mhpT Encodes an Active Transporter Involved in 3-(3-Hydroxyphenyl)propionate Catabolism by Escherichia coli K-12

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Abbreviations: 3HPP, 3-(3-Hydroxyphenyl)propionate; AAHS, Aromatic Acid/H+ symporter; MFS, Major Facilitator Superfamily; TM, Transmembrane
ABSTRACT

*Escherichia coli* K-12 utilizes 3-(3-hydroxyphenyl)propionate (3HPP) as a sole carbon and energy source. Among its catabolic cluster in the genome, *mhpT* was proposed to encode a hypothetical transporter. Since no transporter for 3HPP uptake has been identified, we investigated whether MhpT is responsible for 3HPP uptake. MhpT fused with green fluorescent protein was located at the periphery of cells under confocal microscopy, consistent with localization to the cytoplasmic membrane. Gene knock-out and complementation studies clearly indicated that *mhpT* was essential for 3HPP catabolism in *E. coli* K-12 W3110 at pH 8.2. Uptake assays with [14C]-labeled substrates demonstrated that strain W3110 and strain W3110Δ*mhpT* containing recombinant MhpT specifically transported 3HPP, but not benzoate, 3-hydroxybenzoate or gentisate, into cells. Energy-dependence assays suggested that MhpT-mediated 3HPP transport was driven by the proton motive force. The change of Ala-272 of MhpT to a histidine surprisingly resulted in an enhanced transport activity and strain W3110Δ*mhpT* containing MhpT mutant A272H had a slightly higher growth rate than wild type strain at pH 8.2. Hence, we demonstrated that MhpT is a specific 3HPP transporter and vital for *E. coli* K-12 W3110 to grow on this substrate under the basic condition.
INTRODUCTION

Microbes play a major role in the degradation of phenylpropanoid compounds, which result largely from the degradation of lignin and other aromatic constituents of plants (1, 2). Among these phenylpropanoid compounds, phenylpropionate and its hydroxylated derivatives have been reported to be degraded by various bacterial strains (3-7). Particularly, a number of *Escherichia coli* strains (strains B, C, W, K-12 and NCTC 5928) were shown to be able to utilize 3-(3-hydroxyphenyl)propionate (3HPP), a major member of phenylpropanoids, as their sole carbon source (7). A 9.8-kb *mhp* cluster conferred the ability to grow on 3HPP to *E. coli* MC4100, *Salmonella typhimurium* LT-2, *Pseudomonas putida* KT2442 and *Rhizobium meliloti* Rm1021Rif, suggesting that this cluster contained all of the catabolic genes necessary for the catabolism of this aromatic acid into Krebs cycle intermediates and also the regulatory element of the pathway (8). The expression of *mhp* catabolic genes was shown to be induced by 3HPP (9). In addition to genes *mhpRABCDFE* encoding regulatory function and 3HPP catabolism (8, 9), *mhpT* (formerly *orfT*) was tentatively proposed to encode a potential transporter (8). Nevertheless, the function of MhpT has so far not been identified genetically or biochemically. As a matter of fact, no report is in the literature on the involvement of any transporter in the microbial degradation of phenylpropionate and its hydroxylated derivatives.

The robust ability of microorganisms to grow on a variety of aromatic compounds partially relies on its multiple transporters for their uptake into cells (10, 11). Among the various categories of transporters classified in Transporter Classification Database (TCDB, http://www.tcdb.org/) (12), the functionally identified aromatic acids transporters (13-18) belong to the aromatic acid/H⁺ symporter (AAHS) family (Cluster 2.A.1.15 in TCDB) except for 4-hydroxyphenylacetate transport HpaX in *E.
coli W (18). In this study, we report that MhpT, sharing 27-37% identities with other AAHS transporters, is specifically involved in 3HPP catabolism of *E. coli* K-12 and transports [\(^{14}\)C]-labeled 3HPP, but not [\(^{14}\)C]-labeled benzoate, 3-hydroxybenzoate or gentisate, into cells.

**MATERIALS AND METHODS**

**Strains, plasmids, media, growth conditions and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. The substrate 3HPP was obtained from the highest purity grade (≥ 98%) commercially available. The tracers, [carboxyl-\(^{14}\)C] 3HPP (55 mCi/mmol), [carboxyl-\(^{14}\)C] gentisate (55 mCi/mmol), [carboxyl-\(^{14}\)C] 3-hydroxybenzoate (55 mCi/mmol) and [ring-UL-\(^{14}\)C] benzoate (70 mCi/mmol) were purchased from American Radiolabeled Chemicals, Inc (St. Louis, MO). Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was purchased from Sigma-Aldrich Co. (St. Louis, MO). Restriction enzymes were purchased from TAKARA Biotechnology Co. Ltd. (Dalian, China). Plasmid DNA extraction and DNA gel extraction kits were purchased from OMEGA BIO-TEK Inc. (Doraville, GA). *E. coli* strains were grown in lysogeny broth (LB) (19, 20) or mineral salts medium (MM) (pH 8.2: Na\(_2\)HPO\(_4\)·12H\(_2\)O 35.6987g, KH\(_2\)PO\(_4\) 0.0362g; pH 7.2: Na\(_2\)HPO\(_4\)·12H\(_2\)O 25.5646g, KH\(_2\)PO\(_4\) 4.2694g; pH 6.2: Na\(_2\)HPO\(_4\)·12H\(_2\)O 7.6000g, KH\(_2\)PO\(_4\) 10.7194g; with addition of 0.28 mg MnSO\(_4\)·H\(_2\)O, 0.3 mg FeSO\(_4\)·7H\(_2\)O, 0.06 mg MgSO\(_4\)·7H\(_2\)O, 1 mg CaCl\(_2\), 0.05 mg CuSO\(_4\), 0.05 mg ZnSO\(_4\) and 0.05 mg H\(_3\)BO\(_3\); and finally H\(_2\)O to a total volume of 1L) with 2 mM 3HPP and 6 mM (NH\(_4\))\(_2\)SO\(_4\), on a rotary shaker (200 rpm) at 37°C. When necessary, ampicillin, kanamycin and tetracycline were used at final concentrations of 100, 50 and 25 μg/ml, respectively.

Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 3HPP were used at final concentrations of 0.1 and 0.5 mM, respectively, as inducers.
**General molecular biology methods.** Plasmid DNA was isolated using the alkaline lysis method (21), except for plasmid pVLT31 and its derivatives being isolated by the boiling lysis method (22). Restriction endonuclease digestion and ligation with T4 DNA ligase were conducted in accordance with the manufacturer’s instructions. *E. coli* BL21 (DE3) and DH5α were both transformed by standard procedures (21). All inserts were sequenced by Invitrogen Biotechnology Co. Ltd (Shanghai, China). Analyses and comparison of amino acid sequences were performed with BLAST programs on the National Center for Biotechnology Information website.

**Cellular localization of MhpT-GFP fusion protein.** Cultures of *E. coli* BL21 (DE3) carrying pGFPe (23) and its derivatives containing *mhpT* and mutant *mhpT* genes (all without stop codon) (Table 1) were harvested and washed twice with 50 mM phosphate buffer (pH 7.4) after being induced with IPTG at 37°C for 4 h. Then cells were resuspended in the same buffer mixing with agarose (0.3%) to immobilize cells for imaging under a confocal microscope with a 490 nm excitation filter and a 520 nm emission filter (24). The imaging experiments were performed using a TCS SP2 Leica laser scanning spectral confocal microscope equipped with a cooled CCD camera (Leica TCS SP2, Leica Microsystems, Mannheim, Germany). The background cell fluorescence was subtracted.

**mhpT disruption.** Temperature-sensitive vector pKD46 (25) was introduced into *E. coli* K-12 W3110 (26) for preparation of competent cells. Primers mhpT1F and mhpT1R (Table 2), respectively incorporated with a 51-bp 5’ and 3’ homologous arms of *mhpT*, were used to amplify the kanamycin gene using pKD4 (25) as the template by polymerase chain reaction (PCR). The resulting PCR fragment was electroporated to *E. coli* K-12 W3110[pKD46] for deleting *mhpT* gene as described...
Site-directed mutagenesis. The desired mutants of MhpT were obtained by overlap extension PCR (27). The outer primers for amplification were mhpT1 and mhpT2 (Table 2). The inner primers were designed to incorporate one codon change. The overlap extension PCR products were digested before ligating into the similarly digested pVLT31, resulting in the expression constructs (Table 1). All mutant mhpT genes were verified by DNA sequencing to ensure that only the desired mutations had occurred.

Measurement of bacterial growth on 3HPP. The bacterial growths of E. coli K-12 W3110 and its variants with 2 mM 3HPP were measured at an optical density at 600 nm (OD_{600}). Such experiments were repeated three times and similar results were obtained. The data acquired were transformed to corresponding number of cells. Then growth curves were fitted by the modified Gompertz equation (28) with OriginPro 8 software, and all points represent the mean value of triplicate trials. Their maximum specific growth rates (\( \mu_{max} \), h\(^{-1} \)) were calculated, which were expressed with the mean values of triplicate trials.

Uptake assays. The uptake assays of aromatic acids were performed with [\(^{14}\text{C}\)]-labeled substrates as described (16) with minor modifications. LB-grown cells of E. coli K-12 W3110 and its variants, with 3HPP and/or IPTG induction at 37°C, were washed twice and resuspended in 100 mM Na\(_2\)HPO\(_4\)/KH\(_2\)PO\(_4\) buffer (pH 6.2, 7.2 or 8.2) to OD\(_{600}\) of 1-2. Before the uptake assay, cells were incubated at 37°C for 3 min with 10 mM glucose for energy generation (14). The [\(^{14}\text{C}\)]-labeled substrates’ uptake was measured.
concentration for uptake assay system in *E. coli* was 10 μM. The amount of substrate accumulated in the cells on the filters was determined in a scintillation counter (1450 MicroBeta TriLux, PerkinElmer Life Sciences, Boston, MA). All assays were performed at least in triplicate. The transport activity was expressed as nanomoles of substrates taken up per milligram of protein. Cell protein contents were determined as described (14).

**Cellular starvation and inhibitor treatments.** The uptake assay by starved or reenergized cells of strain W3110Δ*mhpT* [pVLT31-*mhpT*] (LB-grown with IPTG induction) were carried out as described (14) with a slight modification. Cellular energy stores were depleted by washing and resuspending cells in an original volume of media using Na₂HPO₄/KH₂PO₄ buffer (100 mM, pH 8.2), and the cells were incubated with shaking at 37°C for 18 h. Then starved cells were washed and divided into three aliquots in the same buffer, and one of them was treated with CCCP (50 μM) at 37°C for 1 min. After inhibitor treatment, the treated aliquot together with another aliquot was reenergized with 10 mM glucose. Then all three aliquots were incubated at 37°C for an additional 10 min before uptake assay.

**RESULTS**

**Bioinformatics analysis and cellular localization of MhpT.** MhpT was indicated to contain 12 predicted α-helix transmembrane spanners (TM1-TM12), a typical feature of the AAHS family within the MFS (11), through each of four following topology prediction methods of TMHMM (http://www.cbs.dtu.dk/services/TMHMM/), MEMSAT (http://saier-144-21.ucsd.edu/barmemsat.html), PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) and HMMTOP(http://www.enzim.hu/hmmtop/).

By amino acid sequences comparison, MhpT exhibited 27-37% identities with other AAHS members, including 3-hydroxybenzoate transporter MhbT from *Klebsiella*.
pneumoniae M5a1 (16), 4-hydroxybenzoate and protocatechuate transporter PcaK from *P. putida* (14), benzoate transporter BenK from *Acinetobacter sp. ADP1* (13), 2,4-dichlorophenoxyacetate (2,4-D) transporter TfdK from *Ralstonia eutropha JMP134* (15) and gentisate transporter GenK from *Corynebacterium glutamicum* (17).

In order to confirm the prediction that MhpT was a membrane protein, *mhpT* was tagged with *gfp* in plasmid pGFPe-*mhpT* (Table 1) to facilitate the detection of its localization in bacterial cells. Cells examined using confocal microscopy clearly showed that MhpT-GFP fusion protein in *E. coli* BL21 (DE3) [pGFPe-*mhpT*] was located at the periphery of cells (Fig. 1A), consistent with a cytoplasmic membrane location of MhpT predicted by bioinformatics. In contrast, GFP was distributed in the cytoplasm in the negative control of *E. coli* BL21 (DE3) [pGFPe] (Fig. 1H).

**MhpT is essential for 3HPP catabolism under the basic condition.** To identify the possible involvement of *mhpT* in 3HPP catabolism, this gene was deleted to create a mutant strain W3110Δ*mhpT*. Strain W3110, mutant strain W3110Δ*mhpT* and complemented strain W3110Δ*mhpT* [pVLT31-*mhpT*] were grown on 3HPP at three pH values. Growth study data were represented in semi-log plots (Fig. 2), and the maximum specific growth rates (μm, h⁻¹) of these strains were indicated in Table 3. As shown in Fig 2A, strain W3110Δ*mhpT* virtually lost the ability to utilize 3HPP at pH 8.2, but its ability to grow on 3HPP was restored after pVLT31-*mhpT* was introduced, with a similar growth rate to the wild type strain. However, at pH 6.2 (Fig. 2C), all three above strains were able to grow on 3HPP with similar growth rates, regardless of the presence of *mhpT*. Despite three *E. coli* strains had the ability to utilize 3HPP at pH 7.2 (Fig. 2B), the growth rate of mutant strain was slightly lower than those of wild type and complemented ones. This clearly indicated that *mhpT* was essential for 3HPP catabolism in *E. coli* K-12 W3110 under the basic condition.
MhpT specifically transports 3HPP. To detect the ability to transport aromatic acids by MhpT, the intracellular radiolabeled accumulation was measured at pH 8.2 by the resting cells of *E. coli* K-12 W3110 and its variants. As shown in Fig. 3, it was evident that, when grown in LB with 3HPP and IPTG induction, strain W3110 was able to accumulate $[^{14}\text{C}]$-labeled 3HPP, but strain W3110ΔmhpT virtually lost this ability. However, this ability was restored in strain W3110ΔmhpT [pVLT31- mhpT].

On the other hand, 3HPP-induced strain W3110 cells also had a similar 3HPP accumulation activity to that of 3HPP- and IPTG-induced ones, but IPTG-induced ones had no such uptake activity (data not shown). These indicate that the expression of transporter gene *mhpT* in strain W3110 was also induced by 3HPP, together with other *mhp* catabolic genes as described (9). On the other hand, with IPTG induction only, LB-grown strain W3110ΔmhpT [pVLT31- mhpT] was able to accumulate $[^{14}\text{C}]$-labeled 3HPP, but strain W3110ΔmhpT [pVLT31] was not (Fig. 4). Notably, the 3HPP- and IPTG-induced cells, in which all the 3HPP catabolic genes were expressed, had a much higher transport activity than IPTG-induced cells. This observation was similar to those in previous studies on 3-hydroxybenzoate transporter (16) and 4-hydroxybenzoate transporter (14). Accumulation detections were also performed with available $[^{14}\text{C}]$-labeled 3HPP analogues benzoate, 3-hydroxybenzoate and gentisate in the above systems, but no uptake of these three aromatic acids was detected by 3HPP-induced strain W3110 or IPTG-induced strain W3110ΔmhpT [pVLT31- mhpT] (data not shown).

Environmental pH values affect transport activity. Given the fact that the growth of strain W3110ΔmhpT on 3HPP was affected by pH values (Fig. 2 and Table 3), the intracellular radiolabeled accumulation was then also detected at three pH values to investigate the effect of environmental pH values on the transport activity by
MhpT. Three equal portions of LB-grown cells for each strain were resuspended into buffers at pH 6.2, 7.2 and 8.2. After induction with 3HPP and IPTG for 4 h at 37°C, cells were resuspended in the same buffer before the uptake assays. Transport activities of all strains tested were all decreased with the increase of pH values as shown in Table 3. Strain W3110 was capable of accumulating [14C]-labeled 3HPP at three pH values, but the activities in mutant strain W3110ΔmhpT were decreased dramatically at each pH value, particularly at pH 8.2. On the other hand, this ability was restored in the complemented strain W3110ΔmhpT[pVLT31-mhpT].

MhpT-mediated 3HPP Transport is energized by proton motive force. To investigate the energy dependence of MhpT-mediated 3HPP transport, uptake assays were performed using unstarved, starved and glucose-resupplied starved cells of IPTG-induced strain W3110ΔmhpT[pVLT31-mhpT] at pH 8.2. As shown in Fig. 4, the starved cells did not accumulate 3HPP, but its accumulation ability was restored when they were resupplied with glucose. This indicated that the MhpT-mediated 3HPP transport required energy. CCCP was then introduced into starved cells to determine the type of energy requirement between the two main known sources of energy, the ATP and the proton motive force (PMF). In contrast to the above experiment, the CCCP-treated starved cells with resupplied glucose were unable to accumulate 3HPP (Fig. 4). Meanwhile, addition of CCCP (at 15th second) during the uptake assays by glucose-resupplied starved cells resulted in a rapid loss of accumulated capability and efflux of 3HPP (Fig. 4). CCCP was thought to be able to dissipate the PMF by equilibrating protons across the membrane, but without a significant effect on the intracellular ATP synthesis via glycolysis in glucose reenergized-cells (29). The above evidences in this study suggested that the MhpT-mediated 3HPP transport was driven by PMF and did not correlate with ATP,
Identification of critical residues in MhpT for 3HPP transport. In AAHS family members, two conserved stretches with a motif of GXXXD(R/K)XGR(R/K) located in the cytoplasmic hydrophilic loops (the 2-3 and 8-9 loops) are required for substrate transport (30), and an essential motif DGXD containing aspartate residues located in the first transmembrane segment (TM1) is also conserved (31). Thus four residues in the TM1, and the 2-3 and 8-9 cytoplasmic loops, were chosen for the site-directed mutagenesis to examine the functional significance of these regions for 3HPP transport. Glu-27 in TM1, which was a conserved asparate among the other AAHS members, was changed to an alanine (E27A) and an aspartate (E27D), respectively. Conserved Asp-75 in the 2-3 loop was substituted with an alanine (D75A) and a glutamate (D75E). In the 8-9 loop, less-conserved Ala-272 and conserved Lys-276 were changed to a histidine (A272H) and an aspartate (K276D), respectively. Under confocal microscopy, all these six mutant MhpT proteins fused with GFP were also located at the periphery of cells (Fig. 1 B to G), as wild type MhpT. Strains W3110ΔmhpT containing wild type and mutant MhpT proteins were used for measuring their accumulation of [14C]-labeled 3HPP at pH 8.2. All cells were grown in LB with IPTG induction, the transport activity of strain W3110ΔmhpT [pVLT31-mhpT] was 5.37 ± 0.41 nmol/mg protein at the first minute. As shown in Fig. 5, the uptake assays showed that all mutants were devoid of 3HPP transport activity except for MhpT mutants E27D and A272H, which retained approximately 0.8 and 1.3-fold activities, respectively, in comparison with that of wild type MhpT. In an in vivo experiment, strain W3110ΔmhpT carrying E27D or A272H was able to grow on 3HPP at pH 6.2, 7.2 and 8.2 (as shown in Fig. 2, and the maximum specific growth rates (μmax, h⁻¹) of these two strains were indicated in Table 3). Interestingly, strain
W3110ΔmhpT containing A272H had a slightly higher growth rate than that of wild type strain at pH 8.2.

DISCUSSION

Previous accumulating evidences indicate that active transport of aromatic acids was widespread among bacteria (10, 13, 15, 16, 32-34). In addition to E. coli K-12 (7), Rhodococcus globerulus PWD1 (4), Comamonas testosteroni TA441 (5) and Cupriavidus necator JMP134 (6) were also reported to be able to grow on 3HPP. Several enzymes in 3HPP catabolism in various bacterial strain (4, 5, 35, 36) and its regulation in E. coli K-12 (9) have been thoroughly investigated. However, no biochemical and genetic analyses on 3HPP transport have been reported until this study, in which MhpT from E. coli K-12 was identified as an active 3HPP transporter involved in its catabolism. Among the other identified 3HPP utilizers, putative transporter-encoding genes hppK and mhpT were located on 3HPP catabolic clusters in strain PWD1 (4) and strain JMP134 (6), respectively. HppK (GenBank accession number AAB81315) from strain PWD1 and MhpT (YP_299064) from strain JMP134 showed 26% and 49% identities respectively with MhpT in this study. Considering that both hppK and mhpT were located on the 3HPP catabolic clusters, it is likely that both of them encode 3HPP transporters. If this is the case, active transport driven by a specific transporter in the microbial catabolism of 3HPP probably occurs in phylogenetically divergent genera.

It is generally accepted that the undissociated form aromatic acids were able to diffuse across membranes through passive diffusion (16). In the current study, with the increase of pH values, more and more 3HPP (pKa=4.68, calculated by ACD/Labs Software) is changed to dissociated forms. This subsequently resulted in less 3HPP being able to enter into cells by passive diffusion. Thus the significant decreases in
transport activities (as shown in Table 3) with the increase of pH value for the tested 
strains suggested that 3HPP diffusion was pH value dependent in strain W3110. These 
observations are somewhat different from 3-hydroxybenzoate transport in 
Pseudomonas putida (16) or gentisate transport in Corynebacterium glutamicum (17), 
in which the transport activities in transport deficient strain were decreased with the 
increase of pH values, but the activities were almost the same in the wild type or 
complemented mutant strain at different pH values. On the other hand, the growth 
ability of strain W3110ΔmhpT at pH 6.2 and 7.2, and its inability at pH 8.2 indicated 
that the absence of mhpT in mutant strain resulted in a loss of active transport and a 
subsequent blockage of passive diffusion of 3HPP with the increases of pH value. 
This is due to that less than 0.1% 3HPP was present in the undissociated form at pH 
8.2 and deficient 3HPP in the cells was unable to support the growth of mutant strain. 
It is another example showing that the pH value of the growth media was a major 
factor in deciding whether microbes required certain transporters to grow on aromatic 
acids as suggested previously (16).

Like other AAHS members, MhpT also contains 12 predicted α-helices 
transmembrane regions (TM1 to TM12). It was not surprising that the mutation of 
Glu-27 (in TM1) or Asp-75 (in 2-3 loop) to an uncharged alanine resulted in the loss 
of 3HPP transport activity, similar to the previous studies for PcaK (30, 31), BenK 
(37) and MhbT (16). On the other hand, the change of positive charged lysine to 
negative charged aspartate at site 276 resulted in a complete loss of its transport 
activity. Although Lys-276 (in 8-9 loop) is conserved at the corresponding residues 
of all AAHS members, its importance in substrate transport was not illustrated until 
this study. It can be tentatively proposed that it is also the case in other AAHS 
members. It is also worth to note that the less-conserved Ala-272 in 8-9 loop was
different from the corresponding residues in PcaK (Trp-320), BenK (Trp-305), TfdK (Leu-314), MhbT (Val-311) and GenK (Trp-309). To our surprise, a 30% increase in 3HPP transport activity was observed after the simple and uncharged alanine was changed to a complicated and positive charged histidine. This suggested that, although the characteristic features in the 8-9 loop were partially conserved in the AAHS family (30), the less conserved residue Ala-272 in MhpT may play an important role for transport efficiency during the 3HPP transport. It would be intriguing to see if it is also the case when this corresponding residue in other AAHS members was mutated. Meanwhile, given the fact that strain W3110ΔmhpT [pVLT31-mhpTA272H] had an improved 3HPP transport activity and a slightly higher growth rate, the substrate transport is likely a limiting step for the 3HPP utilization by E. coli K-12 W3110 and MhpT with His-272 is probably a better version of 3HPP transporter for this strain to grow on 3HPP.

ACKNOWLEDGEMENTS

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References


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TABLES

TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tr>
<td>E. coli DH5α</td>
<td>supE44 lacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 Δhi relA1</td>
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<td>E. coli BL21(DE3)</td>
<td>F’ ompT hsdS (r6K mrr”) gal dcm lacY1 (DE3)</td>
<td>Novagen, USA</td>
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<td>E. coli K-12 W3110</td>
<td>3-(3-hydroxyphenyl)propionate utilizer, Km’, Te’</td>
<td>(26)</td>
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<td>E. coli K-12 W3110ΔmhpT</td>
<td>E. coli K-12 W3110 with DNA fragment encoding for MhpT deleted</td>
<td>This study</td>
</tr>
<tr>
<td>pGFPe</td>
<td>Km’, ColE1 replicon, derivative of pWaldo digested with XbaI and EcoRI, yielding a 14 amino acid-long linker sequence (SVPGSENLYFQGQF) followed by GFP</td>
<td>(23)</td>
</tr>
<tr>
<td>pGFPe-mhpT</td>
<td>PCR-amplified fragment containing mhpT without stop codon inserted into pGFPe at Xhol/BamHI restriction sites</td>
<td>This study</td>
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<tr>
<td>pGFPe-mhpTE27A</td>
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<td>This study</td>
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<td>pGFPe-mhpTE27D</td>
<td>PCR-amplified fragment containing mhpT (mhpTE27D) without stop codon inserted into pGFPe at Xhol/BamHI restriction sites</td>
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<td>pGFPe-mhpTD75E</td>
<td>PCR-amplified fragment containing mhpT (mhpTD75E) without stop codon inserted into pGFPe at Xhol/BamHI restriction sites</td>
<td>This study</td>
</tr>
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</table>
pGFPe-mhpTA272H  PCR-amplified fragment containing \textit{mhpT} (\textit{mhpTA272H}) without stop codon inserted into pGFPe at XhoI/BamHI restriction sites  This study

pGFPe-mhpTK276D  PCR-amplified fragment containing \textit{mhpT} (\textit{mhpTK276D}) without stop codon inserted into pGFPe at XhoI/BamHI restriction sites  This study

pVLT31  Broad-host-range expression vector (IncQ, RSF1010 replicon) Te', \textit{P}tac, \textit{lacI}q, \textit{tra}-mob'  (39)

pVLT31-mhpT  PCR fragment containing \textit{mhpT} inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTE27A  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTE27A}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTE27D  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTE27D}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTD75A  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTD75A}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTD75E  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTD75E}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTA272H  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTA272H}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

pVLT31-mhpTK276D  PCR fragment containing mutant \textit{mhpT} (\textit{mhpTK276D}) inserted into pVLT31 at EcoRI/HindIII restriction sites  This study

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Primers} & \textbf{Sequences} & \textbf{Purposes} \\
\hline
\textit{mhpT1} & 5'\text{-CGGAATTC\text{CATGTCGACTCGTACC}CCCTT-3'} & Forward primer for \textit{mhpT} \\
\hline
\textit{mhpT2} & 5'\text{-CCGAGCTTTCAAGGCATCGGCGACGCGCC-3'} & Reverse primer for \textit{mhpT} \\
\hline
\textit{mhpT1D} & 5'\text{-CGGCTCGAG\text{ATGTCGACTCGTACC}CCCTT-3'} & Forward primer for \textit{mhpT} without a stop codon \\
\hline
\textit{mhpT2D} & 5'\text{-CGGGATCCCGCGACGCGACGCGATATT}C-3' & Reverse primer for \textit{mhpT} without a stop codon \\
\hline
\textit{mhpT1F} & 5'\text{-GATGTCGACTCGTACC}CCCCTTCAT\text{CATCTATAT}GATATATCCCTCCTAG\text{-3'} & Forward primer for kanamycin gene with homologous arm of \textit{mhpT} \\
\hline
\end{tabular}
\caption{Primers in this study}
\end{table}
mhpT2R  5’-TCAGGCATCGGCACGGGTATTCGTGATCTCCGGCTCATCAAAATAATGTAGGCTGGAGCTGCTTCG-3’ Reverse primer for kanamycin gene with homologous arm of mhpT

The introduced restriction sites are boldface, and the homologous arms of mhpT are underlined.

### TABLE 3. Effects of pH values on the maximum specific growth rates and the transport activities of wild-type strain W3110 and its variants on 3HPP

<table>
<thead>
<tr>
<th>Strains</th>
<th>pH 6.2</th>
<th>pH 7.2</th>
<th>pH 8.2</th>
<th>pH 6.2</th>
<th>pH 7.2</th>
<th>pH 8.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain W3110</td>
<td>0.18</td>
<td>0.19</td>
<td>0.12</td>
<td>107.37±0.98</td>
<td>46.86±0.71</td>
<td>43.91±0.59</td>
</tr>
<tr>
<td>Strain W3110ΔmhpT</td>
<td>0.20</td>
<td>0.11</td>
<td>0.01</td>
<td>25.29±0.18</td>
<td>15.07±0.87</td>
<td>5.45±0.43</td>
</tr>
<tr>
<td>Strain W3110ΔmhpT [pVLT31-mhpT]</td>
<td>0.21</td>
<td>0.14</td>
<td>0.11</td>
<td>178.33±0.98</td>
<td>75.59±0.71</td>
<td>60.07±0.59</td>
</tr>
<tr>
<td>Strain W3110ΔmhpT [pVLT31-mhpTE27D]</td>
<td>0.19</td>
<td>0.12</td>
<td>0.08</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Strain W3110ΔmhpT [pVLT31-mhpTE272H]</td>
<td>0.22</td>
<td>0.17</td>
<td>0.18</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

3HPP, 3-(3-hydroxyphenyl)propanoate; * N/A, not applicable – no detection carried out.
Figure Legends

FIG. 1. Localization of green fluorescence proteins under confocal microscopy. A: MhpT-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpT]; B: MhpTE27A-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTE27A]; C: MhpTE27D-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTE27D]; D: MhpTD75A-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTD75A]; E: MhpTD75E-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTD75E]; F: MhpTA272H-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTA272H]; G: MhpTK276D-GFP expressed in *E. coli* BL21 (DE3) [pGFP-e-mhpTK276D], showing that the fusion proteins were located at the periphery of the cells. H (negative control): GFP expressed in *E. coli* BL21 (DE3) [pGFPe], showing that GFP was distributed throughout the cytoplasm.

FIG. 2. Cell growth curves of *E. coli* K-12 and its variants on 3HPP. Strains were grown with 2 mM 3HPP in MM at 37°C. A: at pH 8.2; B: at pH 7.2; C: at pH 6.2. N: the number of cells; N₀: the initial number of cells; 3HPP: 3-(3-hydroxyphenyl)propionate. The growth curves were fitted by the modified Gompertz equation (28) with OriginPro 8 software, and all points represent the mean value of triplicate trials. The symbols in C apply to the curves in both A and B.

FIG. 3. MhpT-mediated [¹⁴C]-labeled 3HPP accumulation in *E. coli* K-12. 3HPP uptake assays were performed at pH 8.2 by 3HPP- and IPTG-induced cells of *E. coli* K-12 W3110 and its variants. ■: *E. coli* K-12 W3110; ●: *E. coli* K-12 W3110ΔmhpT; □: *E. coli* K-12 W3110ΔmhpT [pVLT31]; ▲: *E. coli* K-12 W3110ΔmhpT [pVLT31-mhpT]; 3HPP: 3-(3-hydroxyphenyl)propionate. All points represent the mean values of triplicate trials with error bars denoting the standard deviation.

All points represent the mean values of triplicate trials with error bars denoting the standard deviation.

FIG. 5. Accumulation of 3HPP by E. coli K-12 W3110ΔmhpT containing mutant MhpT proteins. All strains were grown in LB with IPTG induction. The activity (5.37 ± 0.41 nmol/mg protein) of 3HPP uptake by strain W3110ΔmhpT [pVLT31-mhpT] at the first minute was set as 100%. Wild Type: strain W3110ΔmhpT [pVLT31-mhpT]; Vector: strain W3110ΔmhpT [pVLT31]; E27A: strain W3110ΔmhpT [pVLT31-mhpTE27A]; E27D: strain W3110ΔmhpT [pVLT31-mhpTE27D]; D75A: strain W3110ΔmhpT [pVLT31-mhpTD75A]; D75E: strain W3110ΔmhpT [pVLT31-mhpTD75E]; A272H: strain W3110ΔmhpT [pVLT31-mhpTA272H]; K276D: strain W3110ΔmhpT [pVLT31-mhpTK276D]; 3HPP: 3-(3-hydroxyphenyl)propionate. All assays were performed in triplicate and standard deviations are represented by error bars.