A TonB-dependent receptor constitutes the outer membrane transport system for a lignin-derived aromatic compound

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TonB-dependent receptors (TBDRs) mediate substrate-specific transport across the outer membrane, utilizing energy derived from the proton motive force transmitted from the TonB−ExbB−ExbD complex located in the inner membrane (TonB system). Although a number of TonB systems involved in the uptake of siderophores, vitamin B12 and saccharides have been identified, their involvement in the uptake and catabolism of aromatic compounds was previously unknown. Here, we show that the outer membrane transport of a biphenyl compound derived from lignin is mediated by the TonB system in a Gram-negative bacterium capable of degrading lignin-derived aromatic compounds, Sphingobium sp. strain SYK-6. Furthermore, we found that overexpression of the corresponding TBDR gene enhanced the uptake of this biphenyl compound, contributing to the improved rate of platform chemical production. Our results will provide an important basis for establishing engineered strains optimized for use in lignin valorisation.

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The cell envelope of Gram-negative bacteria comprises outer and inner membranes; therefore, nutrients must be passed through these two membranes to utilize them in the bacterial cells. In the outer membrane, transport of nutrients is achieved by passive transporters, such as porins and substrate-specific channels, and active transporters, TonB-dependent receptors (TBDRs). TBDRs mediate substrate-specific transport across the outer membrane, utilizing energy derived from the proton motive force transmitted from the TonB–ExbB–ExbD complex (TonB complex) located in the inner membrane (TonB system). A number of TonB systems involved in the uptake of siderophores, vitamin B12 and saccharides have been identified. Although there are examples where the TonB system transports certain siderophores containing aromatic groups, its involvement in the uptake and catabolism of aromatic compounds was previously unknown. It was envisaged that TBDRs would have more diverse functions than is currently known based on the fact that many Gram-negative bacteria have a large number of TBDR-like genes in their genomes, whose functions are unknown.

Recently, novel functions of the active transporters, TonB-dependent receptors, have been documented, such as in the uptake of rare earth metals, membrane homeostasis, and secretion of proteins. Its involvement in the uptake and catabolism of aromatic compounds was previously unknown. It was envisaged that TBDRs would have more diverse functions than is currently known based on the fact that many Gram-negative bacteria have a large number of TBDR-like genes in their genomes, whose functions are unknown.

Bacterial outer membrane transporters of aromatic compounds reported thus far have included passive transporters such as OpdK and OmpW, which are a vanillate-specific channel of Pseudomonas aeruginosa PAO1 and a naphthalene porin of Pseudomonas fluorescens, respectively. Recently, novel functions of the active transporters, TonB-dependent receptors, have been documented, such as in the uptake of rare earth metals, membrane homeostasis, and secretion of proteins. In addition, the upregulation of particular TBDR-like genes has been observed in Sphingomonas wittichii RW1 and a Pseudomonas strain during their growth in the presence of dioxin and vanillin, respectively, evoking the possibility that TBDRs participate in the outer membrane transport and catabolism of aromatic compounds. Generally, TBDRs have a membrane-spanning barrel domain consisting of 22 antiparallel β-strands and an N-terminal region containing a short, conserved motif called the TonB box, which is essential for the interaction between TBDRs and the C-terminal region of TonB. The inside of the β-barrel domain is filled with a plug domain that prevents nonspecific substrate influx.

Lignin, a major component of plant cell walls, is the most abundant aromatic compound on Earth; thus, its industrial use is eagerly anticipated to achieve sustainable development. However, the effective utilization of lignin has not yet been established, mainly due to its structural complexity and recalcitrance. Recently, the production of value-added chemicals from lignin has not yet been established, eagerly anticipated to achieve sustainable development. However, disruption of ompW did not affect the growth of SYK-6 on lignin-derived aromatic compounds (Supplementary Figs. 1–3). A phylogenetic tree was constructed based on the deduced amino acid sequence similarity between the 74 putative TBDR porins reported in other bacteria. However, overexpression of pck and ddk in a PDC-accumulating mutant of SYK-6 improved the rate of growth on substrates, substrate conversion and PDC production. Therefore, increased transporter gene expression should be able to enhance the production of metabolites from lignin derivatives. In contrast, the outer membrane transport of lignin-derived aromatic compounds, the initial stage of catabolism, remains largely unknown.

Here, we show that the outer membrane transport of a biphenyl compound derived from lignin is mediated by the TonB system in SYK-6. To our knowledge, this is the first report experimentally demonstrating the involvement of the TonB system in the outer membrane transport and catabolism of an aromatic compound. Furthermore, we found that overexpression of the corresponding TBDR gene enhanced the uptake of this biphenyl compound, thus contributing to the improved rate of platform chemical production from lignin-derived compounds.

**Results**

**TBDR genes induced by lignin-derived aromatic compounds.** SYK-6 has an ompW-like gene (SLG_38320), which has similarities with aromatic-compound porins reported in other bacteria. However, disruption of ompW did not affect the growth of SYK-6 on lignin-derived aromatic compounds (Supplementary Figs. 1–3). A phylogenetic tree was constructed based on the deduced amino acid sequence similarity between the 74 putative TBDR genes in SYK-6 and known TBDR genes (Supplementary Table 1). This analysis showed that 25 and 28 SYK-6 TBDRs could be classified into two specific clades separated from those including the known TBDRs (Fig. 1a). This fact suggests that these TBDRs may have unknown functions.

The expression patterns of all TBDR genes in SYK-6 cells incubated in Wx minimal medium containing SEMP (10 mM sucrose, 10 mM glutamate, 20 mg l−1 methionine and 10 mM proline) in the presence and absence of lignin-derived aromatic compounds were investigated by DNA microarray analysis. We found that the transcription of 17 TBDR genes was specifically induced and increased 2–35-fold during growth with lignin-derived aromatic compounds, including guaiacylglycerol-β-guaiacyl ether (GGE; β–0–4 type), dehydrodiconiferyl alcohol (DCA; β–5 type) and DDVA (5–5 type) (Fig. 1b, Supplementary Table 2). Among these genes, the expression of SLG_07650 was induced 4.87-fold during growth with DDVA. The SLG_07650 gene is located upstream of the DDVA catabolic genes cluster, which includes genes for catabolic enzymes (ligXa, ligZ and ligY), an inner membrane transporter (ddvK), and a Mar-type transcriptional regulator (ddvR) that negatively regulates ligXa expression (Fig. 2a, b) (1). The Boctopus program predicted that the gene product of SLG_07650 forms a membrane-spanning barrel domain, comprising 22 antiparallel β-strands, which is a typical feature of TBDRs (Supplementary Fig. 4). In addition, the presence of an N-terminal signal sequence and a subsequence plug

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**Table 1.** TA and DDVA catabolic genes in SYK-6 (3). A phylogenetic tree was constructed based on the deduced amino acid sequence similarity between the 74 putative TBDR porins reported in other bacteria. Recently, we identified the major facilitator superfamily transporter genes pck and ddk, which are involved in the inner membrane transport of protocatechuate (PCA) and 5,5′-dehydrodiconiflavine (DDVA), respectively, in SYK-6. Overexpression of pck and ddk in a PDC-accumulating mutant of SYK-6 improved the rate of growth on substrates, substrate conversion and PDC production. Therefore, increased transporter gene expression should be able to enhance the production of metabolites from lignin derivatives. In contrast, the outer membrane transport of lignin-derived aromatic compounds, the initial stage of catabolism, remains largely unknown. In the SYK-6 genome (accession numbers AP012222 and AP012223), only one gene showed any similarity with known aromatic-compound porins, whereas there are 74 putative TBDR genes. Based on the fact that SYK-6 specializes in the degradation of lignin-derived aromatic compounds, we predicted the involvement of TBDR genes in the outer membrane transport of aromatic compounds.

**Results**

**TBDR genes induced by lignin-derived aromatic compounds.** SYK-6 has an ompW-like gene (SLG_38320), which has similarities with aromatic-compound porins reported in other bacteria. However, disruption of ompW did not affect the growth of SYK-6 on lignin-derived aromatic compounds (Supplementary Figs. 1–3). A phylogenetic tree was constructed based on the deduced amino acid sequence similarity between the 74 putative TBDR genes in SYK-6 and known TBDR genes (Supplementary Table 1). This analysis showed that 25 and 28 SYK-6 TBDRs could be classified into two specific clades separated from those including the known TBDRs (Fig. 1a). This fact suggests that these TBDRs may have unknown functions.

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domain-like sequence were predicted. We hypothesized that SLG_07650 is involved in the uptake of DDVA, and designated this gene *ddvT*.

**DdvT mediates the outer membrane transport of DDVA.** We performed western blot analysis using anti-DdvT antibodies against total membrane fractions obtained from SYK-6, *ddvT* mutant (Δ*ddvT*, Supplementary Fig. 2), and *ddvR* mutant (Δ*ddvR*) cells grown with or without 1 mM DDVA. DdvT was not detected in Δ*ddvT* cells, whereas production of DdvT in the wild type was highly induced during growth with DDVA (Fig. 2c, Supplementary Fig. 15a). In Δ*ddvR* cells, DdvT was produced in almost equal quantities to the wild-type cells incubated with
DDVA, regardless of the presence or absence of DDVA, indicating that the expression of *ddvT* is negatively regulated by DdvR.

The growth of Δ*ddvT* cells on lignin-derived aromatic compounds was examined. Growth retardation was observed, specifically with DDVA (Fig. 2d, Supplementary Fig. 5a). Furthermore, resting cells of Δ*ddvT* almost lost the ability to convert DDVA, whereas the same resting cells could convert other lignin-derived aromatic compounds as efficiently as the wild type (Fig. 2e, Supplementary Fig. 5b). Next, we evaluated DDVA uptake by Δ*ddvT* cells using a DDVA-uptake assay, which we had developed using the DdvR transcriptional regulation system with lacZ as the reporter30. When SYK-6 cells harbouring the reporter plasmid carrying *ddvR* and a transcriptional fusion of a ligXα promoter region and lacZ are incubated without DDVA, expression of lacZ is heavily repressed by DdvR. In the presence of DDVA, DDVA is incorporated into cells and the repression by DdvR is ended. Therefore, intercellular DDVA can be indirectly monitored by measuring LacZ activity. This assay showed that DDVA uptake by Δ*ddvT* cells was completely lost when they were incubated with 100 μM DDVA, as was seen with Δ*ddvK* cells (Fig. 2f). However, when Δ*ddvT* cells were incubated with 1 and 5 mM DDVA, the uptake of DDVA was estimated to be around 20% and 40% of that of the wild type, respectively. These results suggest that other transporter(s) are also involved in the outer membrane transport of DDVA under high concentrations. The introduction of *ddvT* into Δ*ddvT* cells restored their growth on DDVA, DDVA conversion and DDVA uptake (Fig. 2d, e, g). Additionally, the introduction of *ddvT* into SYK-6 cells considerably enhanced their DDVA conversion and uptake (Fig. 2e, g). Taken together, these results indicate that DdvT is involved in the uptake of DDVA. The growth of Δ*ddvT*-complemented Δ*ddvT* DDVA and its DDVA uptake (1 and 5 mM) were not fully recovered. These phenomena are most likely due to the low expression of *ddvT* resulting from the foreign promoter in the vector (Supplementary Figs. 6, 15b). We also assessed DDVA uptake by mutants of SLG_04460 and SLG_38050, whose transcription was induced 2.64- and 2.00-fold, respectively, during growth with lignin-derived aromatic compounds, indicating that SYK-6 depends on DdvT for the outer membrane transport of low concentrations of DDVA (Fig. 2e–g). Outer membrane transport systems are known to vary among bacteria. For example, substrate uptake by *Pseudomonas* is primarily dependent on substrate-specific channels, and therefore this genus exhibits high levels of antibiotic resistance by suppressing the influx of nonspecific substrates33. SYK-6 may utilize a number of TBDRs for its outer membrane substrate transport, instead of porins and substrate-specific channels. Based on the fact that the transcription of 16 TBDR genes other than *ddvT* was specifically induced during growth with lignin-derived aromatic compounds, it seems likely that these compounds are taken up by TBDRs (Fig. 1). It appears that SYK-6 switches TBDRs via substrate-specific inducible expression in order to facilitate the uptake of necessary nutrients. The genera *Alteromonas*, *Xanthomonas* and *Caulobacter* have large numbers of TBDR-like genes in their genomes, equal to or greater than the number present in SYK-610. Presumably, the presence of various TBDRs with high substrate-affinity and -specificity is advantageous for bacteria, enabling them to survive in natural environments where nutrients are limited.

**Cellular localization of DdvT.** In order to investigate cellular localization of DdvT, a C-terminal His-tagged SLG_34540 encoding a TonB gene was first introduced into SYK-6 cells via a plasmid, so that the SLG_34540 product could act as an inner membrane marker. Using total and outer membrane fractions prepared from the above cells, we performed western blotting with anti-DdvT and anti-His6 antibodies. TonB was detected only in the total membrane fraction, whereas DdvT was detected in both fractions, indicating that DdvT is localized in the outer membrane (Fig. 2h, Supplementary Fig. 15c).

**The TonB box is important for DDVA uptake by DdvT.** At the N-terminus of SYK-6 TBDRs we found a conserved sequence consisting of XXXT (where X is a hydrophobic amino acid), which is similar to the TonB box18. DdvT contains IVVT spanning position 40–43 (Supplementary Fig. 8). Because mutations of the TonB box have been reported to reduce substrate uptake and interactions between TBDR and TonB, alanine mutations were introduced into the highly conserved V42 and T43 residues of DdvT31. Both the V42A mutant and the T43A mutant showed reduced growth on DDVA and rates of DDVA conversion compared with Δ*ddvT + ddvT* (Fig. 3a, b, Supplementary Figs. 9, 15d). The T43A mutant also displayed a substantial decrease in DDVA uptake (Fig. 3c). When alanine mutations were introduced into both V42 and T43, the growth of this double mutant on DDVA, its DDVA conversion and its DDVA uptake decreased to levels comparable with Δ*ddvT*. Thus, V42 and T43 are important residues, which comprise the TonB box of DdvT. Based on all the results obtained above, we concluded that DdvT is the novel outer membrane transporter of DDVA.

In general, substrate uptake by porins is effective under high substrate concentrations because they transport substrates nonspecifically based on the substrate's concentration gradient. In contrast, TBDRs and substrate-specific channels are effective under low substrate concentrations since they show high substrate affinities of the order of nM and μM–mM, respectively1,2,32. The Δ*ddvT* mutant completely lost the ability to uptake and convert DDVA under low concentrations, indicating that SYK-6 depends on DdvT for the outer membrane transport of low concentrations of DDVA (Fig. 2e–g). Outer membrane transport systems are known to vary among bacteria. For example, substrate uptake by *Pseudomonas* is primarily dependent on substrate-specific channels, and therefore this genus exhibits high levels of antibiotic resistance by suppressing the influx of nonspecific substrates33. SYK-6 may utilize a number of TBDRs for its outer membrane substrate transport, instead of porins and substrate-specific channels. Based on the fact that the transcription of 16 TBDR genes other than *ddvT* was specifically induced during growth with lignin-derived aromatic compounds, it seems likely that these compounds are taken up by TBDRs (Fig. 1). It appears that SYK-6 switches TBDRs via substrate-specific inducible expression in order to facilitate the uptake of necessary nutrients. The genera *Alteromonas*, *Xanthomonas* and *Caulobacter* have large numbers of TBDR-like genes in their genomes, equal to or greater than the number present in SYK-610. Presumably, the presence of various TBDRs with high substrate-affinity and -specificity is advantageous for bacteria, enabling them to survive in natural environments where nutrients are limited.

**Component genes of the TonB complex for DDVA uptake.** SYK-6 has six genes encoding putative TonB8s (*tonB1–tonB6*), which transmit energy derived from the proton motive force to TBDRs (Supplementary Figs. 10 and 11). In addition, we found...
two exbB-like genes (exbB1 and exbB2 [previously annotated as tolQ]) and three exbD-like genes (exbD1, exbD2 and exbD3 [previously annotated as tolR]). The gene products of tonB, exbB and exbD may constitute the TonB complex. Between them, tonB1, exbB1, exbD1 and exbD2 comprise an operon (Supplementary Fig. 12). We attempted to disrupt each of the tonB genes and succeeded in obtaining tonB2–tonB6 mutants (Supplementary Fig. 2). Since a tonB1 mutant was not obtained, tonB1 may be essential for the growth of SYK-6. Among ΔtonB2–ΔtonB6, ΔtonB2 exhibited reductions in growth on DDVA and in DDVA conversion (Fig. 4a, b). However, ΔtonB2 also showed similar reductions in growth on other lignin-derived aromatic compounds, lysogeny broth (LB) and SEMP (Supplementary Fig. 13). In the DDVA-uptake assay, ΔtonB2 cells exhibited considerably higher LacZ activity than SYK-6 cells, implying the accumulation of DDVA in ΔtonB2 cytoplasm (Fig. 4c). Because SLG_34550, just downstream of tonB2, has similarity with the E. coli TBDR gene (fiu) that encodes a siderophore transporter, tonB2 appears to be involved in the uptake of iron (Supplementary Fig. 10). The disruption of tonB2 seems to result in a reduction in iron uptake; this affects the activity of DDVA O-demethylase which contains a ferrous ion in its active centre34.

Considering the possibility that DdvT interacts with multiple TonBs, we evaluated the growth of tonB3–4–5–6 quadruple mutants in DDVA and SEMP (Supplementary Fig. 13). These mutants grew worse than SYK-6 cells on DDVA, suggesting that the TonB complex functions to transport DDVA in E. coli.
mutant (ΔtonB3456) cells on DDVA and their DDVA uptake (Supplementary Fig. 14a, b). However, ΔtonB3456 grew as well as the wild type, with DDVA uptake equivalent to the level seen in the wild type. In addition, the growth of tonB2-3-4-5-6 quintuple mutant cells on DDVA and their DDVA uptake were comparable to those of ΔtonB2 cells (Supplementary Fig. 14c, d). These results suggest that tonB1 plays a major role in the outer membrane transport of DDVA. To clarify the involvement of TonB1 in the uptake of DDVA, we evaluated the ability of resting SYK-6 cells harbouring a plasmid carrying tonB1 to convert DDVA. The amount of DDVA converted in tonB1-overexpressing cells increased ca. 1.8-fold (61 ± 3.2 µM) compared with the amount converted by wild-type cells (35 ± 5.7 µM) after 5 h (Fig. 4d). Furthermore, the amount of DDVA converted by cells overexpressing tonB1–exbB1–exbD1 and tonB1–exbB1–exbD1–exbD2 was ca. 1.2- (77 ± 0.4 µM) and 1.4-fold (91 ± 7.1 µM) higher, respectively, than that converted by cells overexpressing tonB1 only. On the other hand, the overexpression of tonB2 had no effect on DDVA conversion (30 ± 2.1 µM). These results suggest that TonB1, and possibly
ExB1, ExbD1 and ExbD2 are involved in the uptake of DDVA (Fig. 5).

**Overexpression of ddvT enhances production of PDC from DDVA.** A enhancement in the conversion and uptake of DDVA was observed following the overexpression of ddvT in SYK-6 cells (Fig. 2e, g). Based on this result, we evaluated the effect of ddvT overexpression on the production of PDC, which is a promising platform chemical produced from lignin, using an SYK-6 mutant of the PDC hydrolase gene (ΔligI), which accumulates PDC. When ΔligI cells harbouring a plasmid carrying ddvT were grown in Wx-SEMP medium in the presence of 1 mM DDVA, the amount of DDVA converted and PDC produced by these cells after 20 h increased ca. 1.3-fold compared with ΔligI cells (Fig. 6a). The cell yield also increased ca. 1.3-fold, suggesting that the cells efficiently utilized 4-carboxy-2-hydroxyxypenta-2,4-dienoate (CHPD) generated from lignin-derived aromatic compounds. Recently, there have been some reports of observations or at least suggestions of the possibility that TBDRs are involved in the uptake of chitin, cellulose oligomers and dioxin in non-pathogenic Gram-negative bacteria. Our findings will contribute not only to the understanding of the diverse functions of TBDRs but also to the application of TBDRs for improvements in the efficiencies of microbial production of value-added products and bioremediation.

**Methods**

**Bacterial strains, plasmids and culture conditions.** The strains and plasmids used in this study are listed in Supplementary Table 3 and the PCR primers are listed in Supplementary Table 4. Sphingobium sp. SYK-6 and its mutants were grown at 30 °C with shaking (160 rpm) in LB or Wx minimal medium containing SEMP. Media for E. coli transformants and PDC containing antibiotics were cultured in LB at 37 °C. Media for E. coli transformants carrying antibiotic resistance markers was supplemented with 50 mg l⁻¹ kanamycin (Km) or 30 mg l⁻¹ chloramphenicol (Cm). E. coli strains were cultured in LB at 37 °C. Media for E. coli transformants carrying antibiotic resistance markers was supplemented with 50 mg l⁻¹ kanamycin (Km) or 30 mg l⁻¹ chloramphenicol (Cm). DDVA, PR, DCA and HMPPD were chemically synthesized from ethyl vanillate, coniferyl aldehyde, coniferyl aldehyde and homovanillic acid, respectively. PDC was obtained from PCA by incubating with P. putida PrY1100 cells harbouring a plasmid carrying the protocatechuate 4,5-dioxygenase gene (ligAB) and the 4-carboxy-2-hydroxyxypenta-2,4-dienoate (CHPD) generated from the meta-cleavage compound of DDVA in addition to SEMP (Figs. 2a, 2b).

To date, TBDRs have mainly been investigated as a drug discovery target in the fight against pathogens because they function in the initial step of acquiring iron and other nutrients essential for growth. In contrast, to date no attempt has been made to utilize TBDRs for improving microbial conversion. Our results clearly demonstrate, however, that the overexpression of a TBDR gene encoding an outer membrane transporter is an effective means of improving the rate of metabolite production from lignin-derived aromatic compounds. Recently, there have been some reports of observations or at least suggestions of the possibility that TBDRs are involved in the uptake of chitin, cellulose oligomers and dioxin in non-pathogenic Gram-negative bacteria. Our findings will contribute not only to the understanding of the diverse functions of TBDRs but also to the application of TBDRs for improvements in the efficiencies of microbial production of value-added products and bioremediation.

**Construction of mutants.** To construct plasmids for gene disruption, ca. 1-kb fragments carrying upstream and downstream regions of each gene were amplified by PCR using SYK-6 genome DNA as a template and the primer pairs shown in Supplementary Table 4. The resulting fragments were inserted into the BamHI site in pAK405 by In-Fusion cloning (TaKaRa Bio, Inc.). These plasmids were independently introduced into SYK-6 cells and its mutants by triparental mating, and transformants generated by the first homologous recombination were selected on an LB agar medium containing Km and 12.5 mg l⁻¹ nalidixic acid. Selection of candidate mutants was performed according to the reported method with slight modifications described below. A mixture of transformants was cultured in LB liquid medium containing 100 mg l⁻¹ streptomycin, and then plated on an LB agar medium containing 100 mg l⁻¹ streptomycin to select for the second homologous recombination event. Resulting colonies were streaked on both an LB agar medium containing streptomycin and an LB agar medium containing Km, and Km-sensitive colonies were analysed by colony PCR using the primer pairs shown in Supplementary Table 4. The plasmids for gene complementation of ΔddvT and ΔtonB2 (Supplementary Table 3) were introduced into mutants by electroporation.

**Sequence analysis and construction of the phylogenetic tree.** Sequence analysis was performed using the MacVector program version 15.5.2 (MacVector, Inc.). Sequence similarity searches, pairwise alignments and multiple alignments were performed using the BLAST program, the EMBOSS program and the Clustal.
independent experiments.

Each value is the average ± the standard deviation of (Qiagen). Hybridizations were performed in a GeneTac HybStation instrument (pSEVA338 [vector]) and

Growth of ΔligI(pSEVA338) and ΔligI(pS-ddVT) in the above-mentioned medium. Each value is the average ± the standard deviation of n = 3 independent experiments.

Omega program43, respectively. For phylogenetic analysis of TBDRs, multiple alignments were performed using the Clustal W program in MEGA X44, and then a phylogenetic tree was generated using the neighbour-joining algorithm of MEGA X, employing 1000 bootstrap replicates. Putative transmembrane segments were predicted using the TMHMM program45 and the PHCOPUS2 program46, and signal sequence prediction was performed using SignalP-5.049.

Fig. 6 The effect of ddVT overexpression on PDC production. a Cells of ΔligI (pSEVA338 [vector]) and ΔligI(pS-ddVT) were cultured in Wx-SEMP containing 1 mM DDVA and 0.5 mM m-toluate. The amount of DDVA converted and PDC produced in the cultures was measured using HPLC.

Growth of ΔligI(pS-ddVT) and ΔligI(pS-ddVT) in the above-mentioned medium. Each value is the average ± the standard deviation of n = 3 independent experiments.

DNA microarray analysis. SYK-6 cells were grown in Wx-SEMP at 30 °C until OD 600 reached 0.5 at 600 nm (OD 600). Cultures were then incubated with 5 mM DDVA, HMPDD, GGE, FA, AV or VN for 6 h; 5 mM PCA for 2 h; 2 mM DCA or PR for 2 h; or without substrates for 2 h. Total RNA was isolated from the resulting cells from three independent cultures and used for DNA microarray analysis after DNase I treatment52. Aminoacyl-tRNA synthesis was performed by reverse transcription using total RNA (6 μg). Mixture of two kinds of random hexamers, normal GC content (Invitrogen) and high GC content (70%) (Sigma), 5-3′-aminolyl]-dUTP (Ambion) and PrimeScript II reverse transcriptase (TaKaRa Bio Inc.). The RNA template was then degraded through incubation with 0.1 N NaOH and 0.1 M EDTA, followed by a neutralization using 1 M HEPES (pH 7.5). For a control of microarray hybridization, 4 μg of fragmented genomic DNA of SYK-6 was labelled using 5′-3′-aminolyl]-dUTP and Klenow fragment. Cy3 and Cy5 dyes were coupled to the aminolyl-dUTP in the cDNA and genomic DNA, respectively, in the presence of 0.1 M sodium bicarbonate (pH 9.0). The unlabelled dyes were removed using QiAquick PCR purification system (Qiagen). Hybridizations were performed in a GeneTac HybStation instrument (Genomic Solutions). Hybridized arrays were scanned using a GenePix 4000B scanner (Axon Instruments), and the spot intensities were quantified using Image 6.1 (BioDiscovery). The expression patterns in cells grown in Wx-SEMP plus each lignin-derived aromatic compound were compared with those of cells grown in Wx-SEMP using an in silico analysis performed with the linear model for microarray analysis loess (subgrid) method using ArrayPipe2.053. Average normalized expression ratios (treatment/control) were calculated for each gene and tested for significant variation between treated (one-way ANOVA with Dunnett’s multiple comparisons post-test). Each value was obtained from n = 3 independent experiments.

RT-PCR analysis. Total RNA was isolated from SYK-6 cells grown in Wx-SEMP for 8 h using an Illumina RNAspin Mini RNA isolation kit (GE Healthcare). To remove any contaminating genomic DNA, the samples were treated with DNase I (TaKaRa Bio Inc.). Total RNA (4 μg) was reverse transcribed using SuperScript IV reverse transcriptase (Invitrogen) with random hexamer primers. The cDNA was purified using a NucleoSpin Gel and PCR Clean-up kit (Takara Bio, Inc.). PCR was performed with the cDNA, specific primers (Supplementary Table 4) and Gilex DNA polymerase (Takara Bio, Inc.). The resulting DNA was subjected to 0.8% agarose gel electrophoresis.

Growth measurement. The cells of SYK-6, its mutants and complemented strains were grown in LB for 24 h, harvested by centrifugation at 4800 × g for 5 min, washed twice with Wx medium and resuspended in 3 ml of the same medium. The cells were then inoculated in Wx medium containing 5 mM DDVA, SA, SN, VA, VN, PCA or FA to an OD 600 of 0.2. SYK-6 exhibits auxotrophy for methionine when grown in a methoxy-group-free substrate, so 20 mg l−1 methionine was added to the medium for growth on PCA. Cells were incubated at 30 °C with shaking (60 rpm) and cell growth was monitored every hour by measuring the OD 600 with a TSV062CA biophotorecorder (Advantec Co., Ltd.). For the analysis of complemented strains of ddVT, cells were grown in Wx medium containing Cm and 0.5 mM m-toluate (an inducer of the Pρn promoter in pSEVA338).

Resting cell assay. The cells of SYK-6 and its mutants were grown in LB for 20 h, harvested by centrifugation at 4800 × g for 5 min, washed twice with 30 mM Tris-HCl buffer (pH 7.5) and resuspended in 1 ml of the same buffer. The cells were then inoculated in 50 mM Tris-HCl buffer (pH 7.5) containing 100 μM DDVA to an OD 600 of 5.0 and incubated for 6 h. For conversion of 200 μM DCA, 200 μM GGE and 100 μM HMPDD, cells were inoculated to an OD 600 of 0.5, 2.0 and 2.0, respectively, and then the mixtures were incubated for 3, 6 and 3 h, respectively. Samples were collected periodically and the reactions were stopped by centrifugation at 18,800 × g for 10 min. The supernatants were diluted fivefold in water, filtered, and analysed by high-performance liquid chromatography (HPLC). For the analysis of the complemented strains of ddVT and SYK-6 harbouring a plasmid carrying tonB or component genes of the TonB complex, the cells grown in LB containing Km or Cm and 0.5 mM m-toluate were employed.

HPLC conditions. HPLC analysis was performed using an Acquity UPLC system (Waters Corporation) with a TSKgel ODS-140HTP column (2.1 by 100 mm; Tosoh Corporation). All analyses were carried out at a flow rate of 0.5 ml min−1 except the analysis of PDC (0.3 ml min−1). The mobile phase was a mixture of A (acetonitrile containing 0.1% formic acid) and B (water containing 0.1% formic acid) under the following conditions. For the analysis of conversion of DDVA, 0–2.5 min, 15% A. For the analysis of conversion of GGE, 0–3.2 min, linear gradient from 5 to 40% A; 3.2–6.0 min, decreasing gradient from 40 to 5% A; 6.0–7.0 min, 5% A. For the analysis of conversion of DCA, 0–3.0 min, 25% A. For the analysis of conversion of HMPDD, 0–2.5 min, 10% A. For the analysis of PDC accumulation, the mobile phase was a mixture of water (85%) and acetonitrile (15%) containing 0.1% phosphoric acid. DDVA, GGE, DCA, HMPDD and PDC were detected at 265, 279, 280, 279 and 315 nm, respectively.

DDVA-uptake assay. Cells of SYK-6 and its mutants harbouring pS-XR grown in LB containing Km for 20 h were harvested by centrifugation at 4800 × g for 5 min, washed twice with Wx medium and resuspended in 1.0 ml Wx-SEMP. The cells were then inoculated in Wx-SEMP with or without DDVA (0.1, 1.0 or 5.0 mM) to an OD 600 of 2.0. Samples were incubated at 30 °C with shaking (1500 rpm) for 3 h. The β-galactosidase activity of the cells was measured using 2-nitrophenyl-β-D-galactopyranoside as the substrate, according to a modified Miller assay (http://openwetware.org/wiki/Beta-Galactosidase_Assay_(A_Better_Miller))50. β-Galactosidase activity is expressed as Miller units. For complementation analysis, cells were grown in LB containing Km, Cm and 0.5 mM m-toluate, and used for the assay.

Alanine mutagenesis. Alanine mutagenesis for the residues in the predicted TonB box was performed by inverse PCR using pS-ddVT as a template and the mutation primers listed in Supplementary Table 4. The plasmids carrying mutated ddVT were
introduced into ΔddvT harbouring pS-XR by electroporation. The growth of these strains on DVA and their ability to uptake and convert DDA were evaluated.

Western blot analysis. A peptide corresponding to residues 67–84 (AER-GATNIAGDFLNEVPSF) of DdvT was synthesized and used as an antigen to obtain antisera against DdvT in rabbits (Cosmo Bio, Inc.). Anti-DDvT-peptide antibodies were obtained by purification of the antisera using peptide affinity chromatography (Cosmo Bio, Inc.). Western blot analysis using anti-DDvT antibodies was performed against total membrane fractions prepared from SYK-6, ΔddvT and ΔddvR cultured in the presence or absence of DDA. The cells were grown in LB and 1 mM DDA was added when the OD600 of the culture reached 0.5; they were then incubated for a further 12 h. Cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer (pH 7.5) and resuspended in the same buffer. The cells were disrupted by sonication and cell lysate was obtained. After the cell lysate was centrifuged at 18,800 × g for 10 min, the resulting supernatant was ultracentrifuged at 120,000 × g for 60 min to obtain the total membrane fraction. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Bio-Rad Laboratories) by electroblotting. The proteins on the membrane were stained with Ponseau S. After destaining with 0.1 M NaOH and washing with water, the PVDF membrane was blocked by incubation with TBS buffer (pH 7.4) containing 0.1% Tween20 (TBST buffer) and 5% blocking agent (GE Healthcare) for 1 h at room temperature, and then incubated with the same buffer containing primary anti-DDvT antibodies (0.25 µg/ml). After incubation for 1 h, the membrane was washed four times with TBST buffer, and then incubated with the same buffer containing horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibodies (Innogen, 0.2 µg/ml) for 1 h. Following incubation, the membrane was washed four times with TBST buffer, and horseradish peroxidase activity was detected by chemiluminescence using the ECL Western Blotting Detection System (GE Healthcare) with a LumiVision PRO image analyser (Asin Seiki Co, Ltd.). For detection of DdvT in the complemented strains and the TonB box mutants, the cells grown in LB containing Cm and 0.5 mM m-toluate were used for the assay. Protein concentrations were determined by the Bradford method using a Bio-Rad protein assay kit or Lowry’s assay with a DC protein assay kit (Bio-Rad Laboratories).

Cellular localization of DdvT in SYK-6. SYK-6 cells harbouring pJB-tonB2His that carried tonB2 fused with a His6 tag at its C-terminus in pJB861 were grown in LB containing Km and 1.0 mM m-toluate to an OD600 of 0.5. The outer membrane fraction was prepared according to the method reported by Hashimoto et al. The cells were harvested by centrifugation at 4800 × g for 10 min, washed twice with water and incubated with 26 mM Tris-HCl buffer (pH 8.3) containing 438 mM sucrose, 1.5 mM EDTA and 0.22 mg/ml lysozyme at 30 °C for 1 h. The resulting solution was centrifuged at 19,000 × g for 60 min to remove spheroplasts, and then the supernatant was ultracentrifuged at 120,000 × g for 60 min to obtain the outer membrane fraction. The total membrane fraction was prepared as described above. Western blot analysis was performed against the prepared outer membrane fraction and total membrane fraction using anti-DDvT and anti-His6 antibodies (Innogen, 1.0 µg ml−1) as primary antibodies. Horseradish peroxidase-conjugated goat anti-mouse IgG antibodies (Innogen, 0.04 µg ml−1) were used as the secondary antibodies for the anti-His6 antibodies.

PDC production. ΔglqI cells harbouring pSEVA338 or pS-ddvT were grown in LB containing Cm for 24 h. The cells were harvested by centrifugation at 4800 × g for 5 min, washed twice with Wx medium and resuspended in 3 ml of the same medium. The cells were inoculated to an OD600 of 0.2 in 5 ml Wx medium containing SEMP, 1 mM DDVA, Cm and 0.5 mM m-toluate and incubated at 30 °C with shaking (60 rpm). Cell growth was periodically measured by monitoring the OD600. Samples were periodically collected by centrifugation, diluted, filtered and analysed by HPLC.

Statistics and reproducibility. All our results were obtained from n = 3 independent experiments. Statistic tests were performed with Graphpad Prism8 (Graphpad software). One-way ANOVA with Dunnett’s multiple comparisons post-test was used as shown in figure legends. *P < 0.05 was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The DNA microarray data have been registered in the Gene Expression Omnibus under accession number GSE134094. All data supporting this study are available within the article and its Supplementary Information or are available from the corresponding author upon request.

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Author contributions
E.M. conceived and supervised the study. M.F., N.K. and E.M. designed the study, performed data analysis and wrote the manuscript. M.F. performed the experiments, with the following exceptions. K.M. constructed the *deB′T* mutant. N.K. and H.H. performed the DNA microarray analysis. S.H. synthesized the lignin-derived aromatic compounds used in this study. K.M. helped to interpret the data and discussed the results. All authors reviewed the paper.

Competing interests
The authors declare no competing interests.

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