INTRODUCTION

The compound 4-aminobenzensulfonate (4-ABS) has been used extensively in the manufacture of azo dyes, brighteners, pesticides and sulfa drugs. 4-ABS is released into the environment either directly from chemical plants or indirectly from the reductive cleavage of azo dyes in textile wastewater that contain 4-ABS as a parent compound (Tan et al., 2005). Exposure to 4-ABS leads to an increase in hyperactivity in developing rat pups (Goldenring et al., 1982) and a decrease in nitrogen transformation in soil (Topaç et al., 2009).

The metabolism of 4-ABS by bacteria has been a subject of investigation for nearly three decades. During the biodegradation of 4-ABS, 4-sulfocatechol was repeatedly proposed or identified as the common intermediate (Feigel & Knackmuss, 1988; Gan et al., 2011a, b; Perei et al., 2001; Wang et al., 2009). The subsequent ring cleavage of 4-sulfocatechol differs significantly among the metabolism of sulfonated aromatics (Hammer et al., 1996). Instead of liberating the sulfonate group before ring cleavage to form catechol, the sulfonate group is retained and 4-sulfocatechol is oxidized to generate a new compound, 3-sulfomuconate. The genes and enzymes involved in the conversion of 4-sulfocatechol to maleylacetate by Hydrogenophaga intermedia S1 have been characterized in great detail via heterologous expression in recombinant Escherichia coli (Halak et al., 2006, 2007; Hammer et al., 1996).

The destabilization of the aromatic ring is a prerequisite in the microbial degradation of aromatic compounds. This is generally accomplished through the introduction of oxygen-containing substituents and is catalysed by a two/three-component dioxygenase system. For example, class I dioxygenase contains two components, namely a homomultimer terminal dioxygenase and a reductase component. The reductase component consists of two domains: (i) an FMN-binding domain for reduction of FMN to FMNH₂ and (ii) a plant-type ferredoxin domain for electron transfer.
transport from the reductase to the terminal oxygenase component. Upon accepting electrons from the reductase, the oxygenase will activate molecular oxygen and incorporate it directly into the target compound (Mason & Cammack, 1992).

The proposed oxidative deamination of 4-ABS to 4-sulfocatechol (Feigel & Knackmuss, 1993) has not been completely elucidated hitherto due to the lack of successful enzyme assays (Locher et al., 1989; Magony et al., 2007). Furthermore, low permeabilization of 4-ABS through the membrane of E. coli, due to its negatively charged sulfonate moiety (Hwang et al., 1989), has made it difficult to obtain detectable in vivo activity even if the required gene cassettes are present and properly expressed in E. coli clones isolated through genomic library construction.

In our recent work, transposon mutagenesis was performed on a 4-ABS-degrading bacterial strain, Hydrogenophaga sp. PBC, isolated from textile wastewater (Gan et al., 2011b), to identify the genetic components affecting 4-ABS degradation. Fortuitously, a mutant containing a Tn5 insertion in the oxygenase component for the 4-ABS 3,4-dioxygenase (also known as sulfanilate dioxygenase) was isolated (Gan et al., 2011a). Even though the expression of the wild-type oxygenase component restored 4-ABS 3,4-dioxygenase activity in the sadA mutant RK40, its activity was significantly lower than that of the wild-type. We hypothesized that in addition to the direct disruption of sadA by the Tn5 insertion, the transcription of essential catabolic genes for 4-ABS degradation located downstream of the oxygenase component was affected by the polar effect of transposon insertion.

In this study, we identified and isolated the additional components of the 4-ABS 3,4-dioxygenase system. The expression of these components in addition to the wild-type sadA gene in RK40 and in the recombinant E. coli host validated the involvement of these genes in 4-ABS degradation. This study provides the final piece of the puzzle for the 4-ABS catabolic pathway in the genus Hydrogenophaga.

METHODS

Bacterial strains, growth conditions and electrocompetent cell preparation. Bacterial strains and plasmids used in this work are listed in Table 1. The Hydrogenophaga strains were grown at 30 °C in either nutrient broth (NB) containing 5 g peptone l−1 and 3 g beef extract l−1, super optimal broth (Hanahan, 1983) or PB medium (Gan et al., 2011b) supplemented with 0.01 % (w/v) yeast extract or vitamins (20 mg p-aminobenzoate l−1 and 2 μg biotin l−1). E. coli strains were grown at 30 or 37 °C in Luria–Bertani (LB) broth or PBN medium (Gan et al., 2011b) supplemented with 0.1 % (w/v) glycerol and 0.01 % (w/v) yeast extract. Antibiotics used for Hydrogenophaga strains were kanamycin (25 μg ml−1) and gentamicin (25 μg ml−1); for E. coli, carbenicillin (50 μg ml−1), kanamycin (25 μg ml−1) and gentamicin (25 μg ml−1) were used. 4-ABS was added at a final concentration of 5–10 mM. The preparation of electrocompetent cells and electroporation using Eppendorf Multiporator were carried out as previously described (Gan et al., 2011a).

DNA manipulation techniques. All molecular manipulations were performed by using standard protocols. DNA was digested with restriction enzymes (Promega) and ligated with T4 DNA ligase (Promega). For routine screening of DNA inserts and most reverse transcriptase PCR (RT-PCR) mixtures, Taq DNA polymerase (Qiagen) was used. For cloning and RT-PCR experiments involving

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrogenophaga sp.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBC</td>
<td>4-ABS+</td>
<td>Gan et al. (2011b)</td>
</tr>
<tr>
<td>RK40</td>
<td>4-ABS−; Tn5 insertion in sadA; Km’</td>
<td>Gan et al. (2011a)</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOP 10</td>
<td>F’ mcrA Δ(mrr-hsdRMS-mcrBC) Δ80lacZAM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (Str’ endA1 nupG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>JM109</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (rK mQ’ λZAM15) relA1 supE44 Δ(lac-proAB) [F’ traD36, proAB, laqλlacZAM15]</td>
<td>Promega</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBBR1MCS-5</td>
<td>Gm’; broad-host-range</td>
<td>Kovach et al. (1995)</td>
</tr>
<tr>
<td>pBAD22</td>
<td>Amp’; arabinose-based expression vector</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pHG6A</td>
<td>Gm’; pBBR1MCS-5 without lac promoter</td>
<td>This work</td>
</tr>
<tr>
<td>pHG7</td>
<td>Gm’; pHG6A carrying sadA</td>
<td>This work</td>
</tr>
<tr>
<td>pHG8</td>
<td>Gm’; pHG6A carrying sadABD</td>
<td>This work</td>
</tr>
<tr>
<td>pHG9</td>
<td>Amp’; sadC cloned into EcoRI/HindIII sites of pBAD22</td>
<td>This work</td>
</tr>
<tr>
<td>pHG10</td>
<td>Gm’; pHG6A carrying sadAB</td>
<td>This work</td>
</tr>
<tr>
<td>pPROBE-NT</td>
<td>Km’; broad-host-range, gfp-based promoter probe</td>
<td>Miller et al. (2000)</td>
</tr>
<tr>
<td>pPROBE-HG</td>
<td>Km’; 221 bp promoter region 14 bp upstream of sadA inserted into EcoRI/HindIII sites of pPROBE-NT</td>
<td>This work</td>
</tr>
</tbody>
</table>
transcripts of more than 1000 bp, Phusion DNA polymerase (Finnzymes) was used. The primers used in this study are described in Table S1, available with the online version of this paper.

RT-PCR. An overnight culture of *Hydrogenophaga* sp. PBC in NB was subcultured (1%, v/v) into PBN minimal salt medium containing 5 mM 4-ABS. Upon reaching OD₆₀₀ 0.6, total RNA was extracted by using the SV total RNA isolation system (Promega) and the synthesis of cDNA was performed using GoScript reverse transcription system (Promega) with random primers. For RT-PCR of the 3069 bp *sad* operon, total RNA was extracted using masterpure complete DNA and RNA purification kit (Epitope) and cDNA synthesis was carried out using MMLV reverse transcriptase 1st-strand cDNA synthesis kit (Epitope) with random primers. The cDNA was used as a template for PCR mixtures with specific primers to amplify the intergenic regions of *sadA–sadB* and *sadB–sadC*, the internal region of *sadA* and the entire *sadA–sadB–sadC* region.

Analytical methods. Microbial growth was monitored by measuring the optical density at 600 nm. 4-ABS was quantified by using Ehrlich’s reagent as described previously (Gan et al., 2011b). The measurement of whole-cell 4-ABS 3,4-dioxygenase activity with an oxygen probe and the HPLC analysis of metabolites have been described elsewhere (Gan et al., 2011a). The 4-ABS metabolite was collected using Waters Fraction Collector II for further analysis. UV-visible absorption spectra of the metabolite were measured with Nano-Drop 1000 (Thermo Scientific). Phenolic products were detected via reaction with 4-aminoantipyrine as described by Omori et al. (1991).

Transcriptional fusion studies and fluorescence measurement. A 221 bp DNA region located 14 bp upstream of *sadA* from strain PBC was amplified with primers ProHin and ProEco and ligated immediately upstream of the promoterless *gfp* gene of the reporter plasmid pPROBE-NT to give pPROBE-HG. This constructed plasmid was electroporated into strain PBC. The transformed PBC cells were grown with different carbon sources and fluorescence was measured on a Perkin-Elmer LS55B Luminescence Spectrometer as described by Miller et al. (2000). Background readings with the vector control were subtracted from the readings for the strains carrying the promoter fusion. Intensity readings are represented by arbitrary units and were normalized to the cell density measured at OD₆₀₀.

Phylogenetic tree construction. Evolutionary history was inferred using the neighbor-joining method (Saitou & Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches (Felsenstein, 1985). Trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Poisson correction method and the units are the number of amino acid substitutions per site. All ambiguous positions were removed for each sequence pair. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011).

Construction of plasmids for complementation of RK40. Primers PmcsF and PmcsR were designed to anneal to the region upstream and downstream, respectively, of the lac promoter in pBBR1MCS-5 and amplify outwards from the promoter to generate a linear pBBR1MCS-5 amplicon lacking the lac promoter and *KpnI* site. Then, the amplicon was digested with *ApaI* and recircularized to give pHG6A. Primer *Dof* was used in combination with primers DoFA, DoFB or DoFC to amplify *sadA, sadAB* or *sadABD*, respectively. The amplicons were directionally cloned into the *HindIII* and EcoRI sites of pHG6A to generate pHG7, pHG10 and pHG8 containing *sadA, sadAB* and *sadABD*, respectively. After verification of the insert via restriction digestion and sequencing, these plasmids were electroporated into the mutant RK40.

Cloning and sequencing of *sadC*. To amplify the gene fragment of the putative dioxygenase reductase, gradient PCR was performed using degenerate primers DredF and DredR with an annealing temperature ranging from 50 to 66°C. Then, additional primers were designed based on the sequence of the amplicon and inverse PCR was performed using *EcoRI*-digested and recircularized genomic DNA from strain PBC as a template. Subsequent sequencing of the amplicon produced the complete sequence of the putative reductase gene. The complete ORF was later coined *sadC* and cloned into pBAD22 to give pHG9.

Expression of 4-ABS 3,4-dioxygenase in *E. coli*. The pBBR1MCS-5-based plasmid pHG8, which was constructed for the complementation of RK40, was used for the expression of *sadABD*. For the expression of *sadC*, a pBAD22-based plasmid, pHG9, was used instead. *E. coli* JM109 cells were then transformed with the plasmids via electroporation. The negative controls for this study were the empty vectors pHG6A and pBAD22. Overnight cultures of JM109 harbouring different sets of plasmids were inoculated (1%, v/v) into 100 ml LB broth with appropriate antibiotics and grown to OD₆₀₀ 1.0. Then, the cultures were harvested, washed twice with 25 mM phosphate buffer (pH 7.0) and resuspended in PBN medium (OD₆₀₀~2.0) supplemented with 0.1% (w/v) glycerol, 0.2% (w/v) arabinose, 0.01% (w/v) yeast extract and 10 mM 4-ABS. After 12 h of incubation with shaking (30°C, 150 r.p.m.), the cell-free supernatant was collected and filtered through a 0.22 μm nylon membrane for HPLC and colorimetric analyses. For the quantification of 4-sulfocatechol, a stock solution of 4-sulfocatechol (500 mM) was prepared and diluted to generate standards from 0 to 5 mM for HPLC quantification (20 μl injection volume). The peak areas obtained for the standards were then used to construct a standard curve. The pelleted cells were resuspended in BugBuster reagent (Novagen) for protein extraction according to the manufacturer’s recommendations. The total protein determination was performed using Bradford’s method (Bradford, 1976).

RESULTS

Transcription of *sadABD* as one operon and phylogenetic analysis of SadA

The *EcoRI* genomic DNA fragment of RK40 containing the Tn5 insertion was cloned into pBBR1-MCS5 and sequenced via primer walking. The Tn5 insertion was located within a dioxygenase gene (subsequently designated *sadA*) with similarity to phthalate dioxygenase (Fig. 1a). Based on BLAST results, *sadB* and *sadD*, located downstream of *sadA*, were predicted to encode a putative glutamine-synthetase-like protein and a ferredoxin, respectively. Previous complementation work of RK40 with plasmid pBBR1MCS-5, containing wild-type *sadA*, enabled minor 4-ABS degradation (Gan et al., 2011a), suggesting suboptimal *sadA* expression and/or low transcription of *sadB* and *sadD*, which may be due to the polar insertion of Tn5 (Berg et al., 1979, 1980). To verify the possibility of *sadABD* being transcribed as an operon, RT-PCR was performed on purified RNA from the 4-ABS-grown strain PBC. The amplicons for the DNA region in *sadA, sadA–sadB, sadB–sadD* and *sadA–sadB–sadD* were observed in the cDNA sample and were similar to the positive control which used genomic DNA as a template (Fig. 1b). According to phylogenetic tree analysis, the oxygenase component of the 4-ABS 3,4-dioxygenase system (SadA) is
clustered with the Group I dioxygenase (Fig. 1c). In addition, the presence of 16 amino acids between the first His residue and the second Cys residue at the conserved Rieske domain site in this oxygenase (Fig. 1d) further supported the placement of SadA in the Group I dioxygenase group (Nam et al., 2001).

**Complementation of RK40**

The results from RT-PCR prompted a redesign of complementation strategy in RK40 to include sadB and sadD as well in the complementation plasmid construction. The presence of wild-type sadABD in trans allowed RK40(pHG8) to completely biotransform 5 mM 4-ABS (Fig. 2a, b) and grow on 4-ABS as a sole carbon and nitrogen source (Fig. 2c). Nevertheless, its 4-ABS 3,4-dioxygenase activity was still lower than that of the wild-type strain PBC, suggesting a partial complementation (Fig. 2d). RK40(pHG7) with only wild-type sadA in trans displayed significantly weaker 4-ABS biodegradation ability as indicated by the partial 4-ABS removal in NB, a longer lag phase in 4-ABS-containing minimal medium and lower 4-ABS 3,4-dioxygenase activity (Fig. 2).
To better understand the transcriptional regulation of the sadABD operon, pPROBE-HG containing the native promoter upstream of sadABD was constructed and transformed into the wild-type strain PBC. During the construction of pPROBE-HG, E. coli cells harbouring this plasmid were fluorescent (data not shown), suggesting that this promoter could be recognized by the RNA polymerase of E. coli. The transcription of the sadABD operon in strain PBC was not constitutive and could be induced by the substrate and the first metabolite of the 4-ABS pathway (Fig. 3). The induction level of this operon by 4-ABS was twofold higher than 4-sulfoacetate. In addition, the presence of an alternative nitrogen source in the form of ammonium did not affect transcription.

**Identification and cloning of sadC**

Based on phylogenetic tree analysis (Fig. 1c), SadA was shown to be closely related to phenoxybenzoate dioxygenase and isophthalate dioxygenase. Therefore, in an attempt to clone the gene encoding the reductase of SadA, degenerate primers DredF and DredR were designed based on the conserved regions obtained from the amino acid sequence alignment of the reductases of these closely related dioxygenases. PCR using these primers generated a single band of approximately 400 bp with decreasing band intensity upon increasing annealing temperature (data not shown). Subsequent inverse PCR led to the generation of an additional 1000 bp sequence flanking the initial amplified sequence. The putative ORF coded for a 319 amino acid protein which shared significant similarity with known type I dioxygenase reductases (PobB, 97%; IphD, 49%; VanB, 47%) and contained an FMN-binding domain at the N-terminus and a plant-type ferredoxin domain at the C-terminus.

**Expression of sadABD and sadC in E. coli**

To identify the genetic components which are required for the reconstitution of 4-ABS 3,4-dioxygenase activity, E. coli strains harbouring different combinations of sad genes were constructed and tested for dioxygenase activity via quantification of the metabolite after incubation with 4-ABS (Table 2). In each positive strain, 4-ABS was partially biotransformed to a metabolite which shared a similar UV/vis profile and co-eluted with 4-sulfoacetate (Fig. S1a, b). The metabolite also tested positive for the presence of a phenolic group similar to 4-sulfocatechol via a reaction with 4-aminoantipyrine (Fig. S1c). The complementation of sadC from the expression system caused a 38% decrease in 4-ABS 3,4-dioxygenase activity, suggesting that sadC was not absolutely required for the reconstitution of 4-ABS dioxygenase activity. The genetic components downstream of sadA were removed one at a time to identify the component(s) in addition to sadA which is absolutely required for the reconstitution of 4-ABS-oxidation activity in E. coli. Similar to sadC, the deletion of sadD still allowed 4-ABS oxidation albeit at a lower rate. Together, these results

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**Fig. 2.** Complementation of RK40 with wild-type sad genes. (a) Final concentration of the remaining 4-ABS in NB supplemented with 5 mM 4-ABS after 72 h incubation with strains harbouring different plasmids. (b) 4-ABS concentration during bacterial growth in PB medium supplemented with 5 mM 4-ABS. (c) Growth profile of wild-type and complemented strains in PB medium supplemented with 5 mM 4-ABS. (d) 4-ABS-dependent oxygen consumption rate of the cell suspension after 24 and/or 36 h exposure to 4-ABS. Values were obtained after subtraction of endogenous respiration. All data are means of two replicates and the error bars represent SD. The P-values were calculated by comparing different strains with RK40(pHG6A). *P<0.05; **P<0.01.

**Transcriptional analysis of the sad promoter**

To better understand the transcriptional regulation of the sadABD operon in vivo, pPROBE-HG containing the native
suggested that *E. coli* contained endogenous electron carriers which could partially replace the specific reductase and ferredoxin components of the 4-ABS dioxygenase system. Interestingly, the exclusion of sadB completely inhibited dioxygenase activity, indicating that its requirement was far greater than the requirement for sadC or sadD.

**Phylogenetic analysis of SadB**

SadB formed a monophyletic group with proteins involved in the oxidative deamination of aniline such as TdnQ, TadQ and AtdA1 (Fukumori & Saint, 1997; Liang *et al.*, 2005; Murakami *et al.*, 2003; Urata *et al.*, 2004; Zhuang *et al.*, 2007) and some uncharacterized putative glutamine-synthetase-like proteins from betaproteobacteria (Fig. 4a). The gene arrangement of oxygenase–glutamine-synthetase-like protein–ferredoxin in the sad operon is not similar to that of the aniline dioxygenase gene cluster (Fig. 4b), suggesting a different regulatory mechanism in these two pathways. A BLAST search of the nucleotide sequence containing sadABD showed that such gene arrangement is present in some genomes of betaproteobacteria that are well known for aromatic compound biodegradation such as *Burkholderia* sp. CCGE1001, *Polaromonas* sp. JS666 and *Variovorax paradoxus* EPS (Pérez-Pantoja *et al.*, 2011). However, it is worth noting that none of the putative sadABD homologues in these bacteria have been functionally validated.

**DISCUSSION**

The isolation of complete genetic components for 4-ABS bioconversion has been unsuccessful hitherto mainly due to the failure in obtaining significant *in vitro* activity and the inherent poor permeabilization of 4-ABS through the *E. coli* cell wall. The identification of the component of 4-ABS 3,4-dioxygenase system paved a new route for the study of this system (Gan *et al.*, 2011a). The subsequent identification of its additional components provided a very crucial piece of information to elucidate the 4-ABS degradation pathway. Combining the data from several 4-ABS-biodegradation studies (Contzen *et al.*, 2001; Feigel & Knackmuss, 1993; Gan *et al.*, 2011a; Halak *et al.*, 2006, 2007; Hammer *et al.*, 1996) and the data from this study, a complete pathway for 4-ABS biodegradation is proposed (Fig. 5).

The presence of the gene coding for a glutamine-synthetase-like protein is rather common in the aniline dioxygenase gene clusters (Fukumori & Saint, 1997; Liang *et al.*, 2005; Murakami *et al.*, 2003; Urata *et al.*, 2004). However, proof that this glutamine-synthetase-like protein is absolutely required in aromatic ring hydroxylation has rarely been demonstrated except in the study of aniline degradation in *Pseudomonas putida* UCC22 whereby the deletion of tdnQ completely eliminated oxygen uptake and ammonia release during whole cell incubation with aniline (Fukumori & Saint, 1997). Currently, the aniline dioxygenase and 4-ABS degradation operons are being explored for their potential to degrade aromatic compounds.

**Table 2. 4-ABS-oxidation activity of recombinant *E. coli* strains harbouring different sets of plasmids**

<table>
<thead>
<tr>
<th>Expression system</th>
<th>Gene product</th>
<th>4-ABS-oxidation activity [nmol 4-SC (μg protein)^(-1^*^h^(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109(pHG6A)(pBAD22)</td>
<td>Negative control</td>
<td>0</td>
</tr>
<tr>
<td>JM109(pHG6A)(pHG9)</td>
<td>sadC</td>
<td>0</td>
</tr>
<tr>
<td>JM109(pHG8)(pBAD22)</td>
<td>sadABD</td>
<td>9.68 ± 0.93</td>
</tr>
<tr>
<td>JM109(pHG7)(pHG9)</td>
<td>sadA + sadC</td>
<td>0</td>
</tr>
<tr>
<td>JM109(pHG9)(pHG10)</td>
<td>sadAB + sadC</td>
<td>9.48 ± 1.23</td>
</tr>
<tr>
<td>JM109(pHG8)(pHG9)</td>
<td>sadABD + sadC</td>
<td>15.55 ± 1.52</td>
</tr>
</tbody>
</table>
the biotransformation, the accumulation of 4-sulfocatechol in to our previous finding. Instead of enhancing 4-ABS pathway (Fig. 3) is rather unexpected and contradictory. The finding of 4-sulfocatechol as an inducer of this synthetase-like component in addition to a dioxygenase 3,4-dioxygenases known to require a glutamine-methylamid synthetase; GSL, glutamine-synthetase-like protein. The evolutionary distances were computed using the Poisson correction method and the units are the number of amino acid substitutions per site. (b) Organization of genes in the immediate gene neighbourhood of glutamine-synthetase-like genes for aniline (TdnQ, BAA12805; TdnQ, BAA12805). GS, Glutamine synthetase; GMAS, (a) SadB is clustered with the glutamine-synthetase-like proteins found in the aniline oxidation pathway (TdnQ, BAA12805; TdnQ, BAA12805). GS, Glutamine synthetase; GMAS, γ-glutamylmethylamide synthetase; GSL, glutamine-synthetase-like protein. The evolutionary distances were computed using the Poisson correction method and the units are the number of amino acid substitutions per site. (b) Organization of genes in the immediate gene neighbourhood of glutamine-synthetase-like genes for aniline (Pseudomonas sp. UC22 and Delfia tsuruhatensis AD9) and 4-ABS (Hydrogenophaga sp. PBC) pathways. Other gene clusters that have not been functionally validated but are similar to the sad gene neighbourhood are included to show the conservation of such gene organization in different bacterial genera.

3,4-dioxygenase systems are the only aromatic ring hydroxylation dioxygenases known to require a glutamine-synthetase-like component in addition to a dioxygenase and electron transport carrier(s).

The finding of 4-sulfocatechol as an inducer of this pathway (Fig. 3) is rather unexpected and contradictory to our previous finding. Instead of enhancing 4-ABS biotransformation, the accumulation of 4-sulfocatechol in the pcaG2 mutant RK1 during 4-ABS bioconversion led to an incomplete 4-ABS biotransformation (Gan et al., 2011a). It is conceivable that instead of being a repressor in the sad operon, a high concentration of 4-sulfocatechol is toxic to the cell, as is observed for some diphenolic compounds such as catechol and protocatechuate (Park et al., 2001; Parke et al., 2000), thereby reducing the overall bacterial fitness and 4-ABS biodegradation efficiency. The results from the transcription study suggest the presence of a transcriptional regulator(s) for the sadABD operon, thus warranting future studies focusing on the identification of an incomplete 4-ABS biotransformation (Gan et al., 2011a). It is conceivable that instead of being a repressor in the sad operon, a high concentration of 4-sulfocatechol is toxic to the cell, as is observed for some diphenolic compounds such as catechol and protocatechuate (Park et al., 2001; Parke et al., 2000), thereby reducing the overall bacterial fitness and 4-ABS biodegradation efficiency. The results from the transcription study suggest the presence of a transcriptional regulator(s) for the sadABD operon, thus warranting future studies focusing on the identification of an incomplete 4-ABS biotransformation (Gan et al., 2011a). It is conceivable that instead of being a repressor in the sad operon, a high concentration of 4-sulfocatechol is toxic to the cell, as is observed for some diphenolic compounds such as catechol and protocatechuate (Park et al., 2001; Parke et al., 2000), thereby reducing the overall bacterial fitness and 4-ABS biodegradation efficiency. The results from the transcription study suggest the presence of a transcriptional regulator(s) for the sadABD operon, thus warranting future studies focusing on the identification of an incomplete 4-ABS biotransformation (Gan et al., 2011a).

**Fig. 4.** SadB plays a key role in the biotransformation of 4-ABS and does not belong to the general glutamine synthetase family. (a) SadB is clustered with the glutamine-synthetase-like proteins found in the aniline oxidation pathway (TdnQ, BAA12805; TdnQ, BAA12805). GS, Glutamine synthetase; GMAS, γ-glutamylmethylamide synthetase; GSL, glutamine-synthetase-like protein. The evolutionary distances were computed using the Poisson correction method and the units are the number of amino acid substitutions per site. (b) Organization of genes in the immediate gene neighbourhood of glutamine-synthetase-like genes for aniline (Pseudomonas sp. UC22 and Delfia tsuruhatensis AD9) and 4-ABS (Hydrogenophaga sp. PBC) pathways. Other gene clusters that have not been functionally validated but are similar to the sad gene neighbourhood are included to show the conservation of such gene organization in different bacterial genera.

**Fig. 5.** The putative degradation pathway of 4-ABS. 4-ABS undergoes several enzymic reactions leading to the production of maleylacetate. The subsequent reduction of maleylacetate led to the formation of β-ketoamide which could be channelled into the citric acid cycle. pcaHG2, the gene encoding type 2 protocatechuate dioxygenase αβ subunits; pcaB2, the gene encoding modified 3-carboxy-cis,cis-muconate-lactonizing enzyme; SLH, 4-sulfomuconolactone hydrolase; 4-SC, 4-sulfocatechol (Contzen et al., 2001; Gan et al., 2011a; Halak et al., 2006, 2007).
this transcriptional regulator(s) and characterization of its DNA-binding site(s) and substrate range.

This study has reported a novel 4-ABS 3,4-dioxygenase system found in *Hydrogenophaga* sp. PBC. It is hoped that this information will be useful for the future development of a successful *in vitro* assay which is still unavailable at this point in time. In addition, the elucidation of the genetic components for this system may aid in the design of probes to assess the distribution of this system in various environments and also in future protein engineering work targeted to improve its stability and substrate range. To our knowledge, this is also the first report of a functional three-gene operon containing the oxygenase–glutamine-synthetase-like protein–ferredoxin arrangement. It will be interesting to investigate the 4-ABS-degrading ability of other betaproteobacterial strains containing these gene homologues (Fig. 4b) and the substrate range of their dioxygenase system.

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**REFERENCES**


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