

Regulation of Expression of Genes Involved in Quinate and Shikimate Utilization in *Corynebacterium glutamicum*[∇]

Haruhiko Teramoto, Masayuki Inui, and Hideaki Yukawa*

Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan

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The utilization of the hydroaromatic compounds quinate and shikimate by *Corynebacterium glutamicum* was investigated. *C. glutamicum* grew well with either quinate or shikimate as the sole carbon source. The disruption of *qsuD*, encoding quinate/shikimate dehydrogenase, completely suppressed growth with either substrate but did not affect growth with glucose, indicating that the enzyme encoded by *qsuD* catalyzes the first step of the catabolism of quinate/shikimate but is not involved in the shikimate pathway required for the biosynthesis of various aromatic compounds. On the chromosome of *C. glutamicum*, the *qsuD* gene is located in a gene cluster also containing *qsuA*, *qsuB*, and *qsuC* genes, which are probably involved in the quinate/shikimate utilization pathway to form protocatechuate. Reverse transcriptase PCR analyses revealed that the expression of the *qsuABCD* genes was markedly induced during growth with either quinate or shikimate relative to expression during growth with glucose. The induction level by shikimate was significantly decreased by the disruption of *qsuR*, which is located immediately upstream of *qsuA* in the opposite direction and encodes a LysR-type transcriptional regulator, suggesting that QsuR acts as an activator of the *qsuABCD* genes. The high level of induction of *qsuABCD* genes by shikimate was still observed in the presence of glucose, and simultaneous consumption of glucose and shikimate during growth was observed.

The abundant plant products quinate and shikimate are utilized as carbon and/or energy sources by various microorganisms. The quinate utilization pathways in the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans* have been well studied (Fig. 1) (12, 14). Quinate is converted to protocatechuate via three enzymatic reactions, catalyzed by quinate dehydrogenase, dehydroquinone dehydratase, and dehydroshikimate dehydratase. Subsequently, protocatechuate is metabolized through the β -ketoacid pathway. The expression of these enzymes is induced during growth on quinate and is subject to carbon catabolite repression (12–14). The first enzyme for the utilization of quinate, quinate dehydrogenase, also converts shikimate to dehydroshikimate. Dehydroquinone and dehydroshikimate are also intermediates of the shikimate pathway, leading to branched pathways of biosynthesis of various aromatic amino acids, vitamins, and quinones. The biosynthetic reactions of dehydroquinone dehydratase and shikimate dehydrogenase are the same as the quinate/shikimate catabolic reactions. However, separately from the inducible catabolic enzymes, a constitutive pentafunctional enzyme containing the dehydroquinone dehydratase and shikimate dehydrogenase activities is involved in the shikimate biosynthetic pathway (12, 14). The pentafunctional enzyme contains regions with amino acid sequence similarity to five shikimate pathway enzymes encoded by separate genes in bacteria, implying multiple gene fusion during evolution.

Among gram-negative bacteria, *Acinetobacter* species utilize quinate and shikimate via reactions similar to those in fungi (4, 6–8, 35, 42). However, the genes involved in quinate/shikimate

utilization (*qui* genes) in *Acinetobacter* have no significant similarity to the corresponding fungal genes, indicating convergent evolution. The *qui* genes are located downstream of a *pca* gene cluster involved in protocatechuate catabolism, and the expression of the *pca-qui* operon is induced during growth on either quinate or shikimate through the intermediate metabolite protocatechuate (4, 6, 35). PcaU, an IclR-type transcriptional activator-repressor, is involved in the regulation of the *pca-qui* operon in *Acinetobacter baylyi* (6, 41). The induction of *qui* gene expression is suppressed in the presence of preferred carbon sources (5, 35).

Understanding of the quinate/shikimate utilization pathways in gram-positive bacteria is very limited. *Amycolatopsis methanolica* can grow on quinate but not on shikimate as the sole carbon source (9). The characterization of *A. methanolica* dehydroquinone dehydratase and aromatic amino acid auxotrophic mutants suggests that the single dehydroquinone dehydratase present in this bacterium is involved in both the quinate catabolic pathway and the shikimate biosynthetic pathway (9). Quinate-inducible quinate/shikimate dehydrogenase in *Rhodococcus rhodochrous* has been purified and characterized previously (3). The features of this enzyme suggest that it is the initial enzyme of the quinate catabolic pathway in this bacterium. However, the relevant genetic information on the quinate utilization pathways in these bacteria remains elusive.

Corynebacterium glutamicum, which is a nonpathogenic gram-positive soil bacterium, is widely used for the industrial production of amino acids such as glutamate and lysine (20, 22). We have developed a bioprocess for the production of lactate, succinate, and ethanol using *C. glutamicum* (17, 18, 26–28). The genome sequence is helpful for the elucidation of various cellular functions of this industrially important microorganism (16, 19, 49). Key enzymes involved in the shikimate pathway for aromatic amino acid production have been char-

* Corresponding author. Mailing address: Research Institute of Innovative Technology for the Earth, 9-2 Kizugawadai, Kizugawa, Kyoto 619-0292, Japan. Phone: 81-774-75-2308. Fax: 81-774-75-2321. E-mail: mmg-lab@rite.or.jp.

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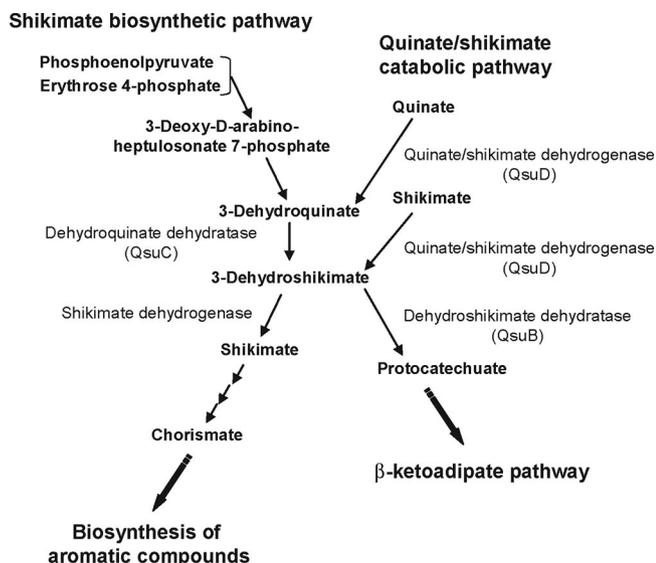


FIG. 1. The quinate/shikimate catabolic and shikimate biosynthetic pathways. Quinate is catabolized to form protocatechuate through the three reactions catalyzed by quinate/shikimate dehydrogenase (*C. glutamicum* QsuD), dehydroquinate dehydratase (QsuC), and dehydroshikimate dehydratase (QsuB). Shikimate is converted to dehydroshikimate by quinate/shikimate dehydrogenase (QsuD). Protocatechuate is subsequently metabolized by the β -ketoacid pathway. Dehydroquinate dehydratase and shikimate dehydrogenase reactions are also involved in the shikimate biosynthetic pathway, which converts phosphoenolpyruvate and erythrose 4-phosphate, intermediate metabolites of glycolysis and the pentose phosphate pathway, respectively, to chorismate. Chorismate is a starting metabolite of branched pathways leading to the biosynthesis of various aromatic compounds.

acterized, and all the relevant biosynthetic genes in the genome sequence of *C. glutamicum* are annotated (15). However, the nature of quinate/shikimate utilization by this bacterium remains unknown.

In this study, we show that *C. glutamicum* can grow with either quinate or shikimate as the sole carbon source and that a gene (designated *qsuD*) encoding quinate/shikimate dehydrogenase (33) is essential for quinate/shikimate utilization. On the chromosome, the *qsuD* gene is clustered with *qsuA*, *qsuB*, and *qsuC*, encoding homologues of proteins identified in other microorganisms, i.e., major facilitator superfamily transporters, dehydroshikimate dehydratase, and dehydroquinate dehydratase, respectively. We also show that the expression of the *qsu* genes is markedly induced during growth in the presence of either quinate or shikimate and that *qsu* gene expression is not subject to glucose repression. Furthermore, the deletion of a LysR-type transcriptional regulator encoded by a gene (designated *qsuR*) located immediately upstream of *qsuABCD* in the opposite direction significantly represses the induction of *qsuABCD* genes.

MATERIALS AND METHODS

Bacterial strains. *C. glutamicum* R (49) was used as a wild-type strain for our experiments. cgR_0495 (*qsuD*)- and cgR_0491 (*qsuR*)-deficient strains were obtained from a single-gene-disruptant mutant library constructed by transposon-mediated mutagenesis (38). Transposons were inserted 646 and 519 bases downstream of the 5' ends of the *qsuD* and *qsuR* genes, respectively.

Culture conditions. For genetic manipulations, *Escherichia coli* and *C. glutamicum* strains were grown as described previously (39).

To evaluate growth with quinate, shikimate, or glucose as the sole carbon source, a *C. glutamicum* cell starter culture was grown aerobically in 10 ml of a medium (18) containing 4% glucose at 33°C in a 100-ml test tube overnight. The cells were harvested by centrifugation at $4,000 \times g$ for 10 min at 4°C. The cell pellet was subsequently washed twice with BT minimal medium (18). The washed cells were suspended in 100 ml of BT medium containing quinate, shikimate, or glucose at 20 or 40 mM and then cultured aerobically at 33°C in 500-ml flasks.

Analytical methods. Cell growth was monitored by measuring the optical density at 610 nm with a spectrophotometer (model DU640; Beckman Coulter, CA).

The cell cultures were centrifuged at $10,000 \times g$ for 10 min at 4°C, and the supernatants were analyzed for shikimate and glucose. Shikimate was quantified by using a high-performance liquid chromatography system (model 8020; Tosoh, Tokyo, Japan) equipped with a UV detector and a TSKgel OAPak column (Tosoh) operating at 40°C with a 0.75 mM H₂SO₄ mobile phase at a flow rate of 1.0 ml min⁻¹. The concentration of glucose was measured by an enzyme electrode glucose sensor (model BF-4; Oji Scientific Instruments, Hyogo, Japan).

DNA techniques. Chromosomal DNA and plasmid DNA were prepared from *C. glutamicum*, and the target DNA regions were amplified by PCR as described previously (39).

C. glutamicum cells were transformed by electroporation as described previously (43). *E. coli* cells were transformed by the CaCl₂ procedure (32).

DNA sequencing was performed with an ABI Prism 3100xl genetic analyzer (Applied Biosystems, Foster City, CA). DNA sequence data were analyzed with the Genetyx program (Software Development, Tokyo, Japan). Sequences were aligned and phylogenetically analyzed by the program CLUSTAL W (40) using the neighbor-joining method (31). The phylogenetic tree was displayed using the program TREEVIEW (29).

Plasmid construction. Plasmids for the expression of the *qsuD* and *qsuR* genes were obtained as follows. The regions of the open reading frames were amplified by PCR from the *C. glutamicum* R chromosomal DNA by using a set of primers with appropriate restriction sites (Table 1). The amplified *qsuD* open reading frame region was digested by the restriction enzymes and was inserted into the corresponding sites of pKK223-3 (Pharmacia). The BamHI fragment containing the resulting *tac* promoter-*qsuD* gene construct was inserted into *E. coli-Corynebacterium* shuttle vector pCRB1 (25), yielding pCRC301 for the expression of *qsuD* under the control of the *tac* promoter. On the other hand, the PCR-amplified *qsuR* open reading frame region was inserted into pCRB1, yielding pCRC302 for the expression of *qsuR* under the control of the *lac* promoter.

Quantitative RT-PCR. Total RNA from *C. glutamicum* cells was prepared using an RNeasy minikit and RNaprotect bacterial reagent (Qiagen, Hilden, Germany), and quantitative reverse transcriptase PCR (RT-PCR) was performed using an Applied Biosystems 7500 fast real-time PCR system as described previously (39). Data on the primers used are summarized in Table 1. Specific amplification of the targeted DNA was confirmed by electrophoresis and sequencing of the PCR product. The relative abundances of the targeted mRNAs were determined based on the cycle threshold value, which is defined as the cycle number required to obtain a fluorescence signal above the background level. To standardize the results, the relative abundance of 16S rRNA was used as the internal standard.

RESULTS

***qsu* gene cluster involved in quinate/shikimate utilization by *C. glutamicum*.** The utilization of quinate and shikimate by *C. glutamicum* strains on culture plates each containing BT minimal medium supplemented with quinate, shikimate, or glucose was examined. The *C. glutamicum* R wild-type strain grew similarly on each of these carbon sources, indicating that this bacterium can utilize both shikimate and quinate as the sole carbon source for growth. A mutant strain deficient in cgR_0495 (designated *qsuD*), encoding quinate/shikimate dehydrogenase (33), was obtained from a mutant library constructed previously by transposon-based insertion of a selection marker (38). The *qsuD*-deficient strain did not grow on quinate- or shikimate-containing plates but grew well on glucose-containing plates.

A plasmid carrying the *qsuD* gene under the control of a constitutive promoter was introduced into the *qsuD*-deficient

TABLE 1. Primers used in this study

Primer	Target gene	Sequence (5'-3')	Overhang restriction site ^a
Primers for construction of expression plasmids			
qsuD-Fw	<i>qsuD</i>	GGAATTCATGAACGACAGTATTCTCC	EcoRI
qsuD-Rv		TCCCCCGGGTCGAGGTTTACTGACTCTT	SmaI
qsuR-Fw	<i>qsuR</i>	GGGGTACCAGTAGAAAGGTTTAGCCAC	KpnI
qsuR-Rv		CGGGATCCTCAGTTTCCGTCAATAAACT	BamHI
Primers for RT-PCR analyses			
16S-F	16S rRNA	TCGATGCAACGCGAAGAAC	
16S-R		GAACCGACCACAAGGGAAAAC	
qsuA-F	<i>qsuA</i>	TTGCGAACTTGGTTGCAC	
qsuA-R		ACGGCATTACCCATGGAG	
qsuB-F	<i>qsuB</i>	CCACTGGACGTTTGGGTGAGA	
qsuB-R		ACATCGCCCTGGGTCCATAC	
qsuC-F	<i>qsuC</i>	ATGCTTGGAAAAATTCTCCTCC	
qsuC-R		GATTAGCTCACCTTCGTGAT	
qsuD-F	<i>qsuD</i>	ATGAACGACAGTATTCTCC	
qsuD-R		ATFGCGGGGTGCGCGATAG	
ptsG-F	<i>ptsG</i>	CTGATCACTTTTGACGCTGACTTC	
ptsG-R		CGAATTTTGC GGCGTTAGAC	

^a The restriction site overhangs used in the cloning procedure have been underlined.

strain. The *qsuD* complemented strain was cultured in liquid medium using glucose, quinate, or shikimate as the sole carbon source, and its growth was compared to that of the *qsuD*-deficient strain and the wild-type strain, both of which were transformed with a vector plasmid without *qsuD* (Fig. 2). The growth of the wild-type strain with the vector plasmid in medium containing either quinate or shikimate was faster than its growth in medium containing glucose (Fig. 2A). Growth in the presence of quinate or shikimate was completely suppressed by the disruption of *qsuD*, whereas the *qsuD*-deficient strain grew in the presence of glucose as well as the wild-type strain (Fig. 2B). The *qsuD* complemented strain grew well with glucose, quinate, or shikimate. These results indicate that quinate/shikimate dehydrogenase encoded by *qsuD* is essential for the utilization of quinate/shikimate in *C. glutamicum*.

A gene cluster containing *qsuD* on the chromosome of *C. glutamicum* R is depicted in Fig. 3. The cgR_0492 gene (designated *qsuA*) encodes a protein exhibiting 40% amino acid sequence identity to the *E. coli* shikimate transporter (45) belonging to the major facilitator superfamily. The cgR_0494 gene (designated *qsuC*) encodes a protein with 60% amino acid identity to type II dehydroquinase dehydratase in *Streptomyces coelicolor* (46). The entire amino acid sequence encoded by cgR_0493 (designated *qsuB*) does not show a high degree of similarity to those of any proteins characterized functionally so far. However, the amino-terminal half of QsuB protein has 25% amino acid sequence identity to dehydroshikimate dehydratases identified in fungi (12, 14), and the carboxy-terminal half has 25% identity to 4-hydroxyphenylpyruvate dioxygenase, which is involved in the tyrosine catabolic pathway in *Pseudomonas fluorescens* (30). Therefore, it is likely that products of the *qsuABCD* gene cluster are involved in the series of reactions of quinate/shikimate metabolism. The cgR_0491 gene (designated *qsuR*), which is located immediately upstream of *qsuA* in the opposite direction, encodes a homologue of LysR-type transcriptional regulators.

QsuR-mediated induction of *qsuABCD* genes in response to quinate/shikimate. In order to examine the expression of the *qsuABCD* genes in response to quinate and shikimate, the *C. glutamicum* R wild-type strain was cultured in liquid medium supplemented with quinate, shikimate, or glucose as the sole carbon source as described above. Total RNA was prepared from the cells cultured for 2, 4, and 6 h and was subjected to quantitative RT-PCR analyses (Fig. 4). The level of *qsuD* mRNA in the quinate-grown cells was markedly upregulated relative to that in the glucose-grown cells, although the level decreased in the late period of the culture. Shikimate induced *qsuD* expression to a higher level than quinate did, and the high expression level of *qsuD* in the shikimate-grown cells remained high throughout the culture period relative to the level in the glucose-grown cells. The patterns of expression of *qsuA*, *qsuB*, and *qsuC* during culture in the presence of quinate, shikimate, and glucose were essentially the same as that of *qsuD* (Fig. 4B, C, D, and E). The *qsuAB*, *qsuBC*, and *qsuCD* intergenic regions were 35, 46, and 14 bp, respectively. Therefore, it is likely that these genes in the cluster are transcribed as an operon. As a control, the expression of a glucose-inducible gene, *ptsG*, encoding a transporter involved in glucose uptake, was also analyzed in these experiments. As expected, the level of *ptsG* mRNA in the quinate- or shikimate-grown cells was lower than that in the glucose-grown cells (Fig. 4F).

The involvement of QsuR, the LysR-type transcriptional regulator, in *qsuABCD* expression was examined using a *qsuR*-deficient strain. The growth of the *qsuR* mutant was slower than that of the wild-type strain when the strains were cultured in minimal medium supplemented with shikimate (Fig. 5B). A plasmid carrying the *qsuR* gene under the control of a constitutive promoter was introduced into the *qsuR*-deficient strain. Complementation with the *qsuR*-containing plasmid enhanced the growth of the *qsuR* mutant to a level comparable to that of the wild-type strain carrying a vector plasmid without *qsuR*.

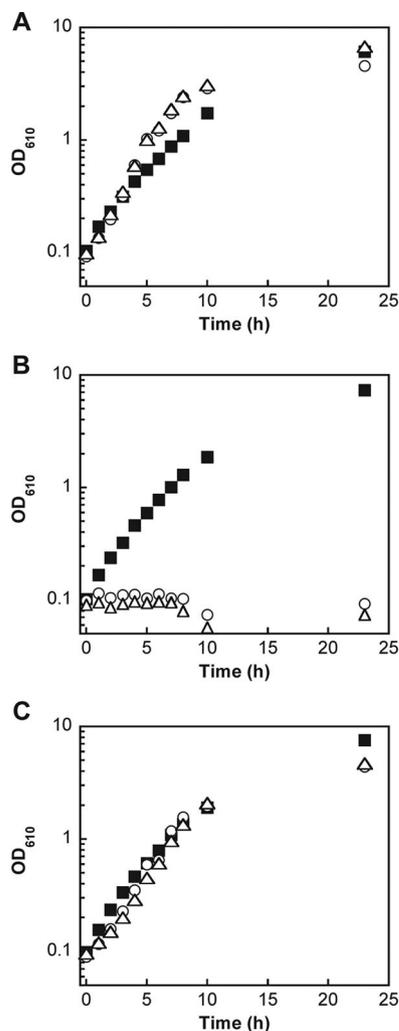


FIG. 2. Effects of disruption of *qsuD* on the growth of *C. glutamicum* cells. The *C. glutamicum* R wild-type strain containing the control vector plasmid pCRB1 (A) and the *qsuD*-deficient strain containing pCRB1 (B) or a plasmid carrying the *tac* promoter-*qsuD* construct (C) were grown in BT minimal medium supplemented with either glucose (black squares), quinate (white circles), or shikimate (white triangles). The optical density at 610 nm (OD₆₁₀) was monitored. Similar results were obtained from two independent experiments, and representative results are shown.

When cultured with glucose as the sole carbon source, the strains grew at almost the same rate (Fig. 5A). Quantitative RT-PCR analyses of the levels of *qsuD* mRNA in the cells cultured for 2 h revealed that for the shikimate-grown cells, the

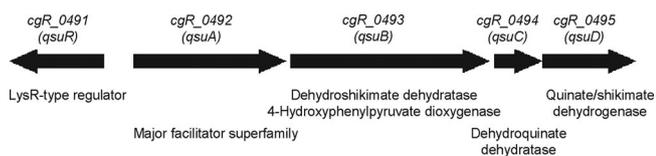


FIG. 3. *qsu* gene cluster of *C. glutamicum* R. cgR_0491 (*qsuR*) encodes a LysR-type transcriptional regulator; cgR_0492 (*qsuA*) encodes a major facilitator superfamily transporter; cgR_0493 (*qsuB*) encodes a fusion protein consisting of dehydroshikimate dehydratase and 4-hydroxyphenylpyruvate dioxygenase; cgR_0494 (*qsuC*) encodes dehydroquininate dehydratase; and cgR_0495 (*qsuD*) encodes quinate/shikimate dehydrogenase.

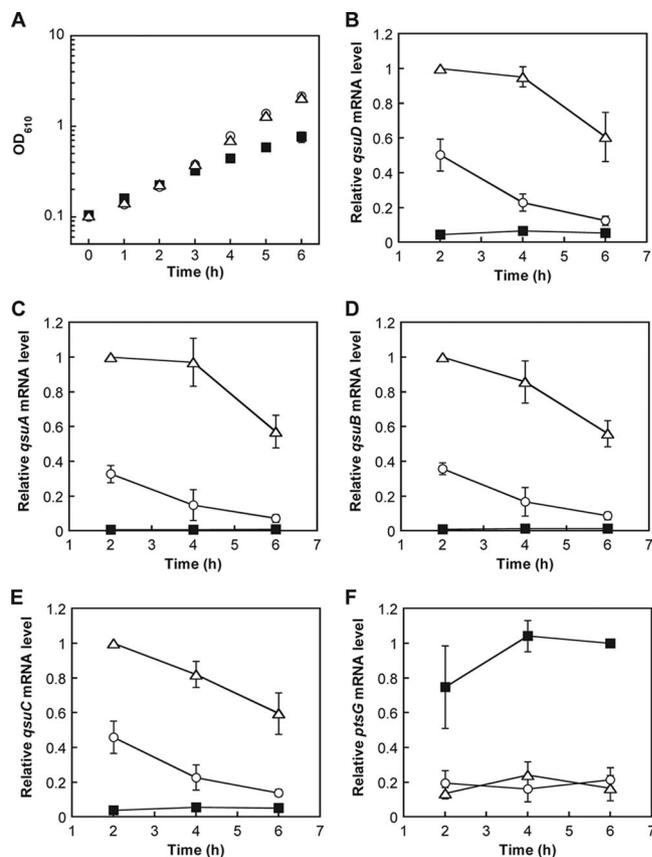


FIG. 4. Expression of the *qsu* genes during growth with different carbon sources. The *C. glutamicum* R wild-type strain was cultured in BT minimal medium supplemented with either glucose (black squares), quinate (white circles), or shikimate (white triangles). (A) The optical density at 610 nm (OD₆₁₀) was monitored. (B to F) The levels of *qsuD* (B), *qsuA* (C), *qsuB* (D), *qsuC* (E), and *ptsG* (F) mRNAs in the cells were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value for the culture in shikimate medium at 2 h, except for *ptsG* mRNA levels, which are presented relative to the value for the culture in glucose medium at 6 h. The values are the means of results from three independent cultivations with standard errors.

qsuD expression level in the *qsuR*-deficient strain was one-fifth of that in the wild-type strain (Fig. 5C). On the other hand, the *qsuR* complemented strain expressed *qsuD* mRNA at a level comparable to that expressed by the wild-type strain. In glucose-grown cells of all the strains, the levels of *qsuD* mRNA were similarly very low. The effects of the disruption and complementation of *qsuR* on the levels of *qsuA*, *qsuB*, *qsuC*, and *qsuD* mRNAs were almost the same (Fig. 5C, D, E, and F). It should be noted that the induction of *qsuABCD* expression by shikimate was still observed in the *qsuR*-deficient strain, although the induction level was lower than that in the wild-type strain, as described above. This result is consistent with the observation that the *qsuR*-deficient strain can still grow with shikimate as the sole carbon source (Fig. 5B).

Simultaneous utilization of glucose and shikimate. The utilization of glucose and shikimate by the *C. glutamicum* R wild-type strain during growth in minimal medium supplemented with both carbon sources was examined. When glucose

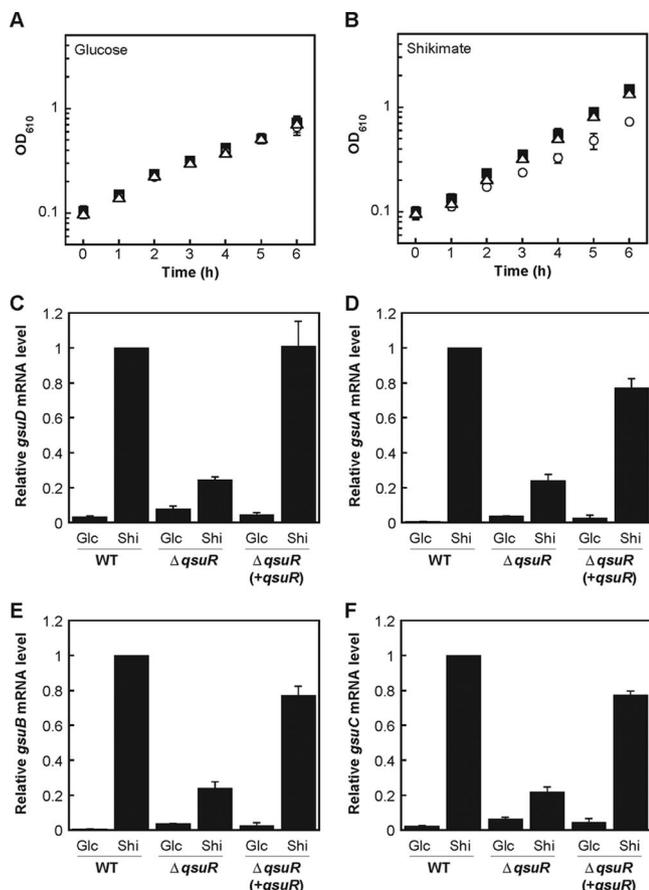


FIG. 5. Effects of the disruption of *qsuR* on the expression of the *qsuABCD* genes. The *C. glutamicum* R wild-type (WT) strain containing the control vector plasmid pCRB1 (black squares) and the *qsuR*-deficient strain containing pCRB1 (Δ *qsuR*; white circles) or a plasmid carrying the *lac* promoter-*qsuR* construct [Δ *qsuR* (+*qsuR*); white triangles] were grown in BT minimal medium supplemented with either glucose (Glc) or shikimate (Shi). (A and B) The optical densities at 610 nm (OD₆₁₀) of the cultures of these strains in the presence of glucose (A) and shikimate (B) are shown. (C to F) The levels of *qsuD* (C), *qsuA* (D), *qsuB* (E), and *qsuC* (F) mRNAs in the cells cultured for 2 h were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value for the pCRB1-containing wild-type strain grown with shikimate. The values are the means of results from three independent experiments with standard errors.

and shikimate at 20 mM each were added together, growth was faster than that of cultures supplemented with either glucose or shikimate alone at 40 mM (Fig. 6A). The concentrations of glucose and shikimate in the medium decreased at similar rates during growth in the presence of both carbon sources, indicating no preference for either carbon source (Fig. 6B).

The effects of supplementation with glucose on the induction of *qsuABCD* expression in response to shikimate were examined. In the presence of both shikimate and glucose, the *qsuD* mRNA level was markedly upregulated relative to that in the absence of shikimate, although the level of induction of *qsuD* expression in response to shikimate was repressed by about 40% by supplementation with glucose (Fig. 6C). The same patterns of expression of *qsuA*, *qsuB*, and *qsuC* were observed in these experiments. The induction of *ptsG* expression in response to glucose in the presence of shikimate was

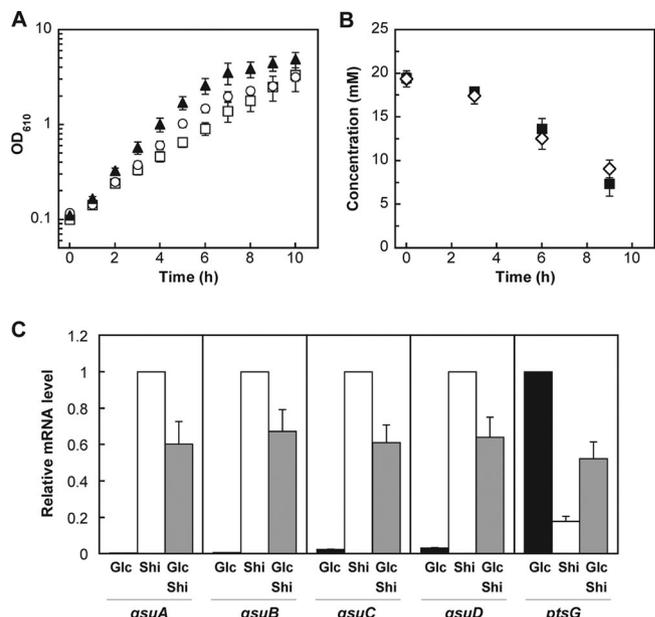


FIG. 6. Effects of supplementation with glucose and shikimate together on growth (A), substrate consumption (B), and the expression of the *qsu* genes (C). The *C. glutamicum* R wild-type strain was cultured in BT minimal medium supplemented with either 40 mM glucose (Glc), 40 mM shikimate (Shi), or both at 20 mM each. (A) The optical densities at 610 nm (OD₆₁₀) of the cell cultures in the presence of glucose (white rectangles), shikimate (white circles), and both glucose and shikimate (black triangles) were monitored. (B) The concentrations of glucose (black rectangles) and shikimate (white diamonds) in medium during growth in the presence of both glucose and shikimate were measured. (C) The levels of *qsuA*, *qsuB*, *qsuC*, *qsuD*, and *ptsG* mRNAs in the cells cultured for 2 h with glucose, shikimate, and both glucose and shikimate were determined by quantitative RT-PCR. The mRNA levels are presented relative to the value for the shikimate-grown cells, except for *ptsG* mRNA levels, which are presented relative to the value for the glucose-grown cells. All the values are the means of results from three independent experiments with standard errors.

observed, although the induction level was about 50% lower than that in the absence of shikimate. These results indicate that the induction of *qsu* genes in response to shikimate even in the presence of glucose allows the cells to utilize both carbon sources simultaneously.

DISCUSSION

In this study, we showed that *C. glutamicum* can readily utilize quinate and shikimate for growth and that *qsuD*, encoding quinate/shikimate dehydrogenase, is essential for utilization. On the *C. glutamicum* chromosome, *qsuD* is located in the *qsu* gene cluster, together with three other genes, *qsuA*, *qsuB*, and *qsuC*, which are probably involved in the utilization of quinate and shikimate. Interestingly, the three proteins QsuD (quinate/shikimate dehydrogenase), QsuC (dehydroquinate dehydratase), and QsuB (dehydroshikimate dehydratase) in *C. glutamicum* have no amino acid sequence similarity to the corresponding bacterial enzymes characterized previously, e.g., *Acinetobacter* species QuiA, QuiB, and QuiC, respectively (7, 8). Instead, they can be classified into the families of fungal enzymes QutB, QutE, and QutC, although the degrees of amino acid sequence identity are very low (approximately

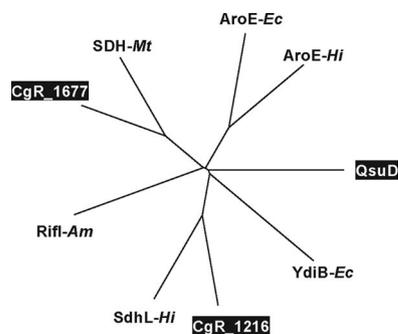


FIG. 7. Unrooted phylogenetic tree showing relationships among bacterial shikimate dehydrogenase family proteins and the homologues in *C. glutamicum*. The proteins analyzed were *E. coli* AroE and YdiB (AroE-Ec and YdiB-Ec) (1, 24), *H. influenzae* AroE and SdhL (AroE-Hi and SdhL-Hi) (36, 47), *M. tuberculosis* shikimate dehydrogenase (SDH-Mt) (10), *Amycolatopsis mediterranei* RifI (RifI-Am) (48), and *C. glutamicum* QsuD, CgR_1216, and CgR_1677, encoded by *qsuD*, *cgR_1216*, and *cgR_1677*, respectively. The amino acid sequences were aligned using the CLUSTAL W program (40), and the tree was constructed using the neighbor-joining method (31).

25%) (14). The findings provide additional insights into the complex evolutionary history of the quinate catabolic pathway and the shikimate biosynthetic pathway.

The shikimate dehydrogenase reaction is common to the shikimate biosynthetic pathway and the shikimate catabolic pathway (Fig. 1). The NADP-dependent shikimate-specific enzyme is involved in the biosynthetic role (1). On the other hand, it is likely that the enzyme involved in the catabolic role acts on both quinate and shikimate as substrates and prefers NAD as a coenzyme in fungi and gram-positive bacteria, although genetic characterization of the bacterial enzymes is obscure (3, 12, 14). Recently, a product of the *C. glutamicum* ATCC 13032 *cgl0424* gene, corresponding to the product of *qsuD* of *C. glutamicum* R (99% amino acid sequence identity), was overexpressed in *E. coli*, purified, and characterized, and the three-dimensional structure was determined (33). The results indicate that the enzyme prefers quinate and NAD, although its physiological function has not been clarified. The enzymatic features support the possibility of its involvement in the quinate/shikimate catabolic role demonstrated in this study. The two homologues of QsuD encoded in the genome of *C. glutamicum* R, i.e., the proteins encoded by *cgR_1216* and *cgR_1677*, correspond to the gene products of *C. glutamicum* ATCC 13032 *cgl1132* and *cgl1629*, respectively. The three quinate/shikimate dehydrogenase family proteins in *C. glutamicum* exhibit approximately 30% amino acid sequence identity to one another. We observed that the *qsuD*-deficient strain could not grow with quinate/shikimate as the sole carbon source but could grow in the presence of glucose without supplementation with aromatic compounds. These results indicate that the two QsuD homologues, the CgR_1216 and CgR_1677 proteins, are not involved in quinate/shikimate utilization but may play another role. Figure 7 shows a comparison of the *C. glutamicum* proteins and representatives of previously identified groups of the bacterial shikimate dehydrogenase family. The CgR_1216 and CgR_1677 proteins exhibit less than 30% amino acid identity to *E. coli* NADP-dependent shikimate dehydrogenase AroE in the shikimate

biosynthetic pathway (1) and to YdiB, with an unknown physiological role (24). CgR_1216 exhibits 49% amino acid sequence identity to a *Haemophilus influenzae* shikimate dehydrogenase-like protein which has much less activity than *E. coli* AroE with shikimate as a substrate and has no activity on quinate (36). CgR_1677 exhibits 49% amino acid identity to *Mycobacterium tuberculosis* NADP-dependent shikimate dehydrogenase (10). It is noteworthy that in gram-negative bacteria, the quinate dehydrogenase involved in the quinate catabolic pathway is a pyrroloquinoline quinone-dependent enzyme and has an evolutionary origin different from that of the NAD(P)-dependent enzymes (7). The functional diversification of these types of enzymes seems to have intricately occurred during their molecular evolution (37).

We showed that the expression of *qsuABCD* genes was markedly induced in response to quinate or shikimate, suggesting that the high level of induction allows the cells to utilize the hydroaromatic compounds efficiently. It has also been reported previously that, in other microorganisms, the quinate/shikimate catabolic enzymes are inducible (4, 6, 12, 14, 35, 42). It should be noted that the actinomycete *A. methanolica* can grow on quinate but not on shikimate, since shikimate does not act as an inducer for the utilization enzymes (9). Our results demonstrated that, in *C. glutamicum*, shikimate acts as a stronger inducer than quinate. The high shikimate inducibility of *qsuD* expression may complement the lower catalytic efficiency of the *qsuD* gene product for shikimate than for quinate as a substrate (33), allowing *C. glutamicum* cells to utilize quinate and shikimate with the same efficiency, as shown in this study.

The disruption of *qsuR*, encoding a LysR-type transcriptional regulator, repressed the *qsuABCD* expression level in the shikimate-grown cells, suggesting that QsuR acts as a transcriptional activator of the quinate/shikimate utilization genes. In *Acinetobacter* species, the *qui* gene cluster for quinate/shikimate utilization forms an operon, together with the *pca* genes involved in the subsequent protocatechuate metabolism (6, 7). The expression of the *pca-qui* operon is regulated by the IclR-type regulator PcaU, dependent on protocatechuate (6, 11, 35). In the *C. glutamicum* chromosome, the *pca* gene cluster is located at a distance from the *qsu* gene cluster (2, 34). The *pca* gene cluster consists of multiple transcription units and contains two genes encoding transcriptional regulators, the IclR-type regulator PcaR and the LuxR-type regulator PcaO (2). However, the regulation of *pca* expression in *C. glutamicum* is not yet fully understood. The multiple regulators may regulate the expression of the *qsu* and *pca* genes in *C. glutamicum* in a complex manner. The induction of *qsuABCD* in response to shikimate still observed in the *qsuR*-deficient strain revealed that another regulator is also involved in the induction.

The expression of *qsuABCD* was markedly induced during growth in the presence of both shikimate and glucose. Simultaneous consumption of glucose and shikimate at the same rates during growth was observed. Synergistic positive effects on the growth rate in the presence of both carbon sources should be noted. This characteristic of *C. glutamicum* is in contrast to those of other microorganisms, in which the expression of quinate/shikimate utilization genes is subject to stringent carbon catabolite repression (5, 12–14, 35). This property of *C. glutamicum* may be related to a unique adaptation strategy of this soil bacterium in response to the fluctuation of

environmental conditions. It is interesting that *C. glutamicum* can simultaneously utilize glucose and another carbon source such as acetate (44) or vanillate (23). A global catabolite repression system, such as the cyclic AMP receptor protein- and CcpA-dependent systems in *E. coli* and *Bacillus subtilis*, respectively, has not been identified in *C. glutamicum*.

Dehydroquinate and dehydroshikimate are common intermediates of the quinate catabolic and shikimate biosynthetic pathways leading to the production of various aromatic compounds. Understanding of the regulation of the inducible *qsu* expression will provide critical insights into the flux control of these pathways in response to extracellular and/or intracellular conditions in this microorganism. *C. glutamicum* has been studied previously for the application of industrial production of aromatic amino acids which are produced via the shikimate pathway (15). Furthermore, metabolic engineering to direct the carbon flow from the shikimate pathway into protocatechuate for aromatic compound production can be achieved by the application of quinate utilization enzymes (21). Therefore, the findings regarding the function and regulation of *qsu* genes in *C. glutamicum* will benefit applications in biotechnology.

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