



Identification of the protocatechuate transporter gene in *Sphingobium* sp. strain SYK-6 and effects of overexpression on production of a value-added metabolite

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Received: 12 January 2018 / Revised: 22 March 2018 / Accepted: 5 April 2018 / Published online: 19 April 2018
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Abstract

Sphingobium sp. strain SYK-6 expresses the best characterized catabolic systems for lignin-derived aromatic compounds. However, the uptake systems for these aromatics remain unknown. In this study, we identified and characterized the protocatechuate (PCA) transporter gene SLG_12880 (*pcaK*) in SYK-6. Sequence comparisons revealed that PcaK belongs to the aromatic acid/H⁺ symporter family of major facilitator superfamily transporters. Further, *pcaK* was highly conserved among *Sphingomonadales* possessing catabolic genes for vanillate and PCA. The growth of an SYK-6 *pcaK* mutant was significantly delayed on PCA medium and PCA uptake rate was only 8% of wild type. In addition, vanillate uptake rate was 78% of wild type, although the *pcaK* mutant grew as well as the wild type on vanillate. When *pcaK* was expressed in *Sphingobium japonicum* UT26S, the transformant acquired the capacity to uptake both PCA and vanillate. These results indicate that *pcaK* is responsible for the major proportion of PCA uptake and a minor fraction of vanillate uptake in SYK-6. The productivity of 2-pyrone-4,6-dicarboxylate (PDC), a building block of functional polymers, was evaluated using a PDC hydrolase SYK-6 mutant harboring a *pcaK* plasmid. The mutant exhibited 1.27-fold greater PCA conversion and 1.24-fold greater PDC production compared to the control strain, suggesting that enhanced expression of transporter genes for lignin-derived aromatics can be used to increase the production of value-added metabolites.

Keywords Lignin · *Sphingobium* · MFS transporter · Protocatechuate · Vanillate · 2-Pyrone-4,6-dicarboxylate

Introduction

Lignin is one of the major components of plant cell walls and the most abundant aromatic compound on earth; hence, it is a promising raw material for the sustainable production of value-added materials and platform chemicals. However, since lignin forms highly complex and stable structures by random polymerization of monolignols, it is difficult to convert into specific compounds, and effective industrial utilization has not yet been achieved. *Sphingobium* sp. strain SYK-6, first isolated as a

5,5'-dehydrodivanillate degrader from pulp effluent (Katayama et al. 1987), is the bacterium with the best characterized catabolic systems for lignin-derived aromatics (Kamimura et al. 2017; Masai et al. 2007). SYK-6 is able to utilize various lignin-derived biaryls (β -aryl ether, phenylcoumaran, biphenyl, and diarylpropane) and monoaryls (ferulate, vanillin, and syringaldehyde) as a sole source of carbon and energy. Bacterial catabolic systems for lignin-derived aromatics hold great potential for industrial-scale production of value-added metabolites, so there is a concerted effort to characterize these systems. In SYK-6, lignin-derived aromatics with guaiacyl moieties are funneled into vanillate. Vanillate is then converted to protocatechuate (PCA) by tetrahydrofolate-dependent *O*-demethylase (LigM) and catabolized via the PCA 4,5-cleavage pathway (Fig. 1) (Abe et al. 2005; Kamimura and Masai 2014). In this pathway, the aromatic ring of PCA is cleaved by PCA 4,5-dioxygenase (LigAB) (Noda et al. 1990) to generate 4-carboxy-2-hydroxymuconate-6-semialdehyde (CHMS), which is then converted to 2-pyrone-4,6-dicarboxylate (PDC)

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00253-018-8988-3>) contains supplementary material, which is available to authorized users.

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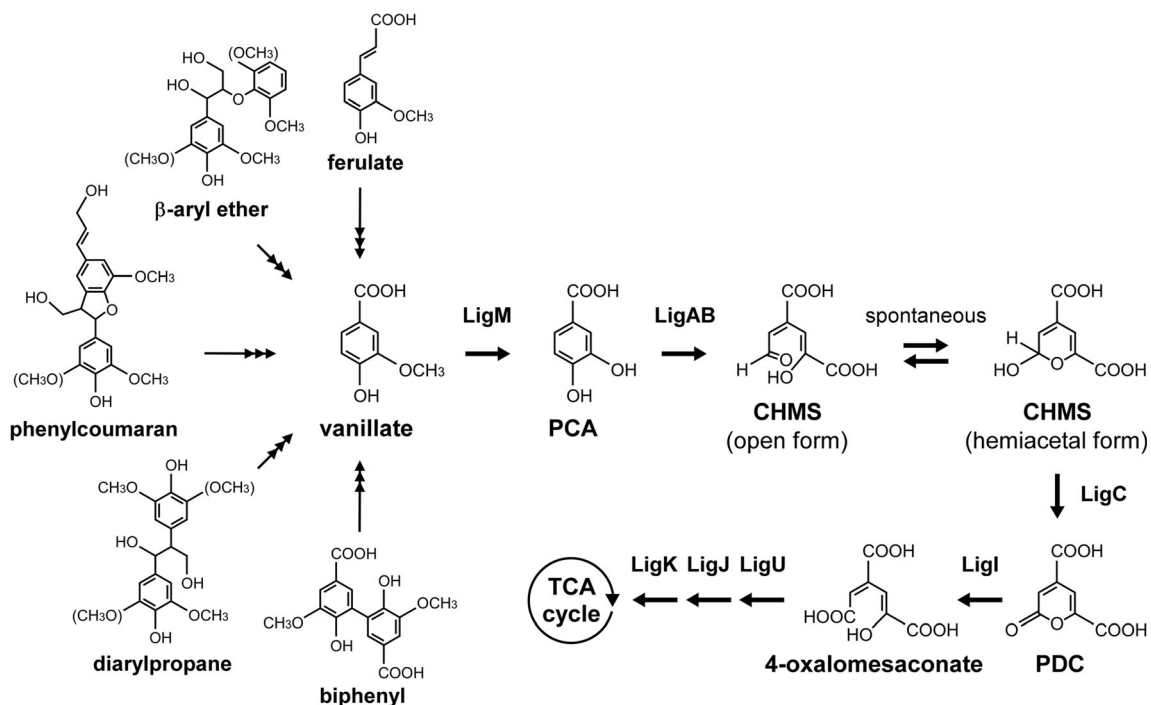


Fig. 1 Catabolic pathways for lignin-derived aromatic compounds by *Sphingobium* sp. SYK-6. Lignin-derived aromatic compounds with guaiacyl moieties are converted into vanillate and catabolized via the PCA 4,5-cleavage pathway. Abbreviations: PCA, protocatechuate; CHMS, 4-carboxy-2-hydroxymuconate-6-semialdehyde; PDC, 2-

pyrone-4,6-dicarboxylate. Enzymes: LigM, vanillate *O*-demethylase; LigAB, PCA 4,5-dioxygenase; LigC, CHMS dehydrogenase; LigI, PDC hydrolase; LigU, putative 4-oxalomesaconate tautomerase; LigJ 4-oxalomesaconate hydratase; LigK, 4-carboxy-4-hydroxy-2-oxoadipate aldolase

by CHMS dehydrogenase (LigC) (Masai et al. 2000). PDC is further catabolized to oxaloacetate and pyruvate through the reactions catalyzed by LigI, LigU, LigJ, and LigK (Kamimura and Masai 2014; Masai et al. 1999). Based on the presence of two carboxylic acid groups and a pseudo-aromatic ring in PDC, functional PDC-based polyamide, polyester, and polyurethane have been developed (Hishida et al. 2009; Michinobu et al. 2009; Shigehara et al. 1998). In addition, production of PDC from PCA was reported using *Pseudomonas putida* PpY1100 harboring a SYK-6 *ligABC* gene plasmid (Otsuka et al. 2006). More recently, PDC production was achieved from vanillin and vanillate derived from Kraft lignin, Japanese cedar, and birch using a *P. putida* PpY1100 strain carrying the upstream catabolic genes in addition to *ligABC* (Qian et al. 2016).

While SYK-6 expresses various specific catabolic enzymes useful for conversion of lignin-derived aromatics, the mechanisms mediating uptake into the cell remain largely unknown. Aromatic acids cannot readily diffuse across cell membranes at physiological pH due to ionization of their carboxyl groups (Kashket 1985). Therefore, bacteria employ active transporters to efficiently uptake aromatic acids, such as the aromatic acid/ H^+ symporter (AAHS) family within the major facilitator superfamily (MFS) transporters (Pao et al. 1998). The AAHS family transporters generally possess 12 α -helix transmembrane (TM) segments and utilize the proton motive

force (PMF) as energy to transport aromatic acids (Nichols and Harwood 1997; Xu et al. 2012b). Two AAHS family transporters of lignin-derived aromatics have been characterized both in vivo and in vitro, the 4-hydroxybenzoate (4HBA)/PCA transporter PcaK of *P. putida* PRS2000 and *Acinetobacter* sp. ADP1, and the gallate/PCA transporter GalT of *P. putida* KTGAL (Nichols and Harwood 1997; Nogales et al. 2011; Pernstich et al. 2014). After reconstitution in proteoliposomes, the ADP1 PcaK was shown to transport vanillate (Pernstich et al. 2014). However, D'Argenio et al. (1999) reported that mutations of *pcaK* did not affect the growth of ADP1 on vanillate. In *Corynebacterium glutamicum* ATCC 13032 (RES167), *vanK* was shown to be involved in the transport of vanillate (Chaudhry et al. 2007); however, Merkens et al. (2005) reported that a *vanK* mutation did not affect the growth of ATCC 13032 on vanillate. Therefore, the transport system involved in the uptake of vanillate is still unknown. On the other hand, bacterial AAHS family transporters of various other aromatics such as benzoate (Choudhary et al. 2017; Collier et al. 1997; Wang et al. 2011), 2,4-dichlorophenoxyacetate (Leveau et al. 1998), 3-chlorobenzoate (Ledger et al. 2009), 3-hydroxybenzoate (3HBA) (Xu et al. 2012a), gentisate (Xu et al. 2012b), and 3-(3-hydroxyphenyl)propionate (Xu et al. 2013) have been identified by uptake experiments using radiolabeled substrates.

Although so far the PCA transporter genes have been characterized in 4HBA/PCA degrading *P. putida* and *Acinetobacter* strains, there have been no reports on the characterization of this transporter in the degraders of lignin-derived aromatics including SYK-6. In order to uncover the whole catabolic system of lignin-derived aromatics in a model degrader SYK-6, it is essential to characterize their transporters; especially transporters for PCA and vanillate, which are major metabolites of guaiacyl-type lignin. Knowledge of these transporters is expected to help establish bioprocesses that will efficiently convert lignin-derived aromatics into value-added metabolites.

In this study, the PCA transporter gene in SYK-6 was identified and biochemically characterized. We also examined whether enhancement of transporter gene expression is effective for improved PDC production from PCA to provide proof of principle for establishment of more efficient bacteria-based production systems to convert lignin-derived aromatics into value-added metabolites.

Materials and methods

Bacterial strains, plasmids, primers, culture conditions, and chemicals

The strains and plasmids used in this study are listed in Table S1 and the PCR primers are listed in Table S2. *Sphingobium* sp. strain SYK-6 (NBRC 103272/JCM 17495) and its mutant were grown at 30 °C in lysogeny broth (LB) or Wx minimal medium (Kasai et al. 2012) containing 5 mM vanillate or PCA. *Sphingobium japonicum* UT26S and *Escherichia coli* strains were grown in LB at 30 and 37 °C, respectively. The media for *E. coli* transformants carrying antibiotic resistance markers were supplemented with 100 mg·L⁻¹ ampicillin, 25 mg·L⁻¹ kanamycin (Km), and 12.5 mg·L⁻¹ tetracycline (Tc). When necessary, the media for SYK-6, its mutant, and UT26S transformants were supplemented with 50 mg·L⁻¹ Km, 12.5 mg·L⁻¹ Tc, and 100 mg·L⁻¹ streptomycin. Syringate, vanillate, gallate, PCA, 4HBA, 3HBA, and benzoate were purchased from Tokyo Chemical Co., Ltd. (Tokyo, Japan) or Wako Pure Chemical Ind., Ltd. (Osaka, Japan). [carboxy-¹⁴C]PCA (55 mCi·mmol⁻¹) and [carboxy-¹⁴C]vanillate (55 mCi·mmol⁻¹) were purchased from American Radiolabeled Chemicals, Inc. (MO, USA). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Sigma-Aldrich Co., Ltd. (MO, USA).

Uptake assays

SYK-6 and its mutant were grown in LB, inoculated into the same media (final concentration, 1%) with or without 10 mM PCA or 10 mM vanillate, and incubated for an additional 24 h.

Cells were then harvested by centrifugation at 5000×*g* for 5 min, washed twice with 50 mM Tris-HCl buffer (pH 7.5), and resuspended in the same buffer to an optical density at 600 nm (OD₆₀₀) of 5.0. PCA and vanillate uptake was measured at 30 °C in a 180-μL reaction mixture containing cells at OD₆₀₀ = 2.0, 50 mM Tris-HCl buffer (pH 7.5), and 20 μM [¹⁴C]PCA or [¹⁴C]vanillate. Cell suspensions were first incubated for 5 min at 30 °C, and uptake was started by adding the radiolabeled substrate. Samples (30 μL) were collected from the reaction mixture at 1, 5, 10, 30, and 60 min, and filtered through nitrocellulose membranes (0.45 μm pore size; Advantec Co., Ltd., Tokyo, Japan). After the filtrates were washed twice with 2 mL of the same buffer, accumulated radiolabeled substrates inside the cells were measured by liquid scintillation counting (AccuFLEX LSC-7400; Hitachi Aloca Medical, Ltd., Tokyo, Japan). For uptake assays in UT26S cells harboring pJB12880k, stationary-phase cells grown in LB for 18–24 h were incubated as described, and samples collected at 0.25, 0.5, 1, 2, 3, and 5 min. In this case, however, UT26S transformant cells were incubated at 30 °C for 5 min with 10 mM glucose for energy generation prior to tracer addition. Uptake rates were calculated from the linear regression slopes of [¹⁴C]PCA or [¹⁴C]vanillate radioactivity in cells versus time (for SYK-6 cells, 1–10 min; for UT26S cells, 0.25–2.0 min). The uptake rates are expressed as nanomoles of substrate per min per milligram of protein. The protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Inc., CA, USA).

An inhibition experiment was performed by incubating cell suspensions in 100 μM of a protonophore CCCP for 5 min at 30 °C prior to adding a radiolabeled substrate. Since ethanol was used as a solvent for CCCP, ethanol was added to the control assay mixtures without CCCP (final concentration of 1 or 5%). The effects of lignin-derived aromatic acids on PCA and vanillate uptake by PcaK were examined by adding 400 μM syringate, vanillate, gallate, PCA, 4HBA, 3HBA, or benzoate to cell suspensions containing 20 μM [¹⁴C]PCA or [¹⁴C]vanillate. For kinetic determination, the concentration of [¹⁴C]PCA was varied (5, 10, 20, 40, or 80 μM).

Sequence analysis

Sequence analysis was performed with the MacVector program (MacVector, Inc., NC, USA). Sequence similarity searches were carried out using the BLAST program (Johnson et al. 2008). Pairwise and multiple alignments were performed with the EMBOSS alignment tool (Rice et al. 2000) and the ClustalW program (Larkin et al. 2007), respectively. Phylogenetic trees were generated using the FigTree program. Putative TM segments were predicted using the TMHMM program (Krogh et al. 2001). Translation initiation rates were predicted for the mRNA regions between –35 and +35 (+1 is the adenine of the initiation codon) of the original

sequence of *pcaK* in pJB12880k and the pET-21a(+) ribosome binding site (RBS)-fused *pcaK* in pJB12880RBSk using the RBS calculator ver. 2.1 (<https://salislab.net/software/forward>) (Salis et al. 2009).

Construction of mutants

To construct plasmids for disruption of SLG_12880 (pAK12880) and *ligI* (pAK-ligI), ~1 kb regions upstream and downstream of each gene were amplified by PCR using the SYK-6 total DNA and primer pairs listed in Table S2. The resulting fragments were cloned into pAK405 by in-fusion cloning (In-Fusion HD Cloning Kit; Takara Bio, Inc., Shiga, Japan). Plasmids were independently introduced into SYK-6 cells by triparental mating using pRK2013 as a helper plasmid (Ditta et al. 1980), and the mutant strains were selected as described previously (Kaczmarczyk et al. 2012). Disruptions of SLG_12880 and *ligI* were examined by Southern hybridization analysis and colony PCR using the primer pair shown in Table S2, respectively.

Bacterial growth measurements

SYK-6 and SLG_12880 mutant (SME148) cells were grown in 10 mL of LB, harvested by centrifugation at 5000×g for 5 min, washed twice with Wx medium, and resuspended in 3 mL of Wx medium. Cells were then inoculated into Wx medium containing 5 mM vanillate, syringate, PCA, or 4HBA to an optical density of OD₆₆₀ = 0.2. For growth on PCA and 4HBA, 20 mg·L⁻¹ methionine was added to the media because SYK-6 exhibits auxotrophy for methionine when methoxy group-free substrates such as PCA and 4HBA are used for the growth substrates (Abe et al. 2005; Masai et al. 2004). Cell growth was periodically monitored by measuring OD₆₆₀ using a TVS062CA bio-photorecorder (Advantec Co., Ltd., Tokyo, Japan) with shaking (60 rpm) at 30 °C. A complementation plasmid for SLG_12880, pJB12880k, was introduced into SME148 cells by electroporation, and the growth of the transformant cells was examined as described.

PDC production from PCA

ligI mutants (SME002–3) harboring pJB866 + pKT230, pJB12880RBS + pKT230, pJB866 + pVAD4, or pJB12880RBS + pVAD4 were grown in LB containing antibiotics. Each of these strains was inoculated into the same medium (final concentration, 1%) and further incubated to stationary phase at 30 °C. The cells were harvested by centrifugation at 5000×g for 5 min, washed twice with 50 mM Tris-HCl buffer (pH 7.5), and resuspended in 3 mL of the same buffer. Conversion of PCA was measured at 30 °C in a 1-mL mixture containing cells suspended to OD₆₀₀ = 2.0,

50 mM Tris-HCl buffer (pH 7.5), and 1 mM PCA. Cell suspensions were first incubated for 5 min at 30 °C, and then conversion started by adding PCA. The reaction mixtures were incubated with shaking (1500 rpm), and 100 μL samples were collected at the start and after 0.5, 1, 2, 3, and 4 h of incubation for analysis. The reaction was stopped by centrifugation at 5000×g for 10 min, and the supernatant diluted 10-fold in water, filtered, and analyzed by high-performance liquid chromatography (HPLC; Acquity UPLC system; Waters Corporation, MA, USA). HPLC separation was performed using a TSKgel ODS-140HTP column (2.1 by 100 mm; Tosoh Corporation, Tokyo, Japan) and a mobile phase of water (95%) plus acetonitrile (5%) containing 0.1% phosphoric acid (flow rate = 0.3 mL·min⁻¹). PDC and PCA were detected at 315.2 and 259.2 nm with retention times of 1.3 and 2.4 min, respectively.

Results

Characterization of PCA and vanillate uptake by *Shingobium* sp. SYK-6 cells

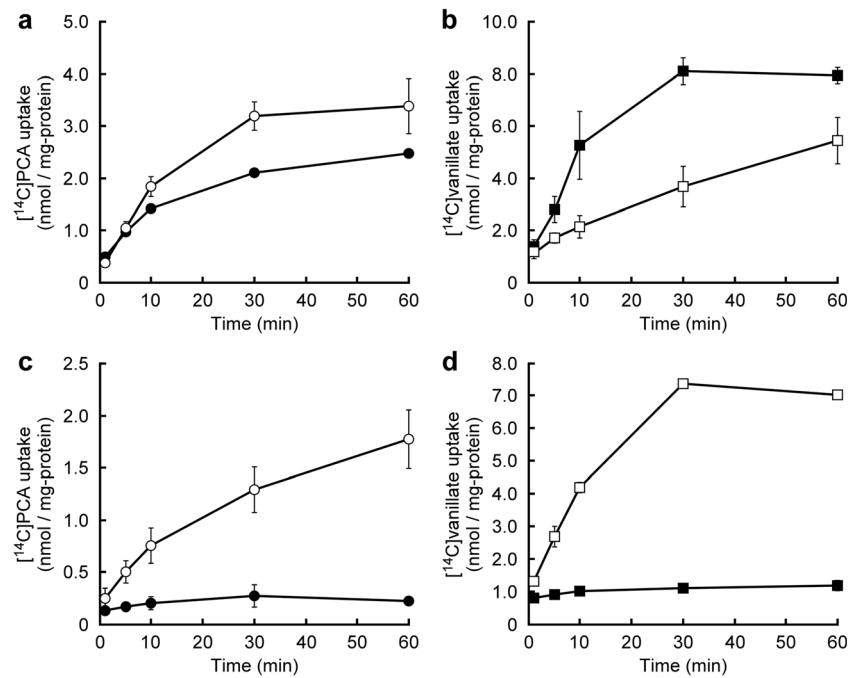
In order to predict the SYK-6 genes which encode PCA and vanillate transporters, the induction profiles and the energy dependence of the uptake of PCA and vanillate by SYK-6 cells were examined. First, the uptake of corresponding [¹⁴C]-labeled substrates was measured during growth in LB + PCA, LB + vanillate, or LB. The PCA uptake by cells grown in LB + PCA was approximately 64% (0.103 ± 0.001 nmol·min⁻¹·mg⁻¹) of that when grown in LB (0.162 ± 0.019 nmol·min⁻¹·mg⁻¹) (Fig. 2a). In contrast, vanillate uptake by cells grown in LB + vanillate (0.432 ± 0.117 nmol·min⁻¹·mg⁻¹) was fourfold higher than when grown in LB (0.108 ± 0.019 nmol·min⁻¹·mg⁻¹) (Fig. 2b). These results suggest that PCA transporter gene expression is partially repressed in SYK-6 cells during degradation of PCA, whereas the expression of the vanillate transporter gene is induced during vanillate catabolism.

The effects of the protonophore CCCP on PCA and vanillate uptake by SYK-6 cells were assessed. Under this treatment condition, SYK-6 cells almost completely lost the ability to uptake PCA and vanillate, suggesting that the PMF is required for transport of both substrates (Fig. 2c, d).

Prediction of the AAHS family transporter gene involved in PCA and vanillate uptake

Since SYK-6 requires a PMF for uptake of PCA and vanillate, we predicted that transporters belonging to the AAHS family of the MFS transporters would be involved. Thus, the 67 putative MFS transporter genes in SYK-6 were searched for

Fig. 2 Uptake of ^{14}C -labeled PCA and vanillate by *Sphingobium* sp. SYK-6. **a** [^{14}C]PCA uptake was measured for SYK-6 cells grown in LB (open circles) or LB + PCA (solid circles). **b** [^{14}C]vanillate uptake was measured for SYK-6 cells grown in LB (open squares) or LB + vanillate (solid squares). **c** [^{14}C]PCA uptake by LB-grown SYK-6 cells in the presence (solid circles) or absence (open circles) of 100 μM CCCP. **d** [^{14}C]vanillate uptake by vanillate-induced SYK-6 cells in the presence (solid squares) or absence (open squares) of 100 μM CCCP. Each value is the average \pm standard deviation of three independent experiments



amino acid sequence similarity with known AAHS family transporters. This analysis demonstrated that SYK-6 possesses 16 AAHS family transporter genes (Fig. S1; Table S3). In particular, SLG_12880, which has relatively high similarity with *vanK* of *Acinetobacter* sp. strain ADP1 and *C. glutamicum* ATCC 13032 (32.1% amino acid sequence identity with that in both species) (Table S4), was found to be well conserved in *Sphingomonadales* strains carrying the putative genes responsible for vanillate catabolism (*ligM*) and the PCA 4,5-cleavage pathway (*ligJABC* and *ligKUD*) (Table S5). Therefore, SLG_12880 was selected as a potential candidate vanillate/PCA transporter gene.

Characterization of a SLG_12880 mutant

To examine the involvement of SLG_12880 in the uptake of PCA and vanillate, SLG_12880 was disrupted in SYK-6 through homologous recombination (Fig. S2a and b). Growth of the resulting mutant (SME148) was markedly reduced when incubated with PCA, but it grew as well as the wild type in vanillate, syringate, or 4HBA (Fig. 3a, b; Fig. S3). The PCA uptake rate of SME148 was only $0.013 \pm 0.004 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, 8% of the wild-type rate (Fig. 3c). On the other hand, the vanillate uptake rate was 78% ($0.292 \pm 0.011 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) of the wild-type rate (Fig. 3d). To verify that diminished growth on PCA and reduced PCA uptake rate were caused by disruption of SLG_12880, we constructed a complementation plasmid, pJB12880k, containing of SLG_12880 and its putative promoter region (279 bp upstream from the initiation codon) downstream of the P_m promoter in pJB861. The

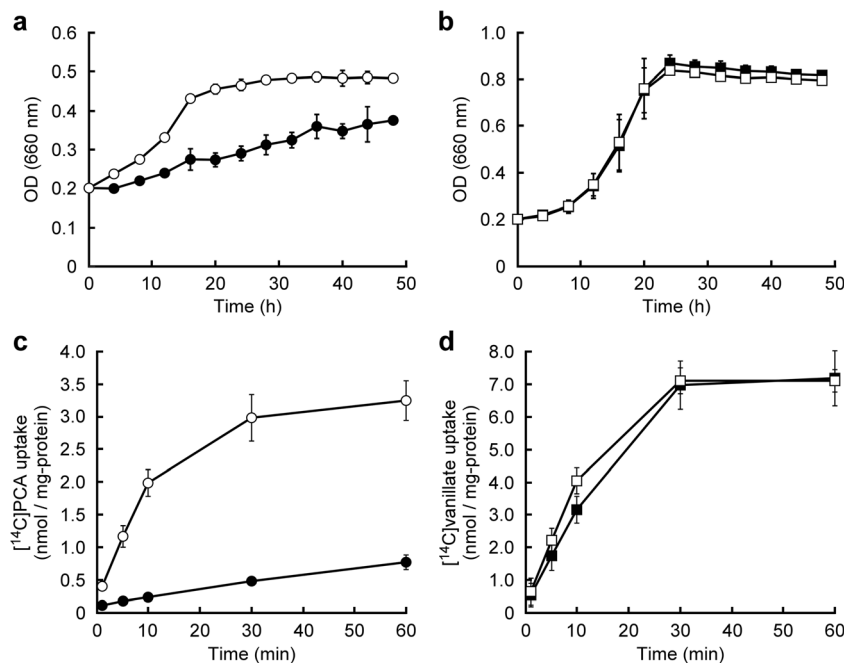
SLG_12880-complemented SME148 strain exhibited growth on PCA and PCA uptake almost equal to the wild type (Fig. S4). These results indicate that SLG_12880 encodes an AAHS family transporter, designated *pcaK*, mediating the major fraction of PCA uptake in SYK-6.

Expression of *pcaK* in a heterologous host and functional analysis

To investigate the biochemical properties of the SYK-6 PcaK, *pcaK* was expressed in *S. japonicum* UT26S. This strain is a γ -hexachlorocyclohexane degrader, which is incapable of utilizing PCA and vanillate as a sole carbon and energy source (Nagata et al. 2010). Introduction of pJB12880k into UT26S cells enabled PCA and vanillate uptake (Fig. 4a, b) at $0.419 \pm 0.089 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ and $0.104 \pm 0.016 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ in 20 μM [^{14}C]PCA and [^{14}C]vanillate, respectively. In addition, PCA uptake by *pcaK*-expressing UT26S cells was inhibited in the presence of the PMF uncoupler CCCP (Fig. 4c).

To characterize substrate recognition by PcaK, the uptake rates of 20 μM [^{14}C]PCA and [^{14}C]vanillate were measured in the presence of a 20-fold excess concentration of a non-radiolabeled substrate (including PCA and vanillate). Compared to control PCA uptake ($0.547 \pm 0.014 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), uptake was reduced to 1% in the presence of 400 μM vanillate, to 3% in syringate, 14% in 4HBA, 21% in PCA, and 84% in 3HBA (Fig. 5a). Control vanillate uptake ($0.119 \pm 0.017 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) was completely inhibited in the presence of 400 μM vanillate, and decreased to 12% of control in the

Fig. 3 Characterization of a SLG_12880 mutant. **a** Growth of control SYK-6 cells (open circles) and mutant SME148 cells (solid circles) in Wx medium containing 5 mM PCA and 20 mg L⁻¹ methionine. **b** Growth of SYK-6 cells (open squares) and SME148 cells (solid squares) in Wx medium containing 5 mM vanillate. **c** [¹⁴C]PCA uptake by SYK-6 cells (open circles) and SME148 cells (solid circles) grown in LB. **d** [¹⁴C]vanillate uptake by SYK-6 cells (open squares) and SME148 cells (solid squares) grown in LB + vanillate. Each value is the average ± standard deviation of three independent experiments



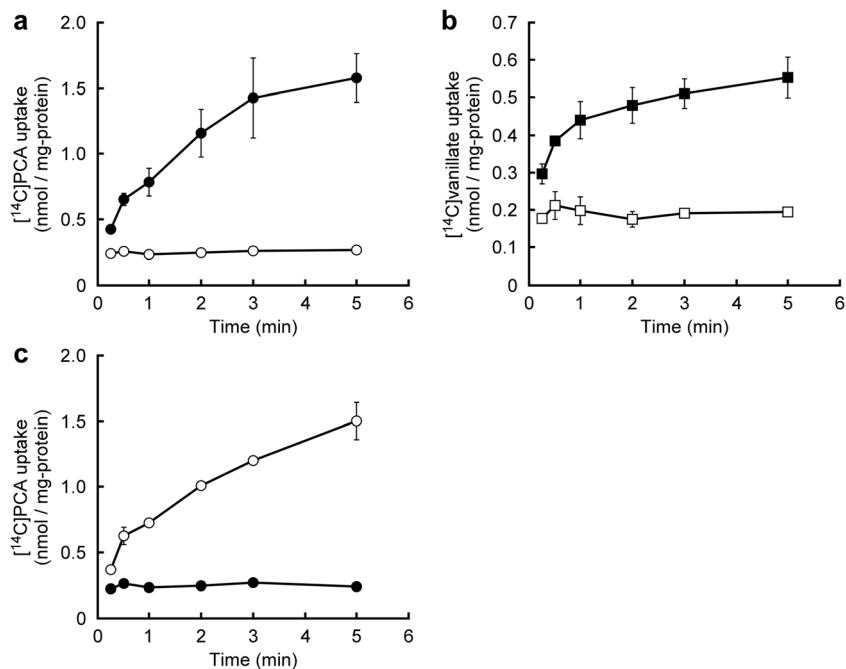
same concentration of syringate, to 14% in PCA, 36% in 4HBA, and 84% in 3HBA (Fig. 5b). In contrast, benzoate and gallate had no inhibitory effect on PCA or vanillate uptake.

To determine the kinetic parameters of PcaK in *pcaK*-expressing UT26S cells, uptake rates were measured using 5–80 μM [¹⁴C]PCA. However, since the uptake rates increased in proportion to the substrate concentrations, kinetic parameters could not be determined (Fig. S5).

Effect of *pcaK* overexpression on production of a value-added metabolite from PCA

We investigated the effect of *pcaK* overexpression on PDC production from PCA using a SYK-6 mutant, SME002–3, harboring a PDC hydrolase gene (*ligI*) disrupted through homologous recombination (Fig. S2c and d). The *pcaK* gene has an AG-rich region (AGGGGA), presumed to be the RBS, located 10-bp upstream of its initiation codon (Fig. S6a);

Fig. 4 Characterization of *pcaK* expressed in *S. japonicum* UT26S. **a** Uptake of [¹⁴C]PCA was examined in UT26S cells harboring pJB12880k (solid circles) or pJB861 (open circles). **b** Uptake of [¹⁴C]vanillate in UT26S cells harboring pJB12880k (solid squares) or pJB861 (open squares). **c** [¹⁴C]PCA uptake by UT26S cells harboring pJB12880k in the presence (solid circles) or absence (open circles) of 100 μM CCCP. Each value is the average ± standard deviation of three independent experiments



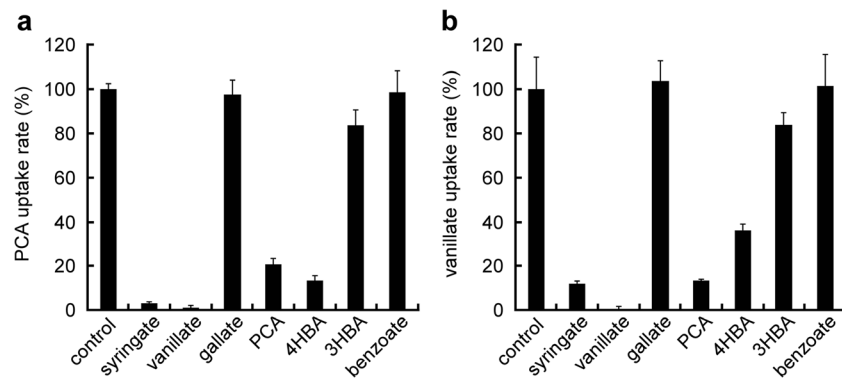


Fig. 5 PCA and vanillate uptake rates by PcaK in the presence of various lignin-derived aromatic acids. The uptake rates of 20 μM [^{14}C]PCA (a) and 20 μM [^{14}C]vanillate (b) by UT26S cells harboring pJB12880k were evaluated in the presence of 400 μM non-radiolabeled lignin-derived aromatic acids (competitors). The PCA and vanillate uptake rates of

UT26S cells harboring pJB12880k without competitors were 0.547 ± 0.014 and 0.119 ± 0.017 $\text{nmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively, which were set as 100% rates (control). Each value is the average \pm standard deviation of three independent experiments. Abbreviations: PCA, protocatechuate; 3HBA, 3-hydroxybenzoate; 4HBA, 4-hydroxybenzoate

however, this sequence has less similarity with the AGGAGG sequence complementary to the 3' terminal sequences of the 16S rRNA from SYK-6 and UT26S. Therefore, we constructed pJB12880RBSk, which contains the RBS from pET-21a(+) and the structural gene of *pcaK* downstream of the P_m promoter in pJB861 (Fig. S6b). Translation initiation rates of *pcaK* of pJB12880k and pJB12880RBSk were predicted using the RBS calculator. The translation initiation rate of *pcaK* in pJB12880RBSk was estimated to be 21-fold higher (22315 arbitrary unit [au]) than that in pJB12880k (1064 au) when the 16S rRNA gene sequences from SYK-6 and UT26S were used for the analysis. The PCA and vanillate uptake rates of UT26S cells harboring pJB12880RBSk were approximately 3.8- and 3.1-fold higher than those of UT26S cells harboring pJB12880k, respectively, suggesting improved translation efficiency by replacement of RBS (Fig. S7; Table S6).

The amount of PCA converted and PDC produced from 1 mM PCA by SME002–3 cells harboring pJB12880RBSk (pJB866 carrying the same insert from pJB12880RBSk) were 1.27-fold (0.84 ± 0.03 mM) and 1.24-fold (0.73 ± 0.02 mM) greater after 4-h incubation, respectively, compared to the corresponding amounts produced by the wild type (0.66 ± 0.01 mM and 0.59 ± 0.01 mM) (Fig. 6; Table S7). To examine if overexpression of genes responsible for PCA to PDC conversion can enhance the conversion in culture, the same cultivations were performed using SME002–3 cells harboring pVAD4, which carries *ligABC*. In this case, the amount of PCA converted was comparable to that of SME002–3 cells harboring pJB12880RBSk (0.85 ± 0.04 mM). However, the amount of PDC produced was lower than that of SME002–3 cells harboring pJB12880RBSk (0.61 ± 0.01 mM). These results suggest that *pcaK* overexpression can improve PDC production efficiency. Therefore, we evaluated the production of PDC from PCA by SME002–3 cells harboring both pJB12880RBSk and pVAD4. This strain converted approximately 1.52–1.56-fold more PCA at 1 h compared to

SME002–3 cells harboring pJB12880RBSk or pVAD4 (Fig. 6a). However, the amount of PDC at 4 h was almost equivalent to that of the wild type (0.62 ± 0.12 mM) (Fig. 6b).

Discussion

In this study, we characterized for the first time a transporter gene involved in the uptake of one of the major lignin-derived aromatics, PCA in SYK-6. Phylogenetic analysis indicated that the SYK-6 PcaK is more phylogenetically related to VanK of *Acinetobacter* sp. strain ADP1 and *C. glutamicum* ATCC 13032 than PcaK of *P. putida* PRS2000 and ADP1. The AAHS family transporters have 12 TM segments (Nichols and Harwood 1997). The conserved DGXD motif in TM1 is known to be important for substrate transport (Ditty and Harwood 2002), while the GXXXD(R/K)XGR(R/K) motif in the cytoplasmic hydrophilic loop between TM2 and TM3 is thought to be the cytoplasmic gate for substrate transport (Ditty and Harwood 1999; Jessen-Marshall et al. 1997; Yamaguchi et al. 1992). An amino acid sequence similar to this motif is also conserved, albeit with greater variation compared to that between TM2 and TM3, in the cytoplasmic loop linking TM8–9 (Ditty and Harwood 1999). Analysis of SYK-6 PcaK using the TMHMM program revealed the presence of 12 TM α -helices like other AAHS family transporters (Fig. S8). In addition, the DGXD and GXXXD(R/K)XGR(R/K) motifs were completely conserved in the putative TM1 and the TM2–3 cytoplasmic loop regions of the SYK-6 PcaK, respectively, and the motif in the TM8–9 cytoplasmic loop was partially conserved (Fig. S9).

The SYK-6 PcaK demonstrated the capacity to transport PCA and vanillate. In addition, syringate, 4HBA, and 3HBA, but not benzoate and gallate, were recognized by PcaK as evidenced by competition assays (Fig. 5). However, since gallate appears to be oxidized after preparation of stock solution

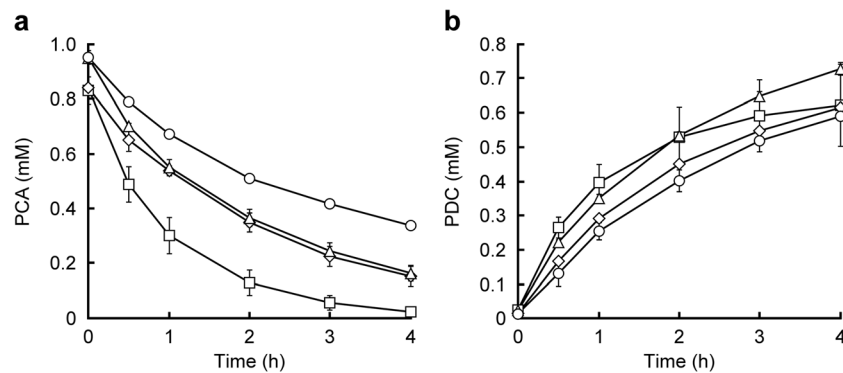


Fig. 6 PDC production from PCA by SME002–3 cells overexpressing *pcaK* and/or *ligABC*. SME002–3 cells harboring pJB866 + pKT230 (vector control; circles), pJB12880RBSt + pKT230 (*pcaK*-RBS; triangles), pJB866 + pVAD4 (*ligABC*; diamonds), or pJB12880RBSt + pVAD4

(*pcaK*-RBS and *ligABC*; squares) were incubated with 1 mM PCA. The amounts of PCA converted (**a**) and PDC produced (**b**) in the cultures were monitored periodically by HPLC. Each value is the average \pm standard deviation of three independent experiments

(the solution turned black) due to chemical instability, further examination is required. Although the actual substrate range of transporters cannot be determined without direct uptake measurements, the strong inhibition of uptake by syringate and 4HBA suggests uptake of these molecules in addition to PCA and vanillate. Similarly, PcaK of *Acinetobacter* sp. ADP1 reconstituted in proteoliposomes showed uptake activities for vanillate, gentisate, 3HBA, 2,4-dihydroxybenzoate, and salicylate as well as PCA and 4HBA (Pernstich et al. 2014). The K_m value of *P. putida* PRS2000 PcaK for 4HBA and of *P. putida* KTGAL GalT for PCA were estimated to be 6 μ M and 9.1 μ M, respectively (Nichols and Harwood 1997; Nogales et al. 2011). Alternatively, the uptake rate for PCA by SYK-6 PcaK was not saturated in the range of 5–80 μ M (Fig. S5), suggesting a relatively lower PCA affinity. In contrast, unlabeled vanillate and syringate more strongly inhibited the uptake of the corresponding labeled molecules than 4HBA and PCA (Fig. 5). Thus, the affinity of SYK-6 PcaK may be higher for vanillate and syringate than for PCA and 4HBA. The relatively low affinity of SYK-6 PcaK for PCA seems to be a distinctive feature from known PcaK.

The *pcaK* mutant of SYK-6 retained the ability to grow on and uptake PCA. Furthermore, *pcaK* disruption did not affect growth on vanillate, syringate, or 4HBA. D'Argenio et al. (1999) reported that the *pcaK*-disrupted ADP1 mutant was able to grow on PCA, while the growth of a *vanK pcaK* double mutant on PCA was significantly retarded. In SYK-6 cells, other AAHS family transporters may be responsible for the residual PCA uptake activity and for the major proportion of vanillate uptake. Further analysis is necessary to identify these transporter genes among the 15 other AAHS family transporter genes in SYK-6.

Development of microbial production processes for value-added metabolites has focused mainly on improving the metabolic pathways, while substrate uptake often depends on the intrinsic ability of the host strain. Recently, application of MFS transporters for improved production of useful

substances has been reported. Zhang et al. (2015) utilized the shikimate transporter gene *shiA* from *E. coli* K-12 to uptake an intermediate metabolite, 3-dehydroshikimate, excreted by the cells during production of *cis,cis*-muconic acid from sugars. In addition, the xylose transporter gene (*araE*) and the D-glucose transporter gene (*iolT1*) were introduced into *C. glutamicum* to produce 3-hydroxypropionic acid from a mixed medium containing xylose and glucose (Chen et al. 2017). More recently, coenzyme Q₁₀ production from 4HBA and glucose was enhanced using a *Rhodobacter* strain expressing the putative 4HBA transporter gene *pcaK* of either *C. glutamicum* or *K. pneumoniae* (Qi et al. 2017). On the other hand, Wu et al. (2018) recently demonstrated that the expression of *couP*, which encodes a substrate-binding protein of an ATP-binding cassette transporter, possibly involved in the uptake of cinnamic acid derivatives in *Rhodospseudomonas palustris* CGA009, improved the efficiency of the production of catechol from vanillin by a genetic-engineered *E. coli* DH1. Here, we examined the effect of *pcaK* on PDC production from PCA. Using SME002–3[pJB12880RBSt], the amount of PCA converted was improved to the levels of SME002–3[pVAD4] (Fig. 6 and Table S7). On the other hand, the yield of PDC from PCA at 4 h of incubation was lower using SME002–3[pVAD4] (73%), whereas the yield using SME002–3[pJB12880RBSt] (87%) was approximately equivalent to that of SME002–3 (89%). Although SME002–3[pJB12880RBSt + pVAD4] rapidly converted PCA, PDC yields from PCA were lower than those of SME002–3 at all sampling time points. After 0.5 h incubation of SME002–3[pJB12880RBSt + pVAD4] with PCA, the culture turned yellow, suggesting the accumulation of CHMS (open form), the ring cleavage product of PCA (Fig. 1). Thus, enhancement of both PCA uptake and conversion of PCA by introduction of pJB12880RBSt and pVAD4 into SME002–3 appears to result in the accumulation of CHMS (open form). The PDC-forming LigC catalyzes the oxidation of the hemiacetal form of CHMS, which is thought to be generated by non-enzymatic

intramolecular cyclization of CHMS (open form), so the low yield of PDC from PCA appears to be caused by the rapid accumulation of CHMS.

While a decrease in PDC yield from PCA was observed in the *ligABC*-overexpressing SME002–3 strain, possibly due to the presence of a rate-limiting step such as autocyclization of semialdehyde to hemiacetal CHMS, it is nonetheless apparent that enhanced expression of transporter genes in addition to enzyme genes can improve the substance conversion rate.

Acknowledgements We thank Yuji Nagata (Tohoku University) for providing *S. japonicum* UT26S. We also thank Kodai Maekawa for assistance with the construction of SME002-3. We are grateful to Yoshihiro Katayama (Nihon University) for insightful discussions and support.

Funding This work was supported in part by JSPS KAKENHI (15H04473) and a research grant from the Institute for Fermentation, Osaka.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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