KT2440 (<http://www.tigr.org>; Fig. 2). The ExaA of *P. putida* ATCC 11172 is a protein of 634 amino acids, with a calculated mass of 69 kDa and a pI of 6.4. The creation of a null mutation in *exaA* slowed, but did not eliminate, *P. putida* ATCC 11172's ability to grow on decanol or ethanol (data not shown).

Discussion

The majority of available information on MCLA utilization is linked to alkane degradation and hence initial efforts to identify and eliminate MCLA utilization involved looking for homologues of *alk* system genes (*alkB*, *alkJ*) within *P. putida* ATCC 11172. The lack of evidence for *alkJ* homologues within this strain indicated either that the sequence of the *alkJ* gene was sufficiently different to prevent detection with consensus primers, or

that a different pathway was responsible. In the absence of known targets, transposon mutagenesis was successfully employed to obtain an alcohol non-utilizing mutant. Unlike the expected ADH-insertion mutant, we identified a mutation in *agmR* which led to the loss of PQQ-linked ADH activity and eliminated the cells' ability to grow on a variety of alcohols and glycerol.

AgmR was first identified by Schweizer (1991) during work on glycerol metabolism in *P. aeruginosa*, in which the protein was shown to positively influence glycerol metabolism. It was subsequently recognized, however, that this was not the prime role of AgmR (Schweizer 1991). The difference between glycerol metabolism (which involves initial phosphorylation and dehydrogenation to dihydroxyacetone phosphate) and alcohol utilization (which proceeds via consecutive oxidations) strongly implies that AgmR has a regulatory influence on more than one pathway within *P. putida*. Furthermore, the observed difficulty in obtaining an alcohol non-utilizing mutant defective in a structural gene suggests the presence of multiple ADHs able to react specifically with MCLAs, all influenced or under the control of a regulatory cascade involving AgmR.

Examination of the unfinished *P. putida* KT2440 genome sequence data revealed the presence of an *exa* cluster (*exaA*–*exaE*) homologous to the gene cluster in *P. aeruginosa*, indicating conservation of this pathway within fluorescent pseudomonads. *agmR* maps adjacent to this region (Fig. 1). Interestingly *P. putida* has a 5.3-kb gap between *agmR* and *exaD*, whereas in *P. aeruginosa* only 547 bp separate these two genes. Furthermore, *P. putida* has a gene encoding a second PQQ-linked ADH (labeled *qadH* in Fig. 1) within the *exa* cluster (between *exaB* and *exaC*). QadH shares approximately 51% amino acid identity with ExaA (Fig. 2). This redundancy potentially explains why our *exaA* mutant still metabolized decanol and explains the initial difficulty in obtaining a decanol non-utilizing mutant.

Examples of enzyme multiplicity (metabolic redundancy) have been alluded to with the *alk* system and are abundant in microbial metabolism, with multiple pathways often acting to degrade even simple substrates (Van Beilen et al. 2001). Toyama et al. (1995) showed the occurrence of three distinct quinoprotein ADHs, with sometimes overlapping substrate specificities, in a single *P. putida* strain. One of these enzymes exhibited a substrate range that included ethanol and a number of longer alcohols (e.g., heptanol, octanol) and appears similar to a quinoprotein ADH previously purified from *P. aeruginosa* (Groen et al. 1984) and *P. putida* (Görisch and Rupp 1989). Evaluation of information in the literature and sequence data show that this dehydrogenase is most likely a homologue of the protein encoded by the *exaA* gene of *P. aeruginosa* (Diehl et al. 1998). One of the other two ADHs identified by Toyama et al. (1995) exhibited a similar alcohol substrate range as the above enzyme, while also utilizing glycerol. Unfortunately

however, no sequence information on this enzyme is available. The partial inhibition of growth on decanol and ethanol of the *P. putida exaA* mutant suggests the occurrence of at least one other PQQ-linked ADH within *P. putida* ATCC 11172. This is further supported by our detection of a second PQQ-linked ADH gene within the *exa* cluster.

Functional equivalencies and sequence similarities between PQQ-linked ADHs and methanol dehydrogenases (MDHs) of methylotrophic bacteria, such as *Methylobacterium extorquens* AM1, *M. organophilum* XX and *P. denitrificans*, led to the conclusion that the PQQ-dependent dehydrogenases are derived from one common ancestor; and it is hypothesized (Stouthamer 1992) that the regulatory mechanisms are also conserved (Cleton-Jansen 1991). Expression of MDHs proceeds via a complex pathway involving at least 26 genes mapping to a number of different loci (Harms et al. 1993; Springer et al. 1997).

In *Methylobacterium* species, MxcQE controls the level of expression of *mxhDM* and MxbDM then in turn controls expression of other *mxh* genes involved in methanol oxidation (Xu et al. 1995; Springer et al. 1997). Similarly, in *P. denitrificans*, two regulator-sensor pairs (MxaXY, FlhRS), potentially acting in concert, have been implicated in the regulation of MDH expression (Harms et al. 2001). The FlhR amino acid sequence shares 26–28% identity with its closest neighbors, which include *P. aeruginosa* AgmR, and 15–21% similarity with other response regulators involved in methanol oxidation from other methylotrophs. The occurrence of at least two, two-component regulatory systems has been demonstrated for MDH regulation in methylotrophs (Springer et al. 1997; Goodwin and Anthony 1998; Harms et al. 2001); and recent work by Schobert and Görisch (2001) has predicted the occurrence of at least four different gene products involved in regulating the *exa* pathway of *P. aeruginosa*. The homology of AgmR to FlhR, the observed phenotype of the *agmR* mutant, and the localization of *agmR* adjacent to the conserved *exa* gene cluster support the hypothesis that AgmR is a component in the complex regulation of PQQ-linked ADH activity in pseudomonads.

Through the application of molecular tools, we addressed a biochemical process issue from a catalyst perspective and eliminated an undesirable characteristic, rather than modifying the process strategy. Mutation in *agmR* allowed us to achieve our goal of creating a mutant unable to grow on a variety of MCLAs and, for our purposes, xenobiotic delivery within TPPBs, potentially presents greater benefits than mutation in a single dehydrogenase with more limited (often duplicated) substrate specificities. Further molecular study will enable elucidation of the structural targets and regulatory cascade involving AgmR.

Acknowledgements We thank Caroline Harwood for providing the mini-Tn5-bearing strain. Funding for this work provided by the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

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