RESEARCH LETTER

Identification of genes involved in the 4-aminobenzenesulfonate degradation pathway of *Hydrogenophaga* sp. PBC via transposon mutagenesis

Han Ming Gan[1], Zaharah Ibrahim[1], Shafinaz Shahir[1] & Adibah Yahya[2]

[1]Department of Biological Sciences, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, Johor, Malaysia; and [2]Research Laboratory 2, Level 2, Building C08, Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, Johor, Malaysia

Correspondence: Adibah Yahya, Research Laboratory 2, Level 2, Building C08, Department of Industrial Biotechnology, Faculty of Biosciences and Bioengineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia. Tel.: +60 7553 4157; fax: +60 7553 1112; e-mail: adibahfbb@gmail.com

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Abstract

Genes involved in the 4-aminobenzenesulfonate (4-ABS) degradation pathway of *Hydrogenophaga* sp. PBC were identified using transposon mutagenesis. The screening of 10 000 mutants for incomplete 4-ABS biotransformation identified four mutants with single transposon insertion. Genes with insertions that impaired the ability to utilize 4-ABS for growth included (1) 4-sulfocatechol 1,2-dioxygenase β-subunit (*pcaH2*) and 3-sulfomuconate cycloisomerase involved in the modified β-ketoadipate pathway; (2) 4-aminobenzenesulfonate 3,4-dioxygenase component (*sadA*) involved in aromatic ring hydroxylation; and (3) transposase gene homolog with a putative *cis*-diol dehydrogenase gene located downstream. The *pcaH2* mutant strain accumulated brown metabolite during growth on 4-ABS which was identified as 4-sulfocatechol through thin layer chromatography and HPLC analyses. Supplementation of wild-type *sadA* gene in *trans* restored the 4-ABS degradation ability of the *sadA* mutant, thus supporting the annotation of its disrupted gene.

Introduction

4-Aminobenzenesulfonate (4-ABS) is commonly used as intermediate in the manufacturing of dyes, brighteners and sulfa drugs. Degradation of 4-ABS is problematic due to poor permeability across the bacterial membrane (Hwang *et al.*, 1989), high C–S bond stability (Wagner & Reid, 1931) and potential bacteriostatic effect (Brown, 1962). Constant exposure of bacteria to 4-ABS induces selection of enzymatic pathways necessary for the utilization of 4-ABS as an energy source. In the last two decades, 4-ABS degradation has been described in the genus *Hydrogenophaga*, *Sphingomonas*, *Agrobacterium* and *Pannonibacter* (Feigel & Knackmuss, 1988; Perei *et al.*, 2001; Singh *et al.*, 2004; Wang *et al.*, 2009).

The first isolated 4-ABS degraders were two-membered co-cultures consisting of *Hydrogenophaga intermedia* S1 and *Agrobacterium radiobacter* S2 (Feigel & Knackmuss, 1988; Contzen *et al.*, 2000). *Hydrogenophaga intermedia* S1 can degrade 4-ABS as a pure culture when vitamins are added to the medium (Dangmann *et al.*, 1996). To date, enzymes involved in the lower pathway of 4-ABS degradation in *H. intermedia* S1 have been characterized through heterologous expression in *Escherichia coli* host (Contzen *et al.*, 2001; Halak *et al.*, 2006; Halak *et al.*, 2007). However, studies focusing on the upper pathway converting 4-ABS to 4-sulfocatechol have hitherto been scarce. Furthermore, the phenotype arising from the individual inactivation of 4-ABS-associated catabolic genes still remains unknown. To determine this and further elucidate the 4-ABS degradation pathway, it is necessary to perform genetic studies in the native microorganism.

So far, the characterization of *Hydrogenophaga* strains involves 16S rRNA gene-based phylogenetic analysis, biochemical tests, DNA G+C content determination and DNA–DNA hybridization (Kampfer *et al.*, 2005; Chung

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et al., 2007; Yoon *et al*., 2008). Although some strains show potential in the degradation of biphenyls and methyl-tert-butyl ether (Hatzinger *et al*., 2001; Lambo & Patel, 2006), the genetic aspects of the degradation pathway for these compounds are still unknown. Furthermore, there are no reports on *in vivo* genetic modification within the genus *Hydrogenophaga*.

Hydrogenophaga sp. PBC is a Gram-negative bacterium isolated from textile wastewater for its ability to degrade 4-ABS (Gan *et al*., 2011). Similar to *H. intermedia* S1, strain PBC can degrade 4-ABS in the presence of vitamins. In this study, we describe the isolation and characterization of genes affecting 4-ABS biotransformation using a transposon mutagenesis approach.

Materials and methods

Culture conditions

Hydrogenophaga sp. PBC was grown at 30 °C in nutrient broth (NB) containing 5 g L^{-1} peptone and 3 g L^{-1} beef extract, super optimal broth (SOB) (Hanathan, 1983) or phosphate-buffered minimal salt (PB) media containing 0.09 mM $MgSO_4$, 0.042 mM KCl, 7.5 mM $NaHPO_4$, 7.5 mM $KHPO_4$, 15 mM KH_2PO_4, 0.0068 mM $FeCl_3$, 0.1 mM $CaCl_2$ and 0.001% w/v yeast extract. $(NH_4)_2SO_4$, 2.5 mM, was included in PB medium to give PBN medium. Kanamycin or gentamycin was used at a concentration of 25 μg mL^{-1} when necessary. 4-ABS was added to a final concentration of 2–6 mM from a filter-sterilized stock solution of 500 mM.

Electrocompetent cell preparation and transposon mutagenesis

To prepare electrocompetent cells of strain PBC, an overnight culture in SOB was diluted (1 : 10 v/v) and cultured for 6 h to early log phase ($OD_{600\,nm}$ of 0.3). Then the culture was cooled on ice for 30 min and washed twice with 10% glycerol (v/v). Electroporation of the electrocompetent cells with EZ-Tn5™ ⟨KAN-2⟩ Tnp Transposome™ (Epicentre) was carried out in a chilled 0.1-cm gap electroporation cuvette at 1.5 kV using an Eppendorf Multiporator. Immediately after pulse delivery, 1 mL of SOB medium was added to the cells. After 3 h of incubation with shaking, cells were plated on nutrient agar supplemented with kanamycin.

Mutant screening

Transposon mutants were individually inoculated using a sterile toothpick into a 96-well plate containing NB, 5 mM 4-ABS and 25 μg mL^{-1} kanamycin followed by incubation for 5 days with shaking at 150 r.p.m. 4-ABS was detected using Ehrlich's reagent (Meyer *et al*., 2005). A 10-μL aliquot of culture was mixed with 90 μL of 10-fold diluted Ehrlich's reagent. Formation of yellow-colored product indicated the presence of 4-ABS, and a potential mutation in a gene involved in 4-ABS degradation.

PCR and Southern blot analyses

Total genomic DNA was isolated using Qiagen DNAeasy Blood and Tissue Kit according to manufacturer's instructions. Presence of transposon was validated with PCR using reverse-complemented transposon mosaic end 5′-CTGTC TCTTATACACATCT-3′ as forward and reverse primers. PCR conditions were an initial denaturation step at 94 °C for 5 min, followed by 30 cycles of 94 °C (1 min), 50 °C (30 s), and 72 °C (1.2 min), plus a final 10-min chain elongation cycle at 72 °C.

For Southern blot analyses, 2 μg of genomic DNA was double digested with restriction enzymes ApaI and SacI for 3 h, separated on 0.75% agarose gel and transferred to positively charged nylon membrane (Roche Applied Science). Hybridization and labeling of probe were performed using DIG High Prime DNA Labeling and Detection Starter Kit 1 according to manufacturer's instructions (Roche Applied Science). Template for the probe was constructed via PCR with the same reverse-complemented mosaic end primer as described above.

Determination of insertion sites

Total genomic DNA was digested using EcoRI, ApaI or SacI (Promega), which does not cut within the transposon site, and was ligated into pUC19 (Yanisch-Perron *et al*., 1985) or pBBR1MCS-5 (Kovach *et al*., 1995). The ligation products were transformed into *E. coli* TOP10 (Invitrogen) and selected on Luria–Bertani agar with kanamycin. DNA sequencing of the insertion site was done using KAN-2 FP-1 forward primer 5′-ACCTACAACAAAGCTCTCATCAACC-3′ and KAN-2 RP-1 reverse primer 5′-GCAATGTAACATCA GAGATTTTGAG-3′ (Epicentre). In some cases, plasmid inserts were further sequenced by primer walking to obtain additional DNA sequence located upstream and downstream of the disrupted gene.

Substrate utilization test

NB-grown mutants were spun down, washed twice with PB medium and inoculated into PBN medium with 3 mM of 4-ABS, 4-sulfocatechol, protocatechuate or *p*-hydroxybenzoate. In addition, utilization of 4-ABS as sole nitrogen source was examined by growing mutants in PB medium with 3 mM of 4-ABS and gluconate. After 5 days of incubation with shaking at 150 r.p.m., growth was quantified by measuring $A_{600\,nm}$.

Identification of brown metabolite secreted by RK1

Cells were grown in PBN medium supplemented with 5 mM of gluconate and 4-ABS. Samples were withdrawn every 48 h, filter sterilized and stored at −20 °C for subsequent analysis. For thin layer chromatography (TLC) analysis, 7.5 μL of sample was spotted onto a C_{18} RP TLC plate (Merck). The plate was allowed to dry and developed in mobile phase of butanol–propanol–acetic acid–water at 8 : 4 : 1 : 1 (Feigel & Knackmuss, 1988). HPLC analysis was performed using Waters 600 equipped with a 4.6 × 250 mm Zorbax SB-Aq column (Agilent, Santa Clara, CA). The mobile phase consisted of 98% water, 1% methanol and 1% phosphoric acid (85%) at a flow rate of 1.0 mL min^{-1}. Detection was carried out at 230 nm. 4-Sulfocatechol standard was synthesized according to published method (Saito & Kawabata, 2006). Chromogenic detection of diphenolic intermediate in catabolism of 4-ABS was done by growing cells on nutrient agar supplemented with 50 μg mL^{-1} *p*-toluidine and 0.5 mM $FeCl_3$ (Parke, 1992).

Construction of plasmids for *trans*-complementation of RK32 and RK40

To complement RK40, the DNA region spanning phthalate dioxygenase-like gene and its putative promoter was amplified from wild-type PBC with primers PDOF 5′-TACTTG CCGGTCTCGTTCG-3′ and PDOR 5′-GTTCGGGGGTGT GCAGTC-3′, cloned into pGEM-T Easy vector (Promega) and subcloned as an EcoRI fragment into pBBR1MCS-5 (Kovach *et al.*, 1995) to give pHG5. A similar approach was applied to RK32 complementation using primers DEHF 5′-GTTGAGACGCTCGTTGACC-3′ and DEHR 5′-TTTG CCTGAGAAATGTGTCG-3′ to amplify the ORFs of transposase and putative dehydrogenase to give pHG6. Plasmids were transformed into mutants via electroporation.

4-ABS-dependent oxygen uptake assay

Oxygen uptake was measured using a Clark-type oxygen electrode (YSI 5905, Yellow Springs Instruments). Cells were pregrown in 20 mL NB medium, harvested by centrifugation and grown in 50 mL 0.5 × NB medium with 5 mM 4-ABS for 36 h to induce 4-aminobenzenesulfonate 3,4-dioxygenase activity. Cells were then harvested, washed twice with 25 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer containing 1 mM 4-ABS ($OD_{600\,nm}$ of 0.15–0.2). Oxygen uptake was measured polarographically at 30 °C for 2 h.

Nucleotide sequence accession numbers

DNA sequences of insertion site in RK1, RK23, RK32 and RK40 were deposited in EMBL Nucleotide Sequence Database and assigned accession numbers FR720595, FR720597, FR720598 and FR720599, respectively.

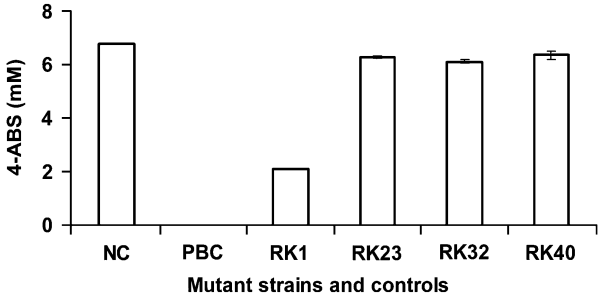

Fig. 1. Final concentration of 4-ABS in NB after 5 days of incubation with mutant and control strains. The data are means of two replications, and error bars represent SDs. NC, no cell control.

Results

Transposon mutagenesis and mutant screening

From three different electroporation experiments, approximately 10 000 kanamycin-resistant colonies were obtained, representing an average transformation efficiency of 1.7×10^5 CFU μg^{-1} transposon. Four mutants, designated RK1, RK23, RK32 and RK40, of 40 mutants from the initial screening were selected for further analysis due to their inability to completely biotransform 4-ABS in NB (Fig. 1). Other mutants were deselected either due to failure to exhibit the same phenotype after a subsequent confirmation test or because they had an insertion in the same gene.

Single transposon insertion and gene disruption in mutants

PCR using the reverse-complemented mosaic end of the transposon on mutant genomic DNA produced a band of approximately 1200 bp which was absent when wild-type genomic DNA was used (data not shown). Southern blot analysis showed that all mutants contained only one copy of transposon, while no hybridized band could be detected in wild-type PBC genomic DNA (Fig. 2).

All DNA fragments containing transposons from the mutants could be cloned into high-copy-number vector, pUC19 except for RK32, in which only ligation with low-copy-number vector, pBBR1MCS-5, was successful. Plasmids were purified and sequenced using primers described in Materials and methods. The disrupted gene in each mutant is shown in Table 1.

Carbon utilization test

The effect of gene disruption in each mutant was investigated by testing the ability of mutants to utilize aromatic compound associated with 4-ABS or the β-ketoadipate pathway (Table 2).

All strains could grow on protocatechuate and 4-hydroxybenzoate. RK32 and RK40 could utilize 4-sulfocatechol but not 4-ABS. In contrast, 4-ABS and 4-sulfocatechol could not serve as sole carbon source for RK1 and RK23. However, RK1 could still utilize 4-ABS as sole nitrogen source with accumulation of brown metabolite during growth.

Identification of metabolite secreted by RK1

Based on the gene disrupted in RK1 and the color of the metabolite, we assumed that the secreted metabolite was 4-sulfocatechol. RK1 was grown on nutrient agar containing *p*-toluidine, $FeCl_3$ and 4-ABS. Within 48 h of incubation, the medium surrounding the patch of RK1 turned purple (Fig. 3a), indicating the presence of diphenolic compound (Parke *et al.*, 1992).

After 48 h of growth, TLC analysis of cell-free supernatant from RK1 grown in 4-ABS and gluconate showed a new spot with an R_f value of 0.49, similar to 4-sulfocatechol standard, which persisted after prolonged incubation (Fig. 3b); this was not detected in wild-type supernatant. A similar trend was observed in HPLC analysis, supporting the identity of the brown metabolite as 4-sulfocatechol (Fig. 3c).

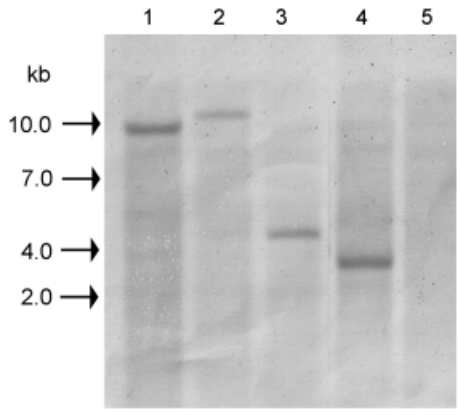

Fig. 2. Southern blot showing transposon as single-copy gene in each mutant. Lanes: 1, RK40; 2, RK32; 3, RK23; 4, RK1; 5, wild-type PBC.

Gene coding for dehydrogenase is located downstream of disrupted integrase gene in RK32

Further sequencing of plasmid containing RK32 EcoRI genomic DNA fragment with transposon insertion revealed an ORF coding for a putative dehydrogenase which overlapped the transposon-disrupted transposase gene by 4 bp and utilized the alternative start codon TTG. The dehydrogenase was 62.8% identical to a short-chain alcohol dehydrogenase/reductase of *Burkholderia* sp. CCGE1002 (ADG17624) and 61.2% identical to the 1,2-dihydroxy-3,5-cyclohexadiene-1,5-dicarboxylate dehydrogenase of *Comamonas* sp. E6 (BAH70271) and *Comamonas* sp. YZW-D (AAX18936).

Trans-complementation of mutations in RK32 and RK40

The ability of plasmids pHG5 and pHG6 to restore the 4-ABS degradation in RK40 and RK32, respectively, was assessed by growing the cells in NB supplemented with 4-ABS. After 5-day incubation, RK40(pHG5) showed partial removal of 4-ABS in NB (Fig. 4a), whereas no 4-ABS removal could be observed for RK32(pHG6) (data not shown). Positive control strain PBC(pBBR1MCS-5), on the contrary, exhibited complete removal of 4-ABS. 4-ABS-dependent oxygen uptake was also measured using cell suspension as an indirect measurement of 4-aminobenzenesulfonate 3,4-dioxygenase activity. RK40(pHG5) showed

Table 1. Insertion site of each mutant and percent identity with published sequences

Strains	Homologs of disrupted ORF	Identity (%)	Accession number
RK1	*Hydrogenophaga intermedia* S1	99.6	AF312376
	Protocatechuate-3,4-dioxygenase type 2 β-subunit (*pcaH2*)		
	Novosphingobium subarcticum	64.0	
	Sulfocatechol 3,4-dioxygenase (*scaE*)		AY700015
RK23	*Hydrogenophaga intermedia* S1	100	AY769868
	3-Carboxy-*cis*,*cis*-muconate lactonizing enzyme (*pcaB2*)		
	Novosphingobium subarcticum	58.2	
	3-Carboxy-*cis*,*cis*-muconate cycloisomerase (*pcaB*)		AY700015
RK32	*Burkholderia multivorans*	63.3	BAG47690
	ISBmu21 transposase		
	Polaromonas sp. JS666	63.3	ABE43514
	Integrase, catalytic region		
RK40	*Polaromonas* sp. JS666	69.8	ABE44060
	Phthalate 4,5-dioxygenase (PhDO)-like subfamily		
	Burkholderia phymatum STM815	65.6	ACC75988
	Rieske domain protein		

Table 2. Carbon utilization of mutants and wild type

	Strains				
Carbon source	PBC	RK1	RK23	RK32	RK40
4-ABS	+	–	–	–	–
4-ABS*	+	–	–	–	–
4-ABS+gluconate*	+	+†	–	–	–
Gluconate	+	+	+	+	+
4-Sulfocatechol	+	–	–	+	+
Protocatechuate	+	+	+	+	+
4-Hydroxybenzoate	+	+	+	+	+
4-Sulfocatechol+succinate	ND	ND	+	ND	ND

*Grown in PB medium.
†Accumulation of brown metabolite.
+, Growth; –, no growth; ND, not determined.

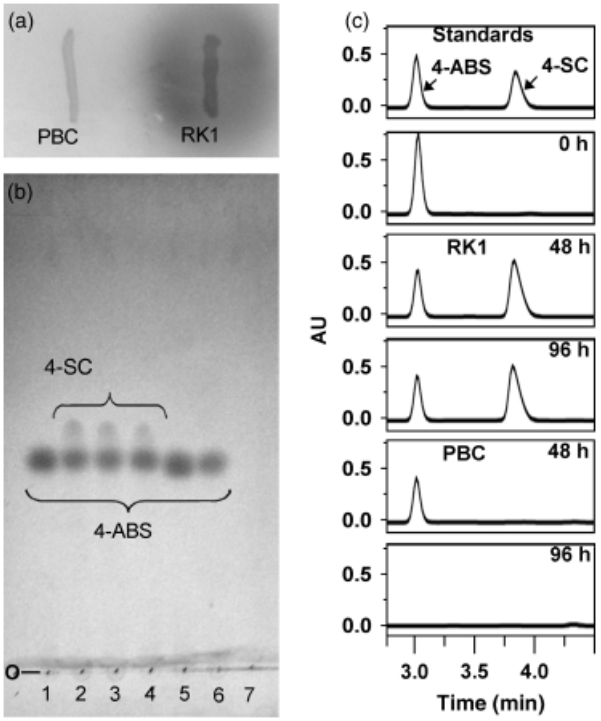

Fig. 3. Identification of metabolite secreted by RK1. (a) Accumulation of diphenolic metabolite by RK1 on nutrient agar supplemented with 5 mM 4-ABS. (b) TLC analysis of cell-free supernatant from RK1 at 0, 48 and 96 h (lanes 1, 2 and 3, respectively); PBC at 0, 48 and 96 h (lanes 5, 6 and 7, respectively); 2.5 nmol of 4-sulfocatechol (4-SC) and 4-ABS standards (lane 4); O, point of origin. (c) Evidence of 4-SC as the accumulated metabolite in RK1 via HPLC analysis.

approximately sevenfold higher 4-ABS-dependent oxygen uptake rate than control strain RK40(pBBR1MCS-5) (Fig. 4b). RK40(pHG5) also regained its ability to grow on 4-ABS as sole carbon and nitrogen source in PB medium, albeit, with an additional 96 h of lag phase compared with PBC(pBBR1MCS-5) (Fig. 4c and d).

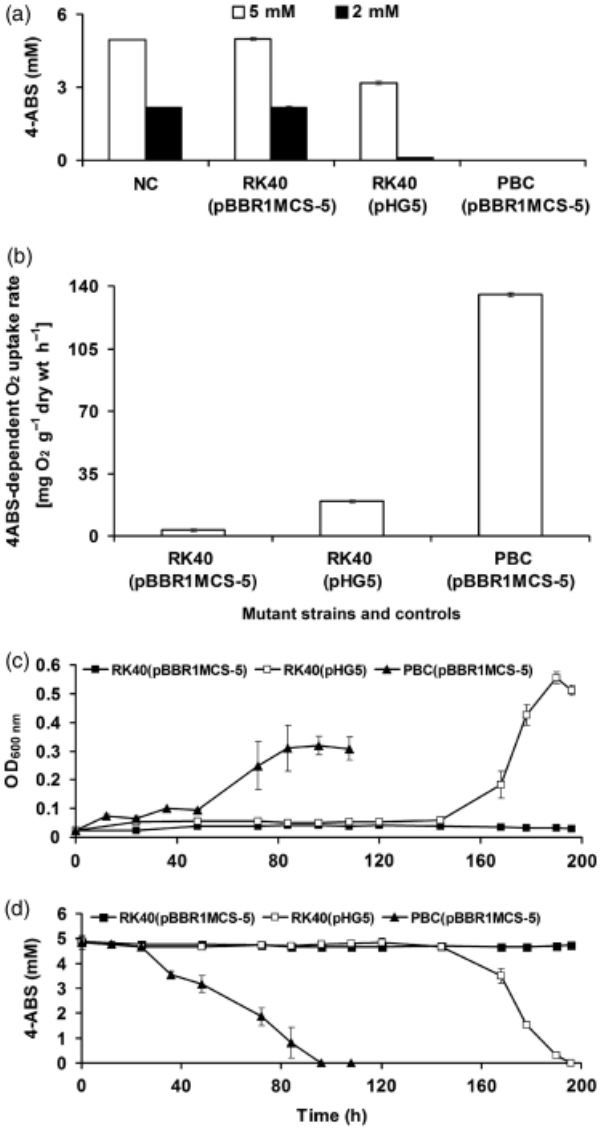

Fig. 4. Complementation of RK40. (a) Quantification of 4-ABS in NB medium after 3 days of incubation with RK40(pHG5), RK40(pBBR1MCS-5) or PBC(pBBR1MCS-5). (b) 4-ABS-dependent oxygen uptake of strains after subtraction with endogenous respiration. (c) Growth profile in minimal media supplemented with 5 mM 4-ABS. (d) Quantification of 4-ABS in media during growth. The data are means of two replications, and error bars represent SDs. NC, no cell control.

Discussion

Study of the 4-ABS metabolic pathway has hitherto been limited to enzymology work focusing on the lower pathway converting 4-sulfocatechol to β-ketoadipate (Contzen *et al.*, 2001; Halak *et al.*, 2006; Halak *et al.*, 2007). In this study, we describe the isolation and characterization of mutants with single insertion in genes affecting 4-ABS degradation of *Hydrogenophaga* sp. PBC.

Several pieces of evidence collected for RK1 point to a mutation in the 4-sulfocatechol 1,2-dioxygenase gene. First, RK1 exhibited no growth with 4-ABS and 4-sulfocatechol as sole carbon source but utilized 4-ABS as sole nitrogen source. Secondly, the secreted brown metabolite was identified as 4-sulfocatechol through HPLC and TLC comparison with authentic standard. The gene annotation was further supported by the strikingly high sequence identity (99.6%) of the disrupted gene to 4-sulfocatechol 1,2-dioxygenase sequence of *H. intermedia* S1 (Contzen *et al.*, 2001). As 4-sulfocatechol 1,2-dioxygenase of *H. intermedia* S1 could oxidize protocatechuate (Contzen *et al.*, 2001), the ability of RK1 to utilize protocatechuate as carbon source was tested. Growth of RK1 on protocatechuate (Table 2) suggests that 4-sulfocatechol 1,2-dioxygenase is not required for protocatechuate utilization and implies the existence of an alternative pathway for the degradation of this phenolic compound.

3-Sulfomuconate cycloisomerase gene is responsible for the conversion of 3-sulfomuconate to 4-sulfomuconolactone in the lower pathway of 4-ABS degradation (Halak *et al.*, 2006). Transposon insertion in the 3-sulfomuconate cycloisomerase gene of RK23 severely impaired its ability to degrade 4-ABS in NB. A similar result was obtained even when it was cultured in minimal media supplemented with protocatechuate as a source of β-ketoadipate, a general inducer of most aromatic compound degradation pathways (data not shown), suggesting that 3-sulfomuconate is a strong repressor and/or its metabolic product, 4-sulfomuconolactone, is an inducer of the 4-ABS biotransformation pathway. The possibility of 3-sulfomuconate being a highly toxic compound, as reported for its analog β-carboxy-*cis*,*cis*-muconate (Parke *et al.*, 2000), is somewhat implausible as RK23 showed growth in 4-sulfocatechol-containing mineral medium using succinate as alternative carbon source (Table 2).

Although various 4-ABS-degrading microorganisms have been isolated in the last two decades (Feigel & Knackmuss, 1988; Perei *et al.*, 2001; Singh *et al.*, 2004; Wang *et al.*, 2009), there are no reports of 4-aminobenzenesulfonate 3,4-dioxygenase *in vitro* activity (Locher *et al.*, 1989; Magony *et al.*, 2007). Restoration of 4-ABS-degrading ability in RK40(pHG5) and sequence similarity of its disrupted gene to various aromatic ring hydroxylating dioxygenases suggest that the disrupted gene in RK40 (Table 1, Fig. 4) encodes for the oxygenase component of 4-aminobenzenesulfonate 3,4-dioxygenase system in *Hydrogenophaga* sp PBC. Low dioxygenase activity, partial 4-ABS degradation in NB and prolonged lag phase during growth with 4-ABS in minimal medium as experienced by RK40(pHG5) may be due to the polar effect of transposon insertion on the expression of the putative downstream component of the 4-aminobenzenesulfonate 3,4-dioxygenase system, which is usually arranged in one operon or in close vicinity with gene for oxygenase component (Butler & Mason, 1996). Preliminary sequencing results showed the presence of a downstream glutamine synthetase-like gene which could be responsible for the amino group transfer of 4-ABS (data not shown) similar to *tdnQ* and *tadQ* genes involved in the aniline degradation pathway of *Pseudomonas putida* UCC22 and *Delftia tsuruhatensis* AD9, respectively (Fukumori & Saint, 2001; Liang *et al.*, 2005).

RK32 contains a transposon insertion in a transposase gene with a putative dehydrogenase gene located downstream. The expression of this gene may be affected by the polar effect of transposon insertion. The similarity of the dehydrogenase gene to 1,2-dihydroxy-3,5-cyclohexadiene-1,5-dicarboxylate dehydrogenase and the growth of RK32 on 4-sulfocatechol but not 4-ABS suggest a possible role of dehydrogenase in catalyzing the conversion of a putative *cis*-diol intermediate typically formed from aromatic ring hydroxylation (Lee *et al.*, 1994; Nakatsu *et al.*, 1997) to 4-sulfocatechol. Failure to complement RK32 may be due to the inefficient expression of the genes in *trans*. Functional expression of the dehydrogenase in *E. coli* harboring the complete 4-aminobenzenesulfonate 3,4-dioxygenase system is necessary to validate its role in 4-ABS degradation.

In this work, we report the effects of various gene mutations on 4-ABS degradation in *Hydrogenophaga* sp. PBC. To our knowledge, this is the first reported application of transposon mutagenesis in the genus *Hydrogenophaga*. This work complements current molecular study of 4-ABS degradation and sheds light on the upper pathway of 4-ABS degradation.

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