Identification and expression analysis of a gene encoding a shikimate transporter of Corynebacterium glutamicum

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Shikimate can be utilized as the sole source of carbon and energy of Corynebacterium glutamicum. Although biosynthesis and degradation of shikimate are well characterized in C. glutamicum, the transport of shikimate has hardly been studied. A mutant strain deficient in cgR_2523 loses the ability to grow on shikimate as well as to consume extracellular shikimate, indicating that the gene is involved in shikimate utilization (designated shiA). The hydropathy profile of the deduced amino acid sequence indicates that ShiA belongs to the metabolite/proton symporter family, which is a member of the major facilitator superfamily. An accumulation assay showed that the uptake of shikimate was hardly detected in the shiA-deficient strain, but was markedly enhanced in a shiA-expressing strain. These results suggested that the uptake of shikimate was mainly mediated by the shikimate transporter encoded by shiA. The level of shiA mRNA induction by shikimate was significantly decreased by the disruption of cgR_2524 (designated shiR), which is located immediately upstream of shiA and encodes a LysR-type transcriptional regulator, suggesting that ShiR acts as an activator of shiA. To our knowledge, this is the first report in Gram-positive bacteria of a shikimate transporter and its regulation.

INTRODUCTION

The cyclic carboxylate shikimate, which is mainly produced by plants, is widely distributed in the biosphere, and a large number of micro-organisms utilize it (Bruce & Cain, 1990; Elsemore & Ornston, 1994; Hawkins et al., 1993; Tresguerres et al., 1970). Shikimate is an important intermediate in the common biosynthetic pathway of aromatic compounds known as the shikimate pathway, and can be utilized as a source of energy through a catabolic pathway (Fig. 1). This shikimate pathway starts from the condensation of glycolysis derived phosphoenolpyruvate and pentose phosphate pathway derived erythrose 4-phosphate to 3-deoxy-D-arabino-heptulosonate 7-phosphate. The shikimate pathway ends with the formation of chorismate via shikimate as an intermediate (Bongaerts et al., 2001; Knaggs, 2003). Quinate is connected to the shikimate pathway by a one-step reaction. Shikimate and quinate are convertible to protocatechuate, which is degraded via the β-ketoacid pathway that generates acetyl-CoA and succinyl-CoA and results in energy production via the tricarboxylic acid cycle. Recently, we reported that a transcriptional regulator, QsuR, upregulates shikimate utilization genes to promote shikimate degradation (Kubota et al., 2014). In contrast to the metabolic pathways involved in formation and/or degradation of shikimate, transporters for the uptake of shikimate have only been barely studied in micro-organisms. A shikimate transporter of Escherichia coli K-12 was cloned and the encoding gene was designated shiA (Whipp et al., 1998). To the best of our knowledge, there have been no other reports of attempts to identify other shikimate transporters.

Corynebacterium glutamicum is a Gram-positive soil bacterium that belongs to the subphylum actinomycetes and has been widely used for the industrial production of various amino acids (Hermann, 2003). C. glutamicum is able to grow on a variety of organic acids as sole carbon sources. Various transporters for organic acids of C. glutamicum have been investigated because it is important to understand the

Abbreviations: CDW, cell dry weight; EMSA, electrophoretic mobility shift assay; LTTR, LysR-type transcriptional regulator; MFS, major facilitator superfamily; qRT-PCR, real-time quantitative reverse transcription PCR.

One supplementary table and five supplementary figures are available with the online Supplementary Material.
In this report, a protein encoded by cgR_2523 (designated shiA) is identified as a shikimate transporter of C. glutamicum by growth tests and accumulation assays. Expression of shiA mRNA is induced in the presence of shikimate, and the induction is eliminated upon deletion of cgR_2524 (designated shiR) located immediately upstream of shiA, indicating that shiR activates shiA in response to shikimate. To our knowledge, ShiA of C. glutamicum is the first shikimate transporter identified in any Gram-positive bacteria.

**METHODS**

**Bacterial strains, media and growth conditions.** Strains and plasmids used in this study are listed in Table 1. C. glutamicum strain R (JCM18229) was used as wild-type (Yukawa et al., 2007). Gene deficient strains were obtained from a single-gene-disruptant mutant library constructed by transposon mediated mutagenesis (Suzuki et al., 2006b). E. coli strains were grown at 37 °C in Luria–Bertani medium with appropriate antibiotics. C. glutamicum strains were routinely grown in nutrient-rich A medium of composition (in 1 l): 2 g yeast extract, 7 g Casamino acids, 2 g urea, 7 g (NH4)2SO4, 0.5 g KH2PO4, 0.5 g K2HPO4, 0.5 g MgSO4·7H2O, 6 mg FeSO4·7H2O, 4.2 mg MnSO4·H2O, 0.2 mg thiamine (Inui et al., 2004). Cells were alternatively grown in minimal BT medium (A medium without yeast extract and Casamino acids). C. glutamicum strains were cultured aerobically with shaking at 180 r.p.m. at 33 °C, and cell starter cultures were grown in 10 ml A medium in 100 ml test tubes overnight, unless otherwise stated. When appropriate, the media contained kanamycin (50 μg ml⁻¹ for C. glutamicum and E. coli), chloramphenicol (5 μg ml⁻¹ for C. glutamicum, 50 μg ml⁻¹ for E. coli) or ampicillin (50 μg ml⁻¹ for E. coli).

To evaluate the growth in liquid medium, the starter cells were harvested by centrifugation and washed twice with BT medium. The cells were suspended to an OD₆₅₀ of 0.2 in 80 ml BT medium containing kanamycin supplemented with 10 mM carbon source and cultured aerobically at 33 °C in 500 ml flasks. Cell growth was monitored by measuring the OD₆₅₀ with a spectrophotometer. Shikimate concentration in medium during cultivation was quantified by using a HPLC system (Prominence; Shimadzu) equipped with a reverse-phase column [Cosmosil 5C18-AR-II packed column (4.6 by 250 mm); Nacalai Tesque] and a photodiode array detector. The mobile phase was methanol/water/60% perchloric acid (1000/4000/5.75, by vol.) and a flow rate of 1.0 ml min⁻¹ was maintained throughout. The setting of the column oven was 40 °C. In this condition, shikimate was eluted at 3.1 min. The wavelength used to detect shikimate was 210 nm.

**Recombinant DNA work.** The oligonucleotides used in this study are listed in Table S1 (available in the online Supplementary Material). Gene deletion mutants were constructed using a suicide vector, pCRA725, carrying the sacB gene encoding the levansucrase of Bacillus subtilis, essentially as described previously (Inui et al., 2004). In the case of shiA, a DNA fragment containing the shiA gene was amplified by PCR with genomic DNA of C. glutamicum as a template, using primers 2523-1 and 2523-2. The PCR product was cloned into the Sall site of pCRA725 with kanamycin resistance, yielding pCRF216. An internal segment of the shiA gene was removed by inverse PCR with pCRF216 as a template, using primers 2523-3 and 2523-4. The PCR product was then digested with Xhol and self-ligated, yielding pCRF217. The resultant plasmid was electroporated into C. glutamicum, and single-crossover cells were isolated based on their kanamycin resistance. Isolated cells were cultivated on a...
medium supplemented with 10% sucrose, and double-crossover cells were isolated. The primers used for *shiR* deletion were 2524-1, 2524-2, 2524-3 and 2524-4. The primers used for *aroK* deletion were 1671-1, 1671-2, 1671-3 and 1671-4. The primers used for *qudI* deletion were 0495-1, 0495-2, 0495-3 and 0495-4. Gene deletion was confirmed by DNA sequencing of PCR products around the deleted region.

For constitutive expression of *shiA*, the region of the ORF of *shiA* was amplified by PCR with genomic DNA of *C. glutamicum* as a template, using primers 2523-5 and 2523-6. The PCR product was inserted into EcoRV site of *E. coli–Corynebacterium* shuttle vector pCR2210, which is derived from pCR22 (Yamamoto et al., 2012), yielding pCRF219 for the expression of *shiA* under the control of the lac promoter. For *shiR*, the PCR product amplified using primers 2524-5 and 2524-6 was digested with *KpnI* and *SalI* and cloned into the corresponding site on pCR22 (Tsuchida et al., 2010), yielding pCRF219 for the expression of *shiR* under the control of the lac promoter.

For fluorescent imaging, a bacterial expression vector pNCS-mTFP1 (Allele Biotechnology) was digested with *BanHI* and *HindIII*, and the fragment containing mTFP1 was cloned into the corresponding site of *E. coli–Corynebacterium* shuttle vector pCRB1 (Nakata et al., 2003), yielding pCRF220 for expressing mTFP1. A C-terminal mTFP1-tagged *ShiA* expression vector, pCRF221, was constructed by cloning the PCR product of *shiA* gene amplified using primers 2523-7 and 2523-8 into the PCR product of *shiA* gene amplified using primers 2523-1, 2523-2, 2523-3 and 2523-4. The PCR product was digested with *KpnI* and *SalI*, and inserted into the corresponding sites of an expression vector, pColdI (Takara), yielding pCRF220 for expressing mTFP1. A C-terminal mTFP1–*shiA* gene fused to a His-tag sequence at the N-terminus was constructed by cloning the PCR product of *shiA* gene. Inui et al. (2004) gene amplified using primers 2523-7 and 2523-8 into the PCR product of *shiA* gene. Inui et al. (2004), yielding pCRF220 for expressing mTFP1. A C-terminal mTFP1–*shiA* gene fused to a His-tag sequence at the N-terminus was constructed by cloning the PCR product of *shiA* gene amplified using primers 2523-7 and 2523-8 into the *SacI* site located upstream of the *mTFP1*-encoding region on pCRF220.

For the electrophoretic mobility shift assay (EMSA), the region of the ORF of *shiA* was amplified by PCR with genomic DNA of *C. glutamicum* as a template, using primers 2524-1 and 2524-2. The PCR product was digested with *KpnI* and *SalI*, and inserted into the corresponding sites of an expression vector, pColdI (Takara), yielding pCRF222 for expressing His-tagged *ShiA*. The promoter region of *shiA* was amplified by PCR with genomic DNA of *C. glutamicum* as a

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>C. glutamicum</em> R</td>
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<tr>
<td>Tn0062</td>
<td>cgR_0062-deficient mutant of strain R constructed by transposon-mediated mutagenesis.</td>
<td>Suzuki et al. (2006a)</td>
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<tr>
<td></td>
<td>Transposon is inserted 1098 bases downstream of the 5′ end of the gene.</td>
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<td>Tn0369</td>
<td>cgR_0369-deficient mutant of strain R constructed by transposon-mediated mutagenesis.</td>
<td>Suzuki et al. (2006a)</td>
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<td>Transposon is inserted 402 bases downstream of the 5′ end of the gene.</td>
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<td>Tn0492</td>
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<td>Suzuki et al. (2006a)</td>
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<tr>
<td></td>
<td>Transposon is inserted 992 bases downstream of the 5′ end of the gene.</td>
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<tr>
<td>Tn2523</td>
<td>cgR_2523-deficient mutant of strain R constructed by transposon-mediated mutagenesis.</td>
<td>Suzuki et al. (2006a)</td>
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<tr>
<td></td>
<td>Transposon is inserted 171 bases downstream of the 5′ end of the gene.</td>
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<td>Tn2542</td>
<td>cgR_2542-deficient mutant of strain R constructed by transposon-mediated mutagenesis.</td>
<td>Suzuki et al. (2006a)</td>
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<tr>
<td></td>
<td>Transposon is inserted 415 bases downstream of the 5′ end of the gene.</td>
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<td>ΔshiA</td>
<td>R with deletion in cgR_2523, lacking an internal 1132 bp segment of the gene.</td>
<td>This study</td>
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<td></td>
<td>Deletion start point is 134 bases downstream of the 5′ end of the gene.</td>
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<tr>
<td>ΔshiA ΔaroK ΔqudI</td>
<td>ΔshiA with deletions in cgR_1671 and cgR_0495, lacking an internal 295 and 446 bp segment of each gene, respectively. Deletion start points are 80 and 194 bases downstream of the 5′ ends of the genes, respectively.</td>
<td>This study</td>
</tr>
<tr>
<td>ΔshiR</td>
<td>R with deletion in cgR_2524, lacking an internal 434 bp segment of the gene.</td>
<td>This study</td>
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<td></td>
<td>Deletion start point is 123 bases downstream of the 5′ end of the gene.</td>
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<tr>
<td>ΔqudI</td>
<td>R with deletion in cgR_0491, lacking an internal 612 bp segment of the gene.</td>
<td>Kubota et al. (2014)</td>
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<tr>
<td></td>
<td>Deletion start point is 57 bases downstream of the 5′ end of the gene.</td>
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<td><strong>Plasmids</strong></td>
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<td>pCRB2</td>
<td><em>E. coli–Corynebacterium</em> shuttle vector with lac promoter and kanamycin-resistance gene.</td>
<td>Tsuchida et al. (2010)</td>
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<td>pCRB22</td>
<td><em>E. coli–Corynebacterium</em> shuttle vector with kanamycin-resistance gene.</td>
<td>Yamamoto et al. (2012)</td>
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<td>pCRB22 with gapA promoter and rmnI terminator.</td>
<td>This study</td>
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<td>pCRF216</td>
<td>pCR7A25 harbouring 3.6 kbp PCR fragment containing the shiA gene.</td>
<td>This study</td>
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<tr>
<td>pCRF217</td>
<td>pCR216 lacking an internal 1132 bp segment of the shiA gene.</td>
<td>This study</td>
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<tr>
<td>pCRF218</td>
<td>pCR210 harbouring shiA under the control of gapA promoter.</td>
<td>This study</td>
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<td>pCRF219</td>
<td>pCR212 harbouring shiR under the control of lac promoter.</td>
<td>This study</td>
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<td>pCRF220</td>
<td>pCR81 harbouring mTFP1 under the control of lac promoter.</td>
<td>This study</td>
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<tr>
<td>pCRF221</td>
<td>pCR81 harbouring shiA–mTFP1 under the control of lac promoter.</td>
<td>This study</td>
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<tr>
<td>pCRF222</td>
<td>pColdI (Takara) harbouring shiA gene fused to a His-tag sequence at the N-terminus.</td>
<td>This study</td>
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<tr>
<td>pCRF223</td>
<td>pUC18 (Takara) harbouring shiA promoter region.</td>
<td>This study</td>
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template, using primers 2523-upFw and 2523-upRv. The product was digested and inserted into the SalI site of cloning vector pUC18 (Takara), yielding pCRF223 for preparing a DNA probe.

**Fluorescent imaging.** *C. glutamicum* cells transformed with pCRF220 or pCRF221 were placed on a 1 % agar-padded slide containing BT medium with 2 % glucose as described previously (Tsuge et al., 2008). Images were acquired with an upright microscope (Olympus BX51) equipped with a ×100 objective (UPlanSapo, NA 1.4, oil immersion) and a CCD camera (DP70; Olympus). Fluorescence was collected with an appropriate filter set (U-MGFPHQ; Ex 460–480, DM 485, Em 495–540).

**Shikimate consumption assay.** Consumption assays were performed using the method of Chaudhry et al. (2007) and Teramoto et al. (2008). *C. glutamicum* starter cell culture was harvested, washed with A medium, and then suspended in 20 ml A medium supplemented with 50 mM of either glucose or shikimate in a 100 ml test tube to grow to an OD\(_{610}\) of 4.0–5.0. Cells were harvested and washed twice with 50 mM Tris/HCl buffer (pH 8.0). Cells were resuspended in the buffer (8 ml) at OD\(_{610}\) 6.0. After precultivation at 33 °C for 5 min with shaking, the cell suspension was supplemented with 10 mM shikimate and incubated with shaking at 180 r.p.m. at 33 °C. Consumption of shikimate, as indicated by a decrease in the concentration in the supernatant, was analysed by using the HPLC system described above.

**Shikimate accumulation assay.** *C. glutamicum* starter cell culture was grown aerobically until mid-exponential phase in 100 ml A medium with 2 % glucose, 25 mg tryptophan l\(^{-1}\), 25 mg phenylalanine l\(^{-1}\), 25 mg tyrosine l\(^{-1}\) and 12.5 mg para-aminobenzoate l\(^{-1}\) at 33 °C in a 500 ml flask. The cells were harvested and washed twice with 50 mM Tris/HCl buffer (pH 8.0), and then suspended with 10 ml of the same buffer at OD\(_{610}\) 5.0 in a 100 ml test tube. After precultivation at 33 °C for 5 min with shaking, the cell suspension was supplemented with 10 mM shikimate and aerobically incubated with shaking at 33 °C. Intracellular shikimate in *C. glutamicum* was extracted by modifying methods described previously (Teramoto et al., 2008). Cells were separated by vacuum filtration (PTFE membrane, 0.5 μm pore size, 47 mm diameter; Advantec) and washed four times with 8 ml ice-cold NaCl solution (0.9%). Subsequently, the filter was plunged into 1 ml methanol (–20 °C) for rapid deactivation of enzymes and simultaneous extraction of intracellular metabolites in *C. glutamicum*. After incubation for 60 min or longer at –20 °C, ice-cold water and –20 °C chloroform were added for extraction. Analyses of upper phase of samples were performed by HPLC as described above. Cell dry weight (CDW) values were calculated from OD\(_{610}\) values using the formula CDW (g l\(^{-1}\)) = 0.334 × OD\(_{610}\).

**RNA preparation and real-time quantitative reverse transcription PCR (qRT-PCR) analysis.** Starter cell culture was harvested and washed with A medium, suspended in 10 ml A medium supplemented with 10 mM carbon source, and then cultured aerobically at 33 °C for 30 min. RNA was isolated using an extraction kit (NucleoSpin RNA II; Machery-Nagel) according to the manufacturer’s protocol for RNA isolation. RNA concentrations were determined spectrophotometrically at OD\(_{260}\). A one-step real-time quantitative reverse transcription PCR (qRT-PCR) was performed with the Power SYBR Green PCR master mix (Applied Biosystems) with 20 ng template RNA by using the 7500 Fast real-time PCR system (Applied Biosystems) as described previously (Kubota et al., 2014). The relative abundances of the target mRNAs were quantified based on the cycle threshold (C\(_t\)) value, which is defined as the number of cycles required in order to obtain a fluorescence signal above the background level. The relative abundance of 16s rRNA was used as the internal standard to standardize the results (ΔC\(_t\)), where

\[ΔC_t = C_{\text{target mRNA}} - C_{\text{16S rRNA}},\]

The expression levels were evaluated using the formula 2(–ΔΔC\(_t\)), where ΔΔC\(_t\) = ΔC\(_t\)sample–mean ΔC\(_t\)control. The primers were RT2523Fw and RT2523Rv for *shiA*, and RT16SFw and RT16SRv for 16s rRNA. Specific amplification of the target gene was confirmed by electrophoresis and sequencing of the PCR product.

**EMSA.** *E. coli* BL21 cells (Takara) were transformed with pCRF222, and His-tagged ShiR was prepared as described previously (Kubota et al., 2014). Protein concentration was determined according to the Bradford method with BSA as a standard. An EMSA was performed as described previously (Kubota et al., 2014) with some modifications. A 340 bp DNA probe containing the *shiA* promoter region was prepared by PCR using primers 2523-upFw and 2523-upRv, with pCRF223 as a template. The purified ShiR protein was incubated with 2 nM DNA probe in 20 μl binding buffer containing 20 mM Tris/HCl (pH 8.0), 100 mM NaCl, 3 mM MgCl\(_2\), 0.1 mM DTT, 0.1 mM EDTA, 50 μg BSA ml\(^{-1}\) and 5 % (v/v) glycerol for 10 min at 25 °C. The binding reaction mixture was subjected to electrophoresis in a 5 % polyacrylamide gel in Tris/borate buffer, and the DNA probe was stained with SYBR Gold (Invitrogen) and detected with an image-analyser LAS-3000 (Fujifilm).

## RESULTS

**cgR\(_{2523}\) of *C. glutamicum* encodes a shikimate transporter**

Basic Local Alignment Search Tool (BLAST) analysis indicated that six proteins of *C. glutamicum* R had over 35 % amino acid identity to *E. coli* shikimate transporter, ShiA. Gene disruption mutants of the six candidate genes (cgR\(_{0062}\), cgR\(_{0369}\), cgR\(_{0492}\), cgR\(_{2523}\), cgR\(_{2542}\) and cgR\(_{2818}\)), which were obtained from our transposon mutant library (Suzuki et al., 2006b), were tested for growth on BT agar plates supplemented with 10 mM shikimate. Five of the six mutant strains and wild-type strain grew at similar rates on the shikimate plate, whereas strain Tn2523 did not grow on the shikimate plate (Fig. 2b). All strains grew at a similar rate on a plate supplemented with 10 mM glucose (Fig. 2a). The gene cgR\(_{2523}\) (GenBank accession no. YP_001139436) encodes a protein that belongs to the major facilitator superfamily (MFS) and has 450 aa residues with a calculated molecular mass of 48.3 kDa. The deduced amino acid sequence of cgR\(_{2523}\) showed 36 % identity to that of *E. coli* shikimate transporter.

To investigate the function of the protein encoded by cgR\(_{2523}\) (designated *shiA*), a gene deletion mutant was constructed and growth was examined in 80 ml BT medium supplemented with 10 mM shikimate. Growth was completely suppressed and shikimate consumption was eliminated upon deletion of *shiA* (Fig. 2c, d). The concentration of shikimate in the medium decreased, accompanied by decreased growth, for wild-type and *shiA*-complemented strains, and shikimate was fully consumed within 8 h. These results indicate that *shiA* is involved in the utilization of extracellular shikimate, potentially as a transporter. The OD\(_{610}\) of the *shiA* deletion mutant did not increase after 24 h cultivation in 10 ml BT medium supplemented with 30 mM or lower concentrations of shikimate, but reached

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the same level as that of the wild-type strain in medium supplemented with 50 mM shikimate (Fig. 2e). These results indicated that the mutant was able to utilize 50 mM shikimate. At high concentration, shikimate might be transported through non-specific or unknown low-affinity shikimate transporters. Then, effects of \( \text{shiA} \) deletion on utilization of other organic acids were examined. Wild-type strain and \( \text{shiA} \) deletion mutant grew at the same rate in 80 ml BT medium supplemented with 10 mM of either quinate or protocatechuate (Fig. 2f). Similar results were obtained using other cyclic organic acids, 4-hydroxybenzoate, vanillate and benzoate (Fig. S1), suggesting that ShiA is mainly involved in shikimate utilization.

Several studies visualized the membrane association of transporters using GFP-tagged proteins (Vogl et al., 2007; Zhao et al., 2011). To confirm whether ShiA associates with the cell membrane or not, a fluorescent protein encoding gene \( \text{mTFP1} \) was fused to \( \text{shiA} \) and subjected to microscopic imaging. Cells transformed with a plasmid carrying \( \text{shiA-mTFP1} \) showed fluorescence that was mainly detected on the cell membrane (Fig. 3a). To confirm whether the tagged ShiA is functional, a \( \text{shiA} \) deletion mutant was transformed with the plasmid and growth on shikimate was examined. Growth of the transformed strain was restored in BT medium supplemented with 10 mM shikimate (Fig. S2). In contrast, fluorescence was evenly

**Fig. 2.** ShiA is involved in shikimate utilization. Wild-type strain transformed with pCRB2 (empty vector) and gene disruption strains were streaked on BT medium plates containing kanamycin supplemented with 10 mM of either glucose (a) or shikimate (b) and incubated at 33 °C for 2 days. (c) Cells were cultured in 80 ml BT medium containing kanamycin supplemented with 10 mM shikimate in 500 ml flasks. Data are mean values with SDs of three independent experiments. (d) Shikimate concentration in the BT medium was monitored during the growth experiment shown in (c). Data are mean values with SDs of three independent experiments. The strains used in (c) and (d) were: wild-type strain containing an empty vector pCRB210 (open circles), \( \text{shiA} \) deletion strain containing an empty vector pCRB210 (filled squares), \( \text{shiA} \) deletion strain containing a constitutively \( \text{shiA} \)-expressing vector pCRF218 (open diamonds). (e) The \( \text{OD}_{610} \) after 24 h cultivation of wild-type and \( \text{shiA} \) deletion strains was measured. Cells were grown in 10 ml BT medium containing kanamycin supplemented with the indicated concentrations of shikimate in 100 ml test tubes. Both strains contained the empty vector (pCRB210). Data are mean values with SDs of three independent experiments. (f) Wild-type and \( \text{shiA} \) deletion strain cells were cultured in 80 ml BT medium containing kanamycin supplemented with 10 mM of either quinate or protocatechuate in 500 ml flasks. Both strains contained an empty vector (pCRB2). Similar results were obtained from two independent experiments, and representative results are shown.
distributed over cell bodies in cells transformed with a plasmid carrying mTFP1 alone (Fig. 3b). Topology prediction on a website (HMMTOP; http://www.enzim.hu/hmmtop/) based on a report by Tusnády & Simon (1998) indicates that ShiA contains 12 transmembrane domains (Fig. S3), supporting the membrane association of ShiA.

The ability of cells to consume shikimate was tested. Cells grown in A medium supplemented with 50 mM glucose were resuspended in 50 mM Tris/HCl buffer (pH 8.0) containing 10 mM shikimate and incubated with shaking at 180 r.p.m. at 33 °C. Shikimate concentrations in the supernatant decreased linearly with time in the constitutively shiA-expressing strain (Fig. S4a), while the concentrations did not change upon deletion of shiA, leading us to the conclusion that the protein encoded by shiA is a shikimate transporter. Essentially similar results were obtained with cells grown on 50 mM shikimate (Fig. S4b). The final shikimate concentrations after a 3 h incubation were 9.5 mM when precultured on glucose and 8.3 mM when precultured on shikimate. This difference in speed of consumption implies shikimate-dependent induction of shiA expression.

Shikimate accumulation was investigated to estimate the uptake ability of ShiA. For this purpose, a mutant strain deficient in a shikimate kinase gene (aroK), a shikimate dehydrogenase gene (qsuD) and shiA was constructed. In our previous reports, the growth of a qsuD-deficient mutant was completely suppressed in BT medium supplemented with 50 mM shikimate (Kubota et al., 2013; Teramoto et al., 2009). This indicates that the triple-deletion mutant is unable to utilize shikimate. Cells grown in A medium supplemented with 2% glucose and a small amount of tryptophan, phenylalanine, tyrosine and para-aminobenzoate were resuspended in 50 mM Tris/HCl buffer (pH 8.0) containing 10 mM shikimate and incubated with shaking at 180 r.p.m. at 33 °C, and then the intracellular metabolite was extracted. HPLC analysis of intracellular shikimate revealed that shikimate accumulated in a constitutively shiA-expressing mutant within 5 min (Fig. 4). As expected, the accumulation was not detected in the mutant containing a control vector.

ShiR increases shiA expression in the presence of shikimate

In order to examine the expression of shiA in response to shikimate, C. glutamicum wild-type strain was cultured in A medium supplemented with 10 mM of either shikimate or glucose and total RNA was prepared. qRT-PCR analysis revealed that shiA mRNA was markedly increased in the presence of shikimate and the expression level in cells grown on shikimate was over 20 times higher at 30 min cultivation than that in cells grown on glucose (Fig. 5a). Quinate, a precursor of shikimate, also induced shiA expression, but other organic acids, protocatechuate, 4-hydroxybenzoate, vanillate or benzoate, had no effect on the induction. To investigate the regulatory mechanisms involved in the shiA induction, the effect of qsuR (cgR_0491) deletion was examined because the corresponding protein QsuR regulates quinate/shikimate utilization genes (cgR_0492–cgR_0495) (Teramoto et al., 2009). However, the shiA mRNA induction in the qsuR-deleted strain was essentially identical to that observed in the wild-type strain (Fig. 5b), suggesting that qsuR is unrelated to the shiA mRNA induction. A gene, cgR_2524 (GenBank accession no. YP_001139437), that is located immediately upstream of shiA in the same orientation encodes a LysR-type transcriptional regulator (LTTR) (Fig. 5b), and this is a second candidate gene for the shiA regulation. Results of qRT-PCR analysis demonstrated that the induction of shiA mRNA was effectively suppressed upon deletion.
of cgR_2524, whereas the cgR_2524-complemented strain showed restored induction (Fig. 5c). Based on this, cgR_2524 was designated shiR, a transcriptional regulator that activates shiA in the presence of shikimate. Growth of the shiR deletion mutant was markedly slower than that of the wild-type strain when the strains were cultured in BT medium supplemented with 10 mM of either glucose, shikimate, quinate, protocatechuate, 4-hydroxybenzoate, vanillate or benzoate. The mRNA levels are presented relative to the mean value for the culture on shikimate. (b) Gene arrangement around shiA is depicted. (c) The levels of shiA mRNA in wild-type (wild-type strain containing an empty vector pCRB2), ΔshiR (shiR deletion strain containing an empty vector pCRB2) or ΔshiR complemented (shiR deletion strain containing a constitutively shiR-expressing vector pCRF219) strains grown in A medium containing kanamycin supplemented with 10 mM of either glucose or shikimate for 30 min were determined. The mRNA levels are presented relative to the mean value of wild-type strain for the culture on shikimate. Data are mean values with sds of at least three independent experiments. (d) Cells were aerobically grown in 80 ml BT medium containing kanamycin supplemented with 10 mM shikimate. Strains: wild-type containing an empty vector pCRB2, shiR deletion strain containing an empty vector pCRB2, shiR deletion strain containing a constitutively shiR-expressing vector pCRF219. Data are mean values with sds of at least three independent experiments.

Since carbohydrate transporters are often targets of carbon catabolite repression (Görke & Stülke, 2008), the effect of glucose on shikimate-induced shiA activation was examined at 30 min cultivation. Although the expression level of shiA mRNA in A medium supplemented with both 10 mM glucose and 10 mM shikimate was about 30% of that in
conclusion that ShiR acts as an activator of eukaryotes (Marger & Saier, 1993; Reddy et al. are ubiquitously distributed in prokaryotes as well as MFS transporters consists of more than 70 families that Comparative analysis of the primary sequence of ShiA with the promoter region of LTTR, ShiR, was necessary for the induction. ShiR binds to mRNA was induced in the presence of shikimate, and an disruption of the shiA that ShiA is a shikimate transporter. Expression of shiA and was restored in the gene. Taken together, we concluded that ShiA is a shikimate transporter. Expression of shiA mRNA was induced in the presence of shikimate, and an LTR, ShiR, was necessary for the induction. ShiR binds to the promoter region of shiA in vitro, supporting the second conclusion that ShiR acts as an activator of shiA.

MFS transporters consists of more than 70 families that are ubiquitously distributed in prokaryotes as well as eukaryotes (Marger & Saier, 1993; Reddy et al., 2012). Comparative analysis of the primary sequence of ShiA with the transport classification database (TCDB; http://www.tcdb.org/) places it in MFS 2.A.1.6, which is the metabolite/ H+ symporter (MHS) family (Pao et al., 1998; Saier et al., 1999). The MHS family includes Kgp of E. coli, for x-ketoglutarate transport (Seol & Shatkin, 1991), CitA of Klebsiella pneumoniae, for citrate (van der Rest et al., 1990), MopB of Burkholderia cepacia, for 4-methyl-o-phthalate (Saint & Romas, 1996), and ShiA of E. coli (Whipp et al., 1998). These members of the MHS family possess a highly conserved motif, GX3DX2GR (amino acids 96–105), is found at a similar position in the sequence of ShiA of C. glutamicum (Fig. S3). A similar sequence, DKIGKR (amino acids 317–322) occurs between predicted transmembrane domains 8 and 9, which is consistent with the model in which the MFS proteins exhibit a repeat of a six-transmembrane-segment unit (Reddy et al., 2012).

A unique post-transcriptional regulation of the E. coli shiA has been reported (Prévost et al., 2007). RyhB, which is a 90 nt small regulatory RNA, directly pairs at the 5′-untranslated region of the E. coli shiA mRNA to disrupt an intrinsic translation inhibition structure, resulting in increased expression of ShiA. The trigger of the activation is iron deprivation. Different from this small RNA-mediated regulation, shiA of C. glutamicum is under the control of an LTTR, ShiR (Figs 5 and 6). A typical LTTR activates its target genes in the presence of effector molecules (Maddocks & Oyston, 2008; Schell, 1993). In most cases, the direct effecter is closely related to the function of regulated genes, such as arginine for ArgP of E. coli to activate arginine-transporter-encoding gene argO (Nandineni & Gowrishankar, 2004). Therefore, it is

**Fig. 6.** In vitro binding of ShiR to the shiA promoter region. (a) The DNA probe (340 bp) used for EMSA is indicated. The DNA probe contained the last base of shiR and the first 15 bases of shiA. (b) Purified His-tagged ShiR was analysed in a 12% SDS-PAGE gel and visualized by Coomassie brilliant blue staining (lane 1, molecular mass marker (Precision Plus Protein All Blue Standards; Bio-Rad); lane 2, 300 ng purified His-tagged ShiR). (c) An EMSA was carried out with the DNA probe (2 nM) and ShiR at various concentrations (lane 1, no protein; lane 2, 37.5 ng; lane 3, 75 ng; lane 4, 150 ng; lane 5, 300 ng) in 20 μl binding buffer. Free DNA and the DNA–protein complex are indicated by white and black arrowheads, respectively.

**Fig. 7.** Effect of supplementation with both glucose and shikimate on the expression of the shiA mRNA. The mRNA levels in wild-type cells grown in A medium supplemented with 10 mM glucose, 10 mM shikimate, or both 10 mM glucose and 10 mM shikimate for 30 min were determined. The levels are presented relative to the mean value for the shikimate-grown cells. Data are mean values with sds of at least three independent experiments.
reasonable to assume that shikimate is an effector of ShiR. However, addition of shikimate in the EMSA buffer did not provoke any difference in migration speed of the DNA–ShiR complex. Further investigations are needed to determine the effector of ShiR. Recently, we reported that an LTTR, QsuR, activates quinate/shikimate utilization (qsu) genes in response to accumulation of chorismate, and this activation promotes degradation of shikimate (Kubota et al., 2014). Deletion experiments revealed that shiA is not regulated by QsuR (Fig. S5), suggesting that genes involved in transportation and utilization of shikimate are independently regulated by different regulators. In addition to the regulation, shiA is likely expressed constitutively at low levels, as is indicated by the result of slower growth of the shiR deletion mutant on shikimate (Fig. 5d). Taken together, extracellular shikimate is transported into the cytosol through ShiA, which is expressed at relatively low levels but constitutively. Subsequently, ShiR recognizes shikimate or an unknown intermediate metabolite then activates shiA expression. In our previous study, cgrR_0492 (qsuA) was presumed to be a quinate/shikimate transporter because qsuA is part of an operon of quinate/shikimate utilization genes. Even though QsuA might act as a low-affinity shikimate transporter, the primary shikimate transporter is ShiA.

Many bacteria utilize a preferred carbon source (frequently glucose) first when they are exposed to more than one carbohydrate (Deutscher, 2008). Unlike this catabolite repression, C. glutamicum can utilize simultaneously glucose and another carbon source, such as arabinose (Kawaguchi et al., 2009), acetate (Wendisch et al., 2000), vanillate (Merkens et al., 2005) or shikimate (Teramoto et al., 2009), in strain ATCC 31831, ATCC 13032 or strain R. Consistently, activation of shiA in the presence of both glucose and shikimate (Fig. 7) indicates the glucose effect for shiA regulation is less stringent. This relatively loose carbon catabolite repression is one of the unique properties of C. glutamicum.

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REFERENCES


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