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MhbT Is a Specific Transporter for 3-Hydroxybenzoate Uptake by Gram-Negative Bacteria

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***Klebsiella pneumoniae* M5a1 is capable of utilizing 3-hydroxybenzoate via gentisate, and the 6.3-kb gene cluster *mhbRTDHIM* conferred the ability to grow on 3-hydroxybenzoate to *Escherichia coli* and *Pseudomonas putida* PaW340. Four of the six genes (*mhbDHIM*) encode enzymes converting 3-hydroxybenzoate to pyruvate and fumarate via gentisate. MhbR is a gene activator, and MhbT is a hypothetical protein belonging to the transporter of the aromatic acid/H^+ symporter family. Since a transporter for 3-hydrxybenzoate uptake has not been characterized to date, we investigated whether MhbT is responsible for the uptake of 3-hydroxybenzoate, its metabolic intermediate gentisate, or both. The MhbT-green fluorescent protein (GFP) fusion protein was located on the cytoplasmic membrane. *P. putida* PaW340 containing *mhbRΔTDHIM* could not grow on 3-hydroxybenzoate; however, supplying *mhbT* in *trans* allowed the bacterium to grow on the substrate. *K. pneumoniae* M5a1 and *P. putida* PaW340 containing recombinant MhbT transported ^{14}C-labeled 3-hydroxybenzoate but not ^{14}C-labeled gentisate and benzoate into the cells. Site-directed mutagenesis of two conserved amino acid residues (Asp-82 and Asp-314) and a less-conserved residue (Val-311) among the members of the symporter family in the hydrophilic cytoplasmic loops resulted in the loss of 3-hydroxybenzoate uptake by *P. putida* PaW340 carrying the mutant proteins. Hence, we demonstrated that MhbT is a specific 3-hydroxybenzoate transporter.**

A substantial number of microbes have been isolated for their capability to utilize aromatic acids as a sole source of carbon and energy. Their degradation pathways have been extensively investigated at both the biochemical and genetic levels in a number of cases (15, 17, 28, 40). The transport of the aromatic acids across the cytoplasmic membrane, an essential precursor to catabolism, has been found to be facilitated by members of the aromatic acid/H^+ symporter (AAHS) family within the major facilitator superfamily (MFS) (34). Three members of the AAHS family involved in the catabolism of corresponding aromatic acids have been functionally identified by uptake assays using the respective ^{14}C-labeled substrates: PcaK (4-hydroxybenzoate and protocatechuate transporter) from the 4-hydroxybenzoate and protocatechuate utilizer *Pseudomonas putida* PRS2000 (33), BenK (benzoate transporter) from the benzoate utilizer *Acinetobacter* sp. strain ADP1 (5), and TfdK (2,4-dichlorophenoxyacetate [2,4-D] transporter) from the 2,4-dichlorophenoxyacetate utilizer *Ralstonia eutropha* JMP134 (24). Nevertheless, the three identified transporters described above exhibit low amino acid sequence identities (28 to 33%) with each another, and no experiments have been conducted to show whether any of them is capable of transporting aromatic acids other than their respective primary substrates.

The microbial catabolism of 3-hydroxybenzoate has been shown to proceed via gentisate in several strains from phylogenetically diverse genera, including *Corynebacterium glutamicum* (40, 46), *Rhodococcus* sp. strain NCIMB 12038 (28), *Polaromonas naphthalenivorans* CJ2 (35), and *Pseudomonas alcaligenes* NCIMB 9867 (12), but no transporter for 3-hydroxybenzoate uptake has been reported so far. Notably, when 3-hydroxybenzoate catabolism in the Gram-positive organism *Corynebacterium glutamicum* was studied, a putative transporter gene within the catabolic cluster turned out to be involved in gentisate catabolism rather than 3-hydroxybenzoate catabolism (40).

Klebsiella pneumoniae M5a1 is a well-known nitrogen-fixing bacterium (10), which is able to metabolize 3-hydroxybenzoate via the gentisate pathway (20). An 8-kb DNA fragment from this strain conferred the ability to grow on 3-hydroxybenzoate to *Escherichia coli* (38). In this study, we report the identification of a specific transporter for 3-hydroxybenzoate uptake and its involvement in 3-hydroxybenzoate catabolism. This *mhbT*-encoded transporter shows 27 to 42% amino acid sequence identities with the three identified AAHS transporters described above and transports ^{14}C-labeled 3-hydroxybenzoate but not ^{14}C-labeled gentisate and benzoate into cells of *Klebsiella pneumoniae* M5a1 and *Pseudomonas putida* PaW340.

MATERIALS AND METHODS

Bacterial strains, plasmids, chemicals, media, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Aromatic compounds were obtained from Sigma-Aldrich Co. (St. Louis, MO). The tracers [carboxyl-^{14}C]3-hydroxybenzoate (55 mCi/mmol), [carboxyl-^{14}C]gentisate (55 mCi/mmol), and [ring-UL-^{14}C]benzoate (70 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Enzymes were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). *Escherichia coli* strains were grown in lysogeny broth (LB) medium at 37°C. *Pseudomonas putida* PaW340 (18, 43) and its variants were grown in LB or minimal medium (MM) (27) supplemented with 2 mM 3-hydroxyben-

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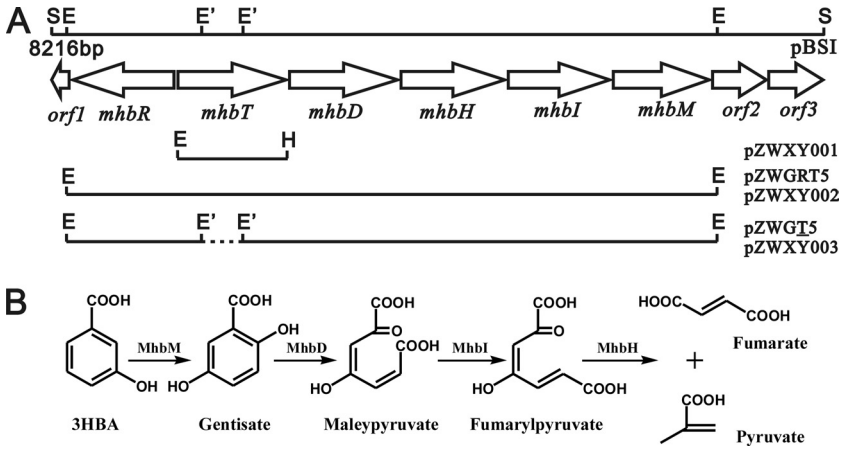

FIG 1 (A) Physical map of the *mhb* gene cluster from pBSI with the 8,216-bp SphI fragment of *K. pneumoniae* M5a1 inserted into pUC18 and inserts of recombinant plasmids constructed from it. Open reading frames are marked by open arrows, with the directions of the arrowheads indicating the transcriptional directions. The restriction sites are designated follows: S, SphI; E, EcoRI; E′, EcoNI; H, HindIII. Both pZWGT5 and pZWXY003 contain a 5.6-kb fragment carrying the *mhb* gene cluster with a 639-bp EcoNI fragment deletion in *mhbT*. (B) Proposed pathway for 3-hydroxybenzoate catabolism in strain M5a1, together with the catabolic reactions catalyzed by *mhb* gene products *in vivo*.

zoate containing 0.015% yeast extract at 30°C. *Klebsiella pneumoniae* M5a1 (11) and its variants were grown in LB or MM supplemented with 2 mM 3-hydroxybenzoate at 30°C. Ampicillin, tetracycline, kanamycin, and chloramphenicol were used at final concentrations of 100, 25, 50, and 34 μg/ml, respectively, when necessary. In the expression experiments, cultures were grown to an optical density at 600 nm (OD_{600}) of 0.6 and then induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for *E. coli* and 1 mM IPTG for *P. putida* PaW340 and *K. pneumoniae* M5a1. A final concentration of 0.5 mM was used when 3-hydroxybenzoate was as an inducer.

General molecular biology methods. Plasmid DNA was isolated by using the alkaline lysis method (39), except in the case of plasmid pVLT33 (7) and its derivatives, which were isolated by using the boiling lysis method (41). Restriction endonuclease digestion and ligation with T4 DNA ligase were conducted in accordance with the manufacturer's instructions. *E. coli* strains were transformed according to standard procedures (39). All the pRK415 (22) and pVLT33 constructs were transferred into *P. putida* PaW340 or *K. pneumoniae* M5a1 cells by triparental mating as described previously (45). Nucleotide sequences of pBSI containing the 8-kb fragment were determined by MWG-Biotech Ltd. (Ebersberg, Germany). Nucleotide sequences of the constructs were verified by Invitrogen Life Technologies Co. Ltd. (Shanghai, China). Analyses and comparison of amino acid sequences (or nucleotide sequences) were performed with BLAST programs at the National Center for Biotechnology Information website.

Site-directed mutagenesis. The desired mutants of MhbT were obtained by overlap extension PCR (36). The outer primers for amplification were *mhbT* forward and *mhbT* reverse (see Table S2 in the supplemental material). The inner primers were designed to incorporate one codon change. The overlap extension PCR products were digested before ligation into similarly digested pVLT33, resulting in the expression constructs (see Table S1 in the supplemental material). The sequences of all mutated *mhbT* gene constructs were verified by DNA sequencing to ensure that only the desired mutations had occurred.

Cloning and expression of *mhbDHIM* genes in *E. coli*. Entire individual genes, including their Shine-Dalgarno sequences, were amplified from pBSI by PCR, and the primers used are listed in Table S2 in the supplemental material. The purified PCR products were digested with EcoRI and ligated into similarly treated pUC18 to generate the desired constructs (see Table S1 in the supplemental material). The inserts in these clones were sequenced to ensure that no mutation had been incorporated during the PCR. Fragments from each of these clones were excised by using EcoRI and NdeI and individually religated into the expression vector pET5a to produce the corresponding constructs (see Table S1 in the supplemental material). The Mhb proteins encoded on the pET5a recombinants were individually expressed in *E. coli* Rosetta(DE3)(pLysS).

Preparation of cell extracts and enzyme assays. *E. coli* cells were lysed by ultrasonic treatment, and the extracts were prepared as described previously (50). All assays were performed with 50 mM phosphate buffer (pH 7.4) at room temperature. The activity of 3-hydroxybenzoate 6-hydroxylase was determined by measuring the decrease in the absorbance at 340 nm due to the substrate-dependent oxidation of NADH, the molar extinction coefficient of which was taken to be 6,220 M^{-1} cm^{-1} (42). The activities of gentisate 1,2-dioxygenase, maleylpyruvate isomerase, and fumarylpyruvate hydrolase were all assayed according to a methods reported previously (50). Protein concentrations were determined by the Bradford method (2), with bovine serum albumin as the standard. One unit of enzyme activity was defined as the amount required for the disappearance (or production) of 1 μmol of substrate (or product) per minute at room temperature. Specific activities are expressed as units per milligram of protein. Analysis of fumarate and pyruvate was performed by using high-performance liquid chromatography (HPLC), as described previously (50).

Cellular localization of MhbT-green fluorescent protein (GFP) fusion proteins. Cultures of *E. coli* BL21(DE3), *K. pneumoniae* M5a1, and *P. putida* PaW340 carrying the appropriate constructs (see Table S1 in the supplemental material) were induced with IPTG for 4 h before cells were harvested. Cells were washed and maintained in 50 mM phosphate buffer (pH 7.4), which was mixed with agarose (0.3%) to immobilize cells and for imaging under a confocal microscope with a 490-nm excitation filter and a 520-nm emission filter (45). The imaging experiments were performed by using a Leica TCS SP2 laser scanning spectral confocal microscope equipped with a cooled charge-coupled-device (CCD) camera (Leica Microsystems, Mannheim, Germany). The background cell fluorescence was subtracted.

***mhbT* disruption.** Plasmid pZWGRT5 was digested with EcoNI within *mhbT* and then religated to produce pZWGT5, resulting in the loss of a 639-bp EcoNI fragment. The complete *mhb* gene cluster of pZWGRT5 and the *mhb* gene cluster with truncated *mhbT* from pZWGT5 were both digested with EcoRI and inserted into pRK415 to produce pZWXY002 and pZWXY003, respectively (Fig. 1A; see also Table S1 in the supplemental material).

Uptake assays. The determination of the uptake rates of aromatic acids, measured by liquid scintillation counting of ^{14}C-labeled substrates,

was performed as described previously (33), with minor modifications. *P. putida* PaW340, *K. pneumoniae* M5a1, and their transconjugants were harvested during the exponential phase by centrifugation. After being washed twice with 50 mM Tris-HCl buffer (pH 8.5), cells were resuspended in the same buffer to an OD_{600} of 5 to 10 and kept on ice. Before the uptake assay, cells were incubated for 3 min at 30°C with 10 mM glucose for energy generation (48). The assays were initiated by mixing 600 μl of 50 mM Tris-HCl buffer (pH 8.5) containing 40 μM ^{14}C-labeled substrates and 400 μl of the cell suspension. Samples (100 μl) were taken at timed intervals and filtered through Nuclepore polycarbonate membranes (0.22-μm pore size; Xinya, Shanghai, China), which were previously soaked with 50 mM Tris-HCl buffer (pH 8.5) containing 400 μM unlabeled substrates. The filters were then immediately washed with 2 ml of cold 0.1 M LiCl. For uptake assays performed at different pH values, cells in 50 mM phosphate buffer (pH 6.5, 7.5, and 8.0) and 50 mM Tris-HCl buffer (pH 7.5 and 8.5) were assayed at 1 min in triplicate. In competition and inhibition experiments, uptake measurements were performed in the presence of saturating substrate concentrations (40 μM 3-hydroxybenzoate) and other possible substrates in a 20-fold excess. The amount of substrate accumulated in the cells on the filters was determined with a scintillation counter (1450 MicroBeta TriLux; Perkin-Elmer Life Sciences, Boston, MA). The uptake activity was expressed as nanomoles of substrates taken up per milligram of protein. Cell protein contents were determined according to a protocol described previously (33). Apparent K_m and V_{max} values were obtained by measuring the uptake of ^{14}C-labeled 3-hydroxybenzoate with nine substrate concentrations at 1 min in triplicate. Data were fitted according to the Michaelis-Menten equation using the least-squares method (4).

Quantitative PCR. cDNA was obtained as previously described (44), and quantitative PCR was conducted according to methods described in a previous study (47). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative changes in gene expression levels (29). The 16S rRNA gene (GenBank accession number FJ482128) from strain M5a1 was used as the internal reference gene.

Nucleotide sequence accession number. The DNA sequence of 8,216 bp of the *mhb* genes has been submitted to GenBank under accession number AY648560.

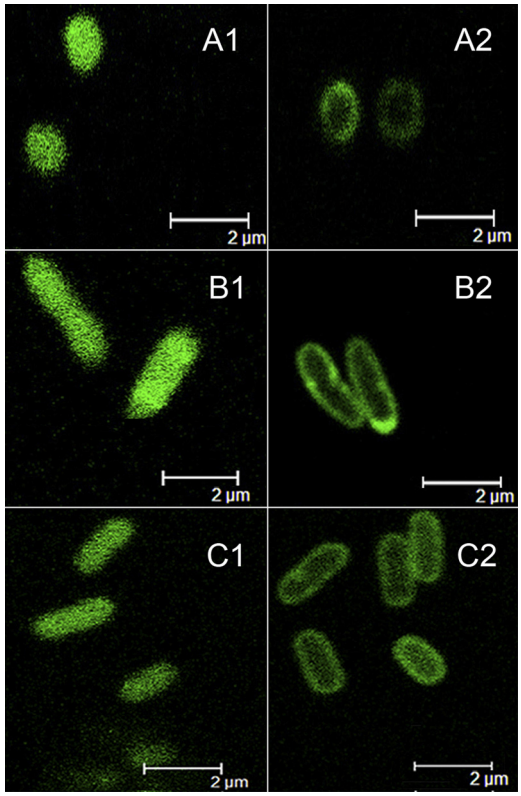

FIG 2 Localization of green fluorescent proteins by confocal microscopy. (A1, B1, and C1) GFP expressed in *K. pneumoniae* M5a1[pZWXY005] (A1), *P. putida* PaW340[pZWXY005] (B1), and *E. coli* BL21(DE3) harboring plasmid pGFPe (37) (C1) (controls), showing that GFP was distributed throughout the cytoplasm. (A2, B2, and C2) MhbT-GFP expressed in *K. pneumoniae* M5a1[pZWXY004] (A2), *P. putida* PaW340[pZWXY004] (B2), and *E. coli* BL21(DE3)[pZWXY006] (C2), showing that the fusion proteins were all located at the periphery of the cells.

RESULTS

Mhb proteins are involved in 3-hydroxybenzoate catabolism and its regulation. The 8-kb DNA fragment of *K. pneumoniae* (38) was sequenced, and a gene cluster (*orf1-mhbRTDHIM-orf2-orf3*) was identified (Fig. 1). In our previous studies, the *mhb* structural genes were demonstrated to be transcribed as an operon, and their expression was regulated by MhbR of the LysR family (25). MhbM and MhbD were shown previously to be 3-hydroxybenzoate 6-hydroxylase (26) and gentisate 1,2-dioxygenase (30), respectively. In this study, cell extracts containing MhbI were found to contain glutathione (GSH)-dependent maleylpyruvate isomerase with a specific activity of 4.99 U/mg. Cell extracts containing MhbH were found to contain fumarylpyruvate hydrolase with a specific activity of 1.45 U/mg. The products from the latter reaction were confirmed to be fumarate and pyruvate by HPLC. Sequentially, MhbM, MhbD, MhbI, and MhbH converted 3-hydroxybenzoate to yield pyruvate and fumarate *in vitro*. It is reasonable to propose that this is also the case for the 3-hydroxybenzoate catabolic pathway *in vivo* (Fig. 1B). Additionally, plasmids pZWGRT5 and pZWXY002, carrying the 6.3-kb EcoRI fragment with the complete *mhbRTDHIM* cluster from the 8-kb fragment in pBSI (Fig. 1A), also conferred the ability to grow on 3-hydroxybenzoate to *E. coli* DH5α and *P. putida* PaW340, respectively.

MhbT is an AAHS family transmembrane protein. As shown in Fig. 1A, *mhbT* was present in the gene cluster between the regulatory-protein-encoding gene *mhbR* and the enzyme genes *mhbDHIM*, encoding a 452-amino-acid polypeptide. Three topology prediction methods, TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), MEMSAT (http://saier-144-37.ucsd.edu/cgi-bin/memsat.cgi), and HMMTOP (http://www.enzim.hu/hmmtop/), indicated that MhbT contained 12 α-helix transmembrane spanners, a typical feature of MFS members (34). By sequence comparison, MhbT exhibited 42% identity with the 4-hydroxybenzoate and protocatechuate transporter PcaK from *P. putida* (33), 27% identity with the benzoate transporter BenK from *Acinetobacter* sp. ADP1 (5), and 30% identity with the 2,4-dichlorophenoxyacetate (2,4-D) transporter TfdK from *Ralstonia eutropha* JMP134 (24). With this degree of identity, the function and substrate specificity of MhbT cannot be convincingly deduced.

Localization of MhbT-GFP to the periphery of cells. To confirm the prediction that MhbT is associated with the membrane, *mhbT* was tagged with *gfp* in plasmids pZWXY004 and pZWXY006 (see Table S1 in the supplemental material) to facilitate the detection of its localization in bacterial cells. *K. pneumoniae* M5a1[pZWXY004], *P. putida* PaW340[pZWXY004], and *E. coli* BL21(DE3)[pZWXY006] (all carrying *mhbT-gfp*) cells were observed by using confocal microscopy. The results clearly showed that MhbT-GFP fusion proteins in these strains from three different genera were located at the periphery of cells (Fig. 2),

consistent with the cytoplasmic membrane location of MhbT predicted by bioinformatics. In the control strains carrying *gfp* only, GFP was distributed in the cytoplasm (Fig. 2).

MhbT is essential for 3-hydroxybenzoate catabolism. In a previous study involving strain M5a1, all four 3-hydroxybenzoate catabolic enzymes exhibited high activities in 3-hydroxybenzoate-grown cells but very low and even undetectable activities in extracts from glycerol-grown cells (20). In this study, the quantitative PCR results indicated that there was a 6.2-fold increase in the transcription level of *mhbT* in the presence of 3-hydroxybenzoate. To identify the possible involvement of *mhbT* in 3-hydroxybenzoate catabolism, unsuccessful attempts to delete *mhbT* in strain M5a1 were conducted with a suicide vector, pZH5 (49), and a temperature-sensitive vector, pKD46 (6). Because of its ability to grow on 3-hydoxybenzoate when carrying the 6.3-kb EcoRI fragment of the *mhb* gene cluster, *P. putida* PaW340 was chosen as a substitute for the determination of the involvement of *mhbT* in the 3-hydoxybenzoate catabolic pathway by the introduction of pZWXY002 (carrying *mhbRTDHIM*) or pZWXY003 (carrying *mhbRΔTDHIM* with a truncated *mhbT* gene). Strain PaW340[pZWXY002] was able to utilize 3-hydroxybenzoate, and its OD_{600} reached 0.4 after a 48-h incubation, whereas strain PaW340[pZWXY003] virtually lost the ability to utilize 3-hydroxybenzoate. However, after pVLT33-based pZWXY001 (carrying *mhbT*) was introduced into strain PaW340[pZWXY003], its capability of growing on 3-hydroxybenzoate was restored. Such experiments were repeated three times, and similar results were obtained in all cases. This clearly indicated that *mhbT* in the *mhb* cluster of *K. pneumoniae* M5a1 was essential for 3-hydroxybenzoate assimilation in *P. putida* PaW340, which is presumably the case for *K. pneumoniae* M5a1.

MhbT transports 3-hydroxybenzoate. To detect the ability for 3-hydroxybenzoate transport by MhbT, the radiolabeled accumulation and the kinetics of 3-hydroxybenzoate transport were measured in resting cells. In a homogenous expression system, when *K. pneumoniae* M5a1 and its variants were grown in LB with IPTG induction, time courses of 3-hydroxybenzoate transport demonstrated that only cells of strain M5a1[pZWXY001] (expressing recombinant MhbT) were able to accumulate ^{14}C-labeled 3-hydroxybenzoate (Fig. 3A), with a V_{max} of 0.92 ± 0.06 nmol/min/mg of protein and a K_m of 4.36 ± 0.12 μM. Wild-type strain M5a1 exhibited no transport activity, apparently because of a lack of *mhbT* transcription in the absence of 3-hydroxybenzoate. However, when they were grown on 3-hydroxybenzoate in MM with IPTG induction, all strains tested had the ability to transport ^{14}C-labeled 3-hydroxybenzoate (Fig. 3B). Under these conditions, the V_{max} was 18.35 ± 0.18 nmol/min/mg of protein and the K_m was 5.57 ± 0.11 μM for 3-hydroxybenzoate-grown strain M5a1, in which all four enzymes for 3-hydroxybenzoate catabolism were active.

Additionally, a heterologous expression system was performed in *P. putida* PaW340, which was shown to be able to grow on 3-hydroxybenzoate after the introduction of the 6.3-kb EcoRI fragment with the complete *mhbRTDHIM* operon. Strain PaW340 and its transconjugants were grown in LB with 3-hydroxybenzoate and IPTG induction. As shown in Fig. 4, it was evident that strain PaW340[pZWXY002] (carrying *mhbRTDHIM*) accumulated ^{14}C-labeled 3-hydroxybenzoate with a V_{max} of 12.05 ± 0.18 nmol/min/mg of protein and a K_m of 4.92 ± 0.08 μM. However, strain PaW340[pZWXY003]

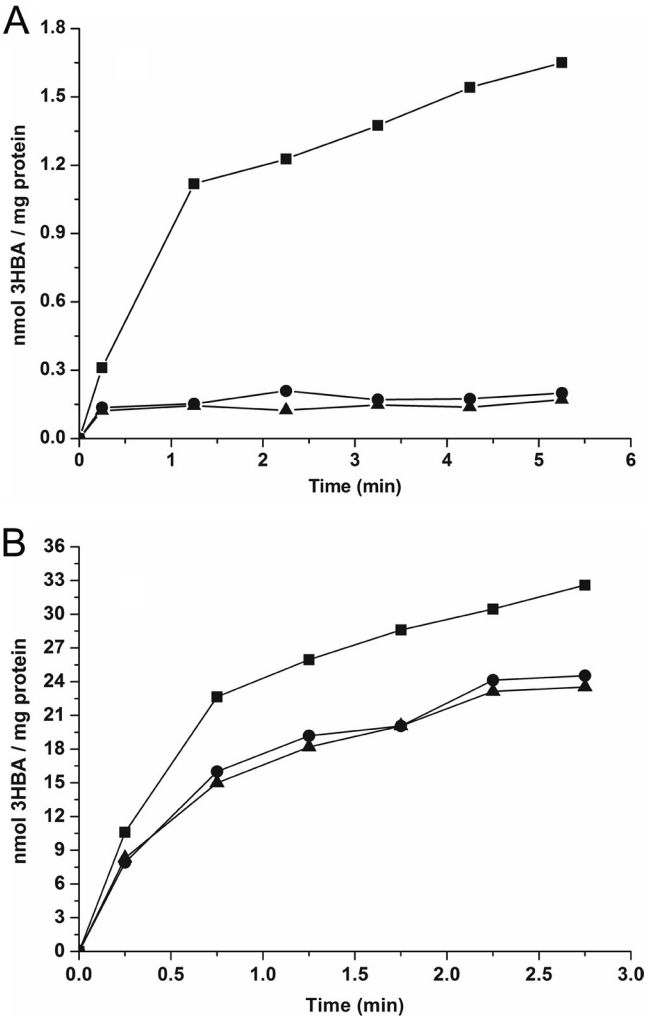

FIG 3 MhbT transports [carboxyl-^{14}C]3-hydroxybenzoate in the homogenous expression system. (A) *K. pneumoniae* M5a1 and its transconjugants grown in LB with IPTG induction at 30°C. (B) *K. pneumoniae* M5a1 and its transconjugants grown on 3-hydroxybenzoate in MM with IPTG induction at 30°C. The same experiments were repeated three times, with the same trends. 3HBA, 3-hydroxybenzoate; ■, *K. pneumoniae* M5a1[pZWXY001] (containing overexpressed MhbT); ●, *K. pneumoniae* M5a1[pVLT33]; ▲, *K. pneumoniae* M5a1.

(carrying *mhb* genes with a truncated *mhbT* gene) showed virtually no ability to transport 3-hydroxybenzoate, similar to plasmid-free strain PaW340. This ability was restored in complemented strain PaW340[pZWXY001,pZWXY003].

As described in the introduction, it is not known whether PcaK, BenK, or TfdK had extended transport activity, in addition to their respective primary substrates. In this study, measurements of uptake by MhbT were also performed with ^{14}C-labeled benzoate and gentisate (a dihydroxylated benzoate) in the above-described systems, but no uptake of these two 3-hydroxybenzoate analogues by MhbT was detected.

Effect of pH values on uptake activity. Strains PaW340 [pZWXY002] (carrying *mhbRTDHIM*) and PaW340[pZWXY003] (carrying *mhb* genes with a truncated *mhbT* gene) were grown in LB with 3-hydroxybenzoate and IPTG induction, and their 3-hydroxybenzoate transport activities were detected at different

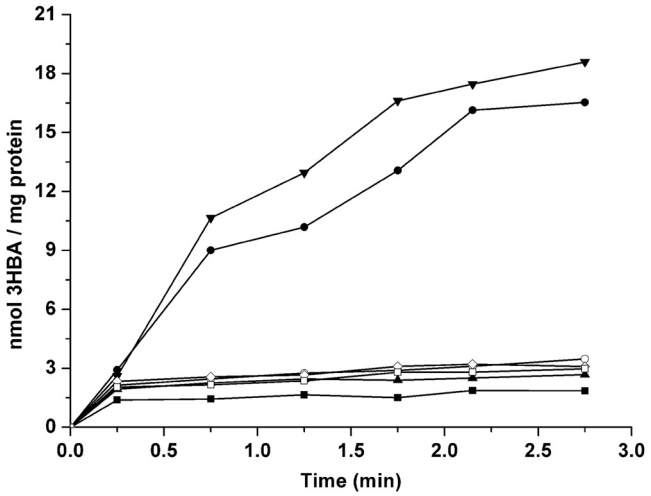

FIG 4 MhbT transports [carboxyl-^{14}C]3-hydroxybenzoate in the heterologous expression system. *P. putida* PaW340 and its transconjugants were grown in LB with 3-hydroxybenzoate and IPTG induction at 30°C. The same experiments were repeated three times, with the same trends. 3HBA, 3-hydroxybenzoate; ■, *P. putida* PaW340; ●, *P. putida* PaW340[pZWXY002] (carrying the *mhb* gene cluster); ▲, *P. putida* PaW340[pZWXY003] (carrying the *mhb* gene cluster with truncated *mhbT*); ▼, *P. putida* PaW340[pZWXY001,pZWXY003] (carrying the *mhb* gene cluster with truncated *mhbT* and recombinant MhbT); ○, *P. putida* PaW340[pZWXY003,pZWXY007] (carrying the *mhb* gene cluster with truncated *mhbT* and the recombinant MhbT D82A mutant); ◇, *P. putida* PaW340[pZWXY003,pZWXY008] (carrying the *mhb* gene cluster with truncated *mhbT* and the recombinant MhbT V311W mutant); □, *P. putida* PaW340[pZWXY003,pZWXY009] (carrying the *mhb* gene cluster with truncated *mhbT* and the recombinant MhbT D314A mutant).

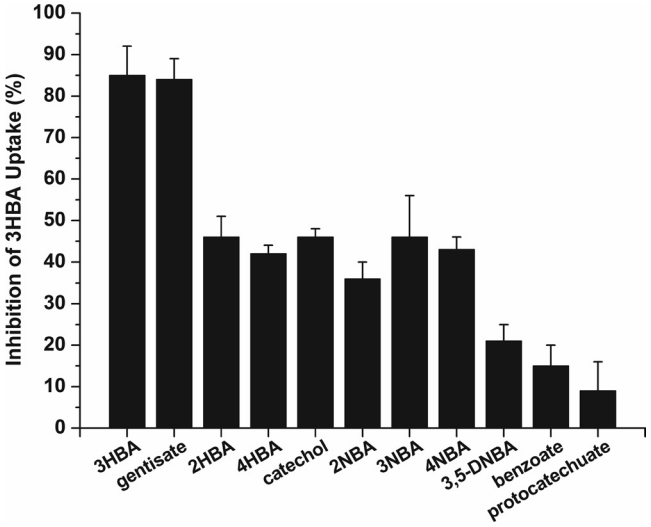

FIG 5 Substrate inhibition of MhbT-mediated 3-hydroxybenzoate uptake in *P. putida* PaW340[pZWXY002]. The concentrations of 3-hydroxybenzoate and the competing substrate were 40 and 800 μM, respectively. The rate of accumulation of 3-hydroxybenzoate (without competing substrates) was 11.86 ± 0.54 nmol/min/mg of protein, which was set as 100% activity. Inhibition was determined by comparing the rates of 3-hydroxybenzoate uptake in the presence and absence of competing substrates. Values are the averages of data from three experiments ± standard deviations. 2HBA, 2-hydroxybenzoate; 3HBA, 3-hydroxybenzoate; 4HBA, 4-hydroxybenzoate; 2NBA, 2-nitrobenzoate; 3NBA, 3-nitrobenzoate; 4NBA, 4-nitrobenzoate; 3,5-DNBA, 3,5-dinitrobenzoate.

pH values, as described in Materials and Methods. Strain PaW340[pZWXY002] accumulated ^{14}C-labeled 3-hydroxybenzoate with similar activities of 10.37 ± 0.48, 12.89 ± 0.30, and 13.57 ± 0.59 nmol/min/mg of protein at pH 6.5, 7.5, and 8.5, respectively. In contrast, while strain PaW340[pZWXY003] exhibited a 3-hydroxybenzoate transport activity of 6.59 ± 0.18 nmol/min/mg of protein at pH 6.5, its activity was almost suppressed at pH 7.5 and 8.5 (1.89 ± 0.43 nmol/min/mg of protein and 2.01 ± 0.28 nmol/min/mg of protein, respectively). This finding suggested that active transport by MhbT played a particular role in substrate uptake under basic conditions.

Identification of residues in MhbT critical for 3-hydroxybenzoate transport. Among the AAHS family members, two conserved stretches of amino acids in the cytoplasmic hydrophilic loops (the 2-3 and 8-9 loops) are known to be required for substrate transport (8, 9). To detect the involvement of the conserved motifs in MhbT in 3-hydroxybenzoate transport, two conserved charged amino acids, Asp-82 and Asp-314, in the 2-3 and 8-9 loops, respectively, were changed to an alanine. Val-311, which was specific in the 8-9 loop of MhbT, was replaced with tryptophan (V311W). The three mutated *mhbT* genes were inserted into pVLT33 to produce pZWXY007, pZWXY008, and pZWXY009, respectively (see Table S1 in the supplemental material). Uptake assays showed that all three mutants lost the ability to transport ^{14}C-labeled 3-hydroxybenzoate (Fig. 4). This indicated that the conserved motifs of MhbT in the cytoplasmic hydrophilic loops were important for 3-hydroxybenzoate transport.

Overexpression of MhbT increases the specific activities of 3-hydroxybenzoate catabolic enzymes. 3-Hydroxybenzoate 6-hydroxylase (MhbM) and gentisate 1,2-dioxygenase (MhbD) catalyze the initial reactions of 3-hydroxybenzoate catabolism in strain M5a1. To investigate whether the overexpression of MhbT would affect the activities of these two enzymes, the specific activities of cell extracts from strain M5a1 and strain M5a1[pZWXY001] (containing overexpressed MhbT) were determined. Both cultures were grown in LB with 3-hydroxybenzoate, with strain M5a1[pZWXY001] being induced with IPTG. The overexpression of MhbT in strain M5a1[pZWXY001] resulted in a 2.5-fold increase of the 3-hydroxybenzoate 6-hydroxylase activity (0.262 U/mg) and a 3.3-fold increase of the gentisate 1,2-dioxygenase activity (0.695 U/mg) in comparison with those measured for a 3-hydroxybenzoate-grown wild-type culture. This observation is similar to that reported in a previous study, in which the overexpression of the styrene transporter, StyE, in *P. putida* CA-3 resulted in a 4.2-fold increase of the styrene monooxygenase activity (32).

Competition and inhibition experiments. In order to detect whether 3-hydroxybenzoate transport by MhbT could be inhibited by other aromatic compounds, ^{14}C-labeled 3-hydroxybenzoate uptake was determined in the presence of the structural analogues of 3-hydroxybenzoate by *P. putida* PaW340[pZWXY002] (carrying *mhbRTDHIM*). The uptake assays for transport were performed in the presence of 20-fold excesses of 3-hydroxybenzoate structural analogues. The impact on 3-hydroxybenzoate transport is shown in Fig. 5. In a control experiment, a 20-fold excess of unlabeled 3-hydroxybenzoate inhibited the rate of transport of radiolabeled 3-hydroxybenzoate by 85%. Gentisate inhibited the transport of 3-hydroxybenzoate by 84%. Catechol, the two monohydroxybenzoate isomers (2-hydroxybenzoate and 4-hy-

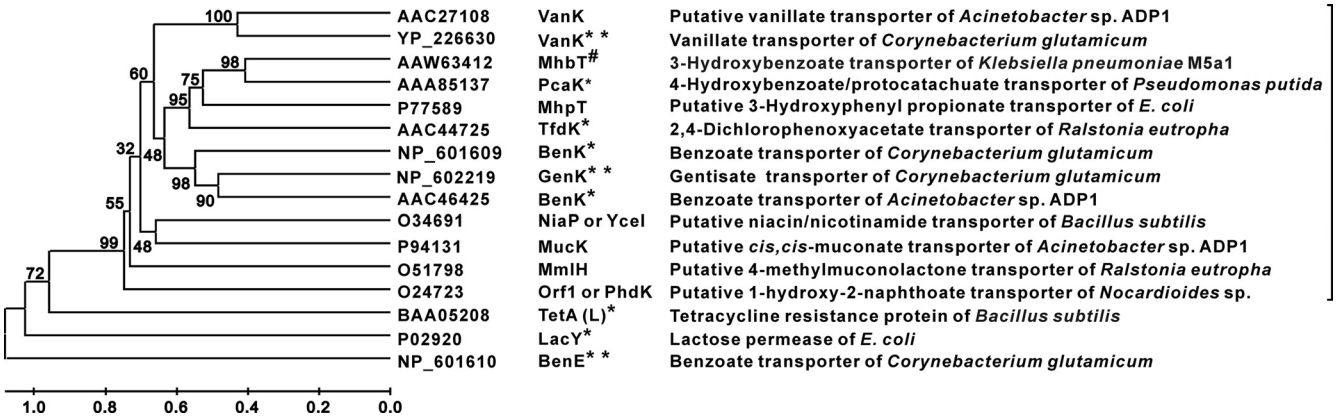

FIG 6 Phylogenetic relationship of the AAHS family transporters. The construction of the phylogenetic tree (using the neighbor-joining method) and multiple-sequence alignments were performed with Mega 3.1 software. The bootstrap confidence limits are indicated at the nodes, and the scale at the bottom indicates sequence divergence. "*" indicates that the function of transporters was examined by genetic disruption/complementation and uptake assays with radioisotopes. "**" indicates that the function of transporters was examined by genetic disruption/complementation. "#" indicates the 3-hydroxybenzoate transporter identified in this study. LacY (13), TetA(L) (14), and BenE (NCgl2326) (3) were used as the outgroups.

droxybenzoate), and the three nitro-substituted benzoates (2-nitrobenzoate, 3-nitrobenzoate, and 4-nitrobenzoate) inhibited approximately 40% of the uptake activity. The addition of benzoate, protocatechuate, and 3,5-dinitrobenzoate had a moderate effect on uptake, with about 20% inhibition.

DISCUSSION

Although 3-hydroxybenzoate catabolism has been studied thoroughly at both the genetic and biochemical levels in phylogenetically diverse strains (28, 35, 40, 46), no report on its transport was documented until MhbT was identified as an active 3-hydroxybenzoate transporter in the current study. MhbT transported ^{14}C-labeled 3-hydroxybenzoate but not ^{14}C-labeled gentisate and benzoate into the cells of two Gram-negative strains. Of the identified 3-hydroxybenzoate catabolic clusters in *Rhodococcus* sp. strain NCIMB 12038 (28) and *Polaromonas naphthalenivorans* CJ2 (35), no putative transporter-encoding genes were found in the vicinity of the catabolic clusters. However, a gentisate transporter gene was found in *Corynebacterium glutamicum* in the 3-hydroxybenzoate catabolic cluster (40). Extensive searching for MhbT homologues in GenBank resulted in the identification of its putative homologues in *Rhodococcus jostii* RHA1 (accession number NC_008268) and *Burkholderia* sp. strain NCIMB 10467 (accession number EU165545), where they are located near putative 3-hydroxybenzoate catabolic clusters. Transport was perhaps theoretically unnecessary, because aromatic acids could diffuse across biological membranes (21), but accumulating evidence indicates that the active transport of aromatic acids is widespread among bacteria (1, 5, 15, 24, 31, 33). The aromatic acids have pK_a values of around 4.0 to 4.5, depending on the substituents on the ring, and the pK_a of 3-hydroxybenzoate is 4.202 (ACD/Labs Software). It is generally accepted that these acids are present in an undissociated form that diffuses across membranes by passive diffusion under acidic conditions. Moreover, under neutral or alkaline conditions, less than 0.1% aromatic acids would be present in the undissociated form, and active transport is necessary for their transport. Therefore, the pH of the medium is a major factor in the decision of whether microbes require certain transporters to grow on aromatic acids. In this study, strain PaW340[pZWXY003] (carrying *mhb* genes and truncated *mhbT*) was unable to grow on 3-hydroxybenzoate at pH 6.5. However, it still retained 64% 3-hydroxybenzoate transport activity compared with that of strain PaW340[pZWXY002] (carrying all *mhb* genes) at this pH value. This is likely because the undissociated form of 3-hydroxybenzoate was capable of entering cells by passive diffusion under acidic conditions. In contrast, the transport activity of strain PaW340[pZWXY003] was almost suppressed at pH 7.5 and 8.5, with a loss of more than 85% of the activity. Although aromatic acids can enter cells by passive diffusion, active transport is thought to increase the efficiency and rate of substrate absorption and may impart a growth advantage in natural environments, where these compounds are present at low concentrations (33).

MhbT showed relatively low amino acid sequence identities (27 to 42%) with three functionally identified AAHS transporters. A phylogenetic tree was generated to further elucidate the relationship among AAHS family members (Fig. 6). Phylogenetic analysis indicated that the AAHS family of transporters (including both putative and functionally identified members) loosely formed one cluster, which was distant from the outgroup. However, the relatively low amino acid sequence identities among the identified AAHS transporters suggest that they may have undergone adaptive evolution to result in the specific transport of the respective aromatic acids in the process of their catabolism. Therefore, the functions and substrate specificities of putative transporters cannot be convincingly deduced without functional analyses using radiolabeled compounds, such as in the current study.

Unlike the broad substrate ranges for many catabolic enzymes in aromatic degradation, the transporters for these substrates exhibited a relatively narrow substrate range. Among the transporters studied in the AAHS family, BenK (5) and TfdK (24) were shown previously to transport benzoate and 2,4-dichlorophenoxyacetate, respectively. PcaK was able to transport both 4-hydroxybenzoate and protocatechuate (33). MhbT specifically transported 3-hydroxybenzoate in the current study. It was thought that the 12 transmembrane α-helices in the MFS transporters packed to form the perimeter of a pore through which the substrate crosses the cell membrane (13, 16, 23). The changing of

a single amino acid residue made conformational changes of the transporters, affecting the ability of the substrate to cross the cell membrane (19). The two conserved domains [GXXXD(R/K)XG R(R/K)] in two identified cytoplasmic hydrophilic loops (the 2-3 and 8-9 loops) were known to be required for substrate transportation in the AAHS family (8, 9). This was also the case for MhbT, in which two strictly conserved residues, Asp-82 and Asp-314 in the 2-3 and 8-9 loops, respectively, were important for 3-hydroxybenzoate transport activity (Fig. 4). The less-conserved Val-311 of MhbT in the 8-9 loop was different from the corresponding residues in PcaK (W-309), BenK (W-305), and TfdK (L-314) but was also necessary for activity. This specific residue in MhbT possibly has an important role in the substrate specificity of this 3-hydroxybenzoate transport.

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