Corynebacterium glutamicum Contains 3-Deoxy-D-Arabino-Heptulosonate 7-Phosphate Synthases That Display Novel Biochemical Features^{∇†}

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3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 2.5.1.54) catalyzes the first step of the shikimate pathway that finally leads to the biosynthesis of aromatic amino acids phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr). In *Corynebacterium glutamicum* ATCC 13032, two chromosomal genes, NCgl0950 (*aroF*) and NCgl2098 (*aroG*), were located that encode two putative DAHP synthases. The deletion of NCgl2098 resulted in the loss of the ability of *C. glutamicum* RES167 (a restriction-deficient strain derived from *C. glutamicum* ATCC 13032) to grow in mineral medium; however, the deletion of NCgl0950 did not result in any observable phenotypic alteration. Analysis of DAHP synthase activities in the wild type and mutants of *C. glutamicum* RES167 indicated that NCgl2098, rather than NCgl0950, was involved in the biosynthesis of aromatic amino acids. Cloning and expression in *Escherichia coli* showed that both NCgl0950 and NCgl2098 encoded active DAHP synthases. Both the NCgl0950 and NCgl2098 DAHP synthases were purified from recombinant *E. coli* cells and characterized. The NCgl0950 DAHP synthase was sensitive to feedback inhibition by Tyr and, to a much lesser extent, by Phe and Trp. The NCgl2098 DAHP synthase was slightly sensitive to feedback inhibition by Trp, but not sensitive to Tyr and Phe, findings that were in contrast to the properties of previously known DAHP synthases from *C. glutamicum* subsp. *flavum*. Both Co²⁺ and Mn²⁺ significantly stimulated the NCgl0950 DAHP synthase's activity, whereas Mn²⁺ was much more stimulatory than Co²⁺ to the NCgl2098 DAHP synthase's activity.

3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase (EC 2.5.1.54) catalyzes the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) to form DAHP. This condensation is the first step of the shikimate pathway that leads to the biosynthesis of aromatic amino acids, vitamin K, folic acid, and ubiquinone (1, 7). The DAHP synthase(s) and its encoding gene(s) were characterized from many bacteria, including the gram-negative *Escherichia coli* (5, 29, 30) and the gram-positive *Bacillus subtilis* (2). *E. coli* has three DAHP allelic synthases that are sensitive to feedback regulation by phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr), respectively (8, 26). In contrast, *B. subtilis* possesses only one DAHP synthase that is regulated by the shikimate pathway intermediates prephenate and chorismate but is insensitive to Phe, Trp, and Tyr (28).

Corynebacterium glutamicum is commercially used for the production of amino acids, including the three aromatic amino acids Phe, Tyr, and Trp (10). As in many other organisms, the biosynthesis of Phe, Trp, and Tyr in *C. glutamicum* starts from the shikimate pathway. The early work on DAHP synthase from *C. glutamicum* subsp. *flavum* (formerly *Brevibacterium flavum* [18]) revealed that Tyr and Phe each inhibited the

DAHP activity slightly but together they caused stronger and synergistic inhibition (21). A DAHP synthase that was purified to electrophoretic homogeneity later had a molecular mass of 55 kDa, and its activity was not affected by Trp (23). Two DAHP synthase genes were cloned from an L-Phe-producing mutant of *C. glutamicum* subsp. *lactofermentum* (formerly *Brevibacterium lactofermentum* [18]), but the DNA sequences of the two DAHP synthase genes were not reported (12). A DAHP synthase (39 kDa) was subsequently found in *C. glutamicum* subsp. *lactofermentum* (4). This DAHP synthase was sensitive to Tyr feedback inhibition (17). Recently, the genome of *C. glutamicum* ATCC 13032 became available (11, 15). Although genome annotation revealed two chromosomal DAHP synthases, encoded by NCgl0950 (*aroF*) and NCgl2098 (*aroG*), respectively, the function of the two genes was not experimentally identified. This current study focused on functional characterization of the genes NCgl0950 and NCgl2098 and the biochemical characterization of the two putative DAHP synthases in *C. glutamicum* ATCC 13032 (strain RES167).

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation. The bacterial strains and plasmids used in this study are listed in Table 1. All *E. coli* strains were grown aerobically on a rotary shaker (150 rpm) at 37°C in Luria-Bertani (LB) broth or on an LB plate with 1.5% (wt/vol) agar. *C. glutamicum* strains were routinely grown at 30°C on a rotary shaker (150 rpm) in LB medium or in mineral salts medium (16) which was adjusted to pH 7.5 and supplemented with biotin (100 µg/liter) and thiamine (500 µg/liter) but omitted yeast extract. Glucose at a final concentration of 2 or 10 mM and aromatic amino acids at a final concentration of 0.2 mM were added, serving as carbon and energy sources and meeting the demands of auxotrophic mutants. Cellular growth was monitored by measuring the turbidity at a wavelength of 600 nm by using a spectrophotometer. For the generation of

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TABLE 1. Bacterial strains, plasmids, and primers used in the study

Strain/plasmid/primer	Relevant characteristics	Source or reference	Notes
Strains			
<i>E. coli</i>			
XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1</i> Δlac F' { <i>proAB</i> ⁺ <i>lacI</i> ^q <i>lacZ</i> ΔM15 Tn10[<i>tet</i> (Ts)]}	Stratagene (catalog no. 200249)	
BL21(DE3)	<i>hsdS gal(λcIts857 ind-1 Sam7 nin-5 lacUV5-T7</i> gene 1)	Novagen (catalog no. 69450-3)	
<i>C. glutamicum</i>			
RES167	Restriction-deficient mutant of ATCC 13032, $\Delta cglIM \Delta cglIR \Delta cglIIR$	University of Bielefeld	
RES167ΔNCgl0950	With disrupted NCgl0950 (amino acids 136–226)	This study	
RES167ΔNCgl2098	With disrupted NCgl2098 (amino acids 76–467)	This study	
RES167ΔNCgl(0950 + 2098)	Both genes NCgl0950 (amino acids 136–226) and NCgl2098 (amino acids 76–467) were disrupted	This study	
Plasmids			
pK18 <i>mobsacB</i>	Mobilizable vector, allows for selection of double crossover in <i>C. glutamicum</i>	20	
pK18 <i>mobsacB</i> -ΔNCgl0950	Carrying NCgl0950 deletion; refer to RES167ΔNCgl0950	This study	
pK18 <i>mobsacB</i> -ΔNCgl2098	Carrying NCgl2098 deletion; refer to RES167ΔNCgl2098	This study	
pXMJ19	Shuttle vector; Cam ^r <i>Ptac lacI</i> ^q pBL1 <i>oriV_{C. glutamicum}</i> , pK18 <i>oriV_{E. coli}</i>	13	
pXMJ19-NCgl0950	Carrying NCgl0950; to generate complementation for NCgl0950	This study	
pXMJ19-NCgl2098	Carrying NCgl2098; to generate complementation for NCgl2098	This study	
pET28a	Expression vector with N-terminal hexahistidine affinity tag	Novagen	
pET28a-NCgl0950	pET28a derivative for expression of NCgl0950	This study	
pET28a-NCgl2098	pET28a derivative for expression of NCgl2098	This study	
Primers^a			
0950F	<u>GCTGCAGAAAGGAGGACACATATGAGT</u> TCTCCAGTCTCAC (PstI/NdeI) ^b		To generate pK18 <i>mobsacB</i> - ΔNCgl0950, pXMJ19- NCgl0950, pET28- NCgl0950 and for RT- PCR
0950R	GACGGATCCACATTTAACAGTCT AGCG (BamHI)		
0950rtF	TCAAGAACGGAAGTACGG		RT-PCR
0950rtR	CTGCGATTTCACGAACAAC		RT-PCR
2098F	CTCTAGAAAAGGAGGACATATGAATAG GGGTGTGAGTTGG (XbaI/NdeI) ^c		To generate pK18 <i>mobsacB</i> - ΔNCgl2098, pXMJ19- NCgl2098, and pET28- NCgl2098
2098R	GTGGATCCTGTGGAGCGGAGTTATC TTGA (BamHI)		

^a Restriction enzyme sites are underlined. Ribosome binding sites are boldfaced.

^b Restriction by PstI was used to generate fragments of NCgl0950 for gene deletion and complementation. Restriction by NdeI was used to generate the fragment of NCgl0950 beginning with ATG for expression of NCgl0950 in *E. coli* and *C. glutamicum*.

^c Restriction by PstI was used to generate fragments of NCgl2098 for gene deletion and complementation. Restriction by NdeI was used to generate the fragment of NCgl2098 beginning with ATG for expression of NCgl2098 in *E. coli* and *C. glutamicum*.

mutants and maintenance of *C. glutamicum*, brain heart broth with 0.5 M sorbitol medium was used. When needed, antibiotics were used at the following concentrations: kanamycin, 50 μg ml⁻¹ for *E. coli* and 25 μg ml⁻¹ for *C. glutamicum*; ampicillin, 100 μg ml⁻¹ for *E. coli*; chloramphenicol, 20 μg ml⁻¹ for *E. coli* and 10 μg ml⁻¹ for *C. glutamicum*; and nalidixic acid, 100 μg ml⁻¹ for *C. glutamicum*.

Analysis of sequence data. The genome sequence of *C. glutamicum* ATCC 13032 (accession no. NCgl0001; <http://www.ncbi.nlm.nih.gov>) was retrieved from

GenBank. Sequence comparisons and database searches were carried out by using the BLAST service at the NCBI website.

DNA extraction and manipulation. The total genomic DNA of *C. glutamicum* was isolated according to the procedure of Tauch et al. (24). DNA restriction enzyme digestion, plasmid isolation, and agarose gel electrophoresis were carried out as described by Sambrook et al. (19). *E. coli* and *C. glutamicum* were transformed by electroporation according to the method of Tauch et al. (25).

Amplification of DNA fragments with PCR and construction of plasmids. PCRs were performed by using *Pfu* DNA polymerase or *Taq* DNA polymerase (Transgen, Beijing, China). The PCR products were purified by using an agarose gel DNA fragment recovery kit (Tiangen, Beijing, China). Cloning of PCR fragments was performed with a pEASY-T1 cloning vector system (Transgen, Beijing, China). Various plasmids (Table 1) for genetic disruption and complementation in *C. glutamicum* and for expression in *E. coli* were constructed with pK18*mobsacB*, pXMJ19, and pET28a. The primers used for amplification of the entire or disrupted target gene fragment are listed in Table 1. In vitro disruption of NCgl0950 or NCgl2098 was performed by removal of its partial region through restriction enzyme digestion. The lengths of the intact NCgl0950 and NCgl2098 genes were 1,101 bp and 1,401 bp, respectively. For NCgl0950, the fragment from bp 401 to bp 672 was removed to generate the Δ NCgl0950 mutant through digestion with BssHII, and the region from bp 219 to bp 1399 of NCgl2098 was deleted to generate the Δ NCgl2098 mutant by using restriction enzyme HindIII. The disrupted genes were fused into pK18*mobsacB* to generate plasmids pK18*mobsacB*- Δ NCgl0950 and pK18*mobsacB*- Δ NCgl2098. For complementation, plasmids pXMJ19-NCgl0950 and pXMJ19-NCgl2098 were constructed by the insertion of either PCR-amplified intact gene, NCgl0950 or NCgl2098, into pXMJ19 (13). Plasmids for the heterologous expression of NCgl0950 and NCgl2098 were created by ligating each PCR-amplified intact gene onto vector pET28a.

Genetic disruption and complementation in *C. glutamicum*. The pK18*mobsacB* derivatives were transformed into *C. glutamicum* RES167 by electroporation (25). Screening for the first and second recombination events, as well as confirmation of the chromosomal deletion, was performed as described previously (20). The resulting strains were designated *C. glutamicum* RES167 Δ NCgl0950, RES167 Δ NCgl2098, and RES167 Δ NCgl(0950 + 2098) (RES167 Δ NCgl0950 Δ NCgl2098) (Table 1). The deletion of the target genes in pK18*mobsacB* derivatives and in *C. glutamicum* mutants was verified by PCR amplification and DNA sequencing. Complementary plasmids pXMJ19-NCgl0950 and pXMJ19-NCgl2098 were introduced into the respective mutants by electroporation. The expression of each gene in *C. glutamicum* was induced by the direct addition of 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to cultures.

RNA isolation and RT-PCR. *C. glutamicum* was cultivated in glucose mineral broth or LB broth. Total RNA was obtained with a TRNzol extraction kit (TIANGEN, Beijing, China). The RNA preparation was first treated with DNase I and then used as a template for reverse transcription with Moloney murine leukemia virus reverse transcriptase (TIANGEN, Beijing, China). The amplification of the NCgl0950 fragment from the reverse transcription PCR (RT-PCR) products (cDNAs) was conducted with the primers listed in Table 1. In order to exclude the possibility that residual DNA was amplified, negative controls were run in parallel, except that the Moloney murine leukemia virus reverse transcriptase was omitted from the reaction mixtures.

Heterologous expression of genes in *E. coli*, preparation of cellular lysate, and protein purification. Plasmids (pET28a-NCgl0950 and pET28a-NCgl2098) were transformed into *E. coli* BL21(DE3) by electroporation. Synthesis of recombinant proteins in *E. coli* BL21(DE3) cells was initiated by the addition of 0.05 mM IPTG for NCgl2098 or 0.6 mM for NCgl0950, and cultivation was continued for an additional 8 to 10 h at 14°C. Cells were harvested by centrifugation at $10,000 \times g$; washed twice with 0.2% of KCl; resuspended in 0.05 M Tris-HCl buffer, pH 7.5, containing 1 mM dithiothreitol (21); and disrupted by sonication in an ice-water bath. Cellular lysates were centrifuged at $20,000 \times g$ for 30 min at 4°C, and the supernatant was used for protein purification. The hexahistidine cascade of pET28a was fused to each target gene at its 5' end during plasmid construction. Recombinant proteins were purified with a His-Bind protein purification kit (Novagen, Madison, WI) according to the manufacturer's instructions.

Assay of DAHP synthase. The DAHP synthase activity was determined according to the method of Siehl (22). In this assay, the enzymatic product DAHP is oxidized with NaIO_4 , and the oxidation product α -keto-butryl aldehyde acid reacts with thiobarbituric acid at 100°C and generates a pink chromophore (22). The assay mixture contained 50 mM Tris-HCl buffer, pH 7.5, 5 mM PEP, 2 mM E4P, and enzyme in a total volume of 75 μ l. The mixture was incubated at 30°C for 10 min. The reaction was initiated by the addition of enzyme and terminated by the addition of 400 μ l of 10% (wt/vol) trichloroacetic acid. After termination, 100 μ l of 25 mM NaIO_4 in 62.5 mM H_2SO_4 was added to the mixture and incubated at 37°C for 30 min. Then, the reaction was stopped by the addition of 100 μ l of 2% (wt/vol) of Na_2SO_3 in 0.5 M HCl to remove any excessive oxidants, and the tubes were mixed before adding 1 ml of 0.36% (wt/vol) thiobarbituric acid. Then, the tubes were incubated at 100°C for 10 min. The tubes were allowed to cool to room temperature, and the absorbance at a wavelength of 549 nm was measured. Reaction mixtures without enzyme were run in parallel and

used as controls. One unit of activity is defined as the amount of enzyme that catalyzes the synthesis of 1 μ mol DAHP per min at 30°C. For calculation, the coefficient of DAHP at 549 nm ($\epsilon_{549 \text{ nm}}$) of $4.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was adopted (14).

Aromatic amino acids (Phe, Tyr, or Trp) were added to the mixture when the feedback inhibition of the enzyme was tested, and the concentrations varied from 0.01 to 5 mM. Various metal cations (2 mM) and EDTA (2 mM) were added to the mixture when the effects of EDTA and divalent cations on enzyme activity were tested.

The Michaelis constant (K_m), maximal initial velocity (V_{max}), and k_{cat} values were obtained by fitting the kinetic data directly to the equation $v = V_{\text{max}} \cdot S / (K_m + S)$, where v is the initial velocity and S is the variable substrate concentration. The inhibition constant (K_i) values of the aromatic amino acids were determined by Dixon plot (6).

SDS-PAGE, determinations of molecular mass of the purified enzymes, and protein estimation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted with 5% stacking gels and 12% resolving gel and was run with a Mini-Protein II electrophoresis cell (Bio-Rad) according to the manufacturer's instructions. After electrophoresis, the protein bands were visualized by Coomassie brilliant blue staining. The apparent molecular masses were estimated according to the relative mobility of protein markers with molecular masses ranging from 12 to 90 kDa. The native molecular masses of the enzymes were estimated by gel filtration chromatography on a prepacked Superdex 200HR column (Pharmacia). The column was equilibrated and eluted with 50 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl at a flow rate of 0.5 ml min⁻¹. The molecular masses were calculated according to the elution volumes and calibrated with a standard molecular mass kit with molecular masses ranging from 48 to 669 kDa. The protein concentrations were determined according to the method of Bradford (3), with bovine serum albumin as the standard.

RESULTS

Genetic disruption of NCgl0950 and NCgl2098 and phenotypes of RES167 Δ NCgl0950, RES167 Δ NCgl2098, and RES167 Δ NCgl(0950 + 2098). According to the KEGG Pathway database and the genome annotation (11, 15), two genes (NCgl0950 and NCgl2098) putatively encode DAHP synthases in *C. glutamicum* ATCC 13032. Three mutants were constructed, and the disruption of each gene was confirmed by PCR amplification and sequencing of the intact and truncated genes (Fig. 1, lanes 1 to 4). Individual mutation of NCgl0950 and NCgl2098 resulted in strains RES167 Δ NCgl0950 and RES167 Δ NCgl2098, respectively. Double mutation of NCgl0950 and NCgl2098 resulted in strain RES167 Δ NCgl(0950 + 2098). Phenotypic characterization indicated that mutant RES167 Δ NCgl0950 did not show any observable change (Table 2), but RES167 Δ NCgl2098 lost the ability to grow on minimal medium (Table 2 and Fig. 2). RES167 Δ NCgl(0950 + 2098) also lost the ability to grow on minimal medium.

The complementation of mutant RES167 Δ NCgl2098 with NCgl2098 restored its growth (Fig. 2). The simultaneous addition of three aromatic amino acids (Phe, Trp, and Tyr, each at 0.2 mM concentration) also completely restored its growth in minimal medium (Table 2). The results indicated that NCgl0950 and each of the amino acids Phe, Trp, and Tyr or the combination of Phe plus Tyr were not able to restore the growth of RES167 Δ NCgl2098 in glucose minimal medium (Table 2). The complementation of mutant RES167 Δ NCgl(0950 + 2098) with either NCgl0950 or NCgl2098 or various aromatic amino acids was also carried out, and the phenotype of RES167 Δ NCgl(0950 + 2098) was similar to that of RES167 Δ NCgl2098 (Table 2).

Enzymatic characterization of mutants RES167 Δ NCgl0950, RES167 Δ NCgl2098, and RES167 Δ NCgl(0950 + 2098). DAHP synthase activity was not detected in RES167 Δ NCgl2098 and

FIG. 1. Confirmation of gene disruption of genes NCgl0950 and NCgl2098 by PCR amplification using chromosomal DNAs from the wild type or mutants as templates (lanes 1 to 4), expression of NCgl0950 and NCgl2098 in *E. coli*, and purification of NCgl0950 DAHP synthase and NCgl2098 DAHP synthase from recombinant *E. coli* cells (lanes 5 to 9). Lanes: 1, NCgl0950; 2, Δ NCgl0950; 3, Δ NCgl2098; 4, NCgl2098; 5, cellular lysate of BL21(DE3)/pET28a; 6, cellular lysate of BL21(DE3)/pET28a-NCgl0950; 7, cellular lysate of BL21(DE3)/pET28a-NCgl2098; 8, purified NCgl0950 product; 9, purified NCgl2098 product; M₁, DNA ladder (5,000, 3,000, 2,000, 1,000, 750, and 500 bp); M₂, protein molecular mass standards (90, 62, 40, 30, 20, and 12 kDa).

RES167 Δ NCgl(0950 + 2098); in contrast, the DAHP synthase activity of mutant RES167 Δ NCgl0950 was similar to that of the parent strain RES167 (Table 2). Complementation with NCgl0950 did not result in DAHP synthase activity, whereas the DAHP synthase activity of RES167 was similar to that obtained via complementation with NCgl2098 (Table 2).

Cloning and expression of NCgl0950 and NCgl2098 and purification of NCgl0950 DAHP synthase and NCgl2098 DAHP synthase from recombinant *E. coli* cells. Ikeda (9, 10) proposed that NCgl0950 and NCgl2098 encoded DAHP synthases. According to sequence alignment and analysis, NCgl0950 encoded a putative protein of 366 amino acid residues with a predicted molecular mass of 39 kDa. This protein (i.e., NCgl0950) was called Tyr-sensitive DAHP synthase (9, 10) and had 47% sequence identity to the functionally identified Tyr-sensitive DAHP synthase (AroF) of *E. coli* and 100% sequence identity to the functionally identified DAHP synthase of *C. glutamicum* strain CCRC 18310 (4). Similarly, NCgl2098 encoded a putative protein of 462 amino acid residues with a predicted molecular mass of 51 kDa. This protein, NCgl2098, was called Phe- and Tyr-sensitive DAHP synthase (9, 10) and had 55% sequence identity to the functionally

identified Trp-sensitive DAHP synthase of *Streptomyces coelicolor* (27).

Both NCgl0950 and NCgl2098 were cloned and expressed in *E. coli* BL21(DE3) (Fig. 1, lanes 6 and 7). The expression of NCgl0950 was easily achieved, and the expression of NCgl2098 indicated that this gene's product tended to form inclusion bodies in recombinant *E. coli* cells, although soluble NCgl2098 DAHP synthase also occurred in cellular lysate. The recombinant NCgl0950 DAHP synthase was purified to electrophoretic homogeneity (Fig. 1, lane 8). The results of SDS-PAGE showed that the purified NCgl0950 DAHP synthase had a molecular mass of ca. 40 kDa. The results of gel filtration chromatography indicated that the native NCgl0950 DAHP synthase had a molecular mass of 84 kDa. These results demonstrated that the NCgl0950 DAHP synthase was a homodimer. The NCgl2098 DAHP synthase was likewise purified (Fig. 1, lane 9). Determination of the molecular mass of native NCgl2098 DAHP synthase was not successful, but the protein was composed of subunits with a molecular mass of ca. 50 kDa. The purified NCgl0950 and NCgl2098 DAHP synthases were active and yielded specific activities of 0.065 and 0.015 units/mg of protein, respectively.

TABLE 2. Phenotypes of various mutants grown in glucose mineral medium with or without the addition of aromatic amino acids and DAHP synthase activities in the wild type and mutants of *C. glutamicum* RES167^a

Strain	Growth with added amino acid(s)						DAHP synthase activity
	None	Phe	Tyr	Trp	Phe and Tyr	Phe, Trp, and Tyr	
RES167	+	+	+	+	+	+	+
RES167 Δ NCgl0950	+	+	+	+	+	+	+
RES167 Δ NCgl2098	—	—	—	—	—	+	—
RES167 Δ NCgl(0950 + 2098)	—	—	—	—	—	+	—
RES167 Δ NCgl0950/pXMJ19-NCgl0950	+	+	+	+	+	+	+
RES167 Δ NCgl2098/pXMJ19-NCgl2098	+	+	+	+	+	+	+
RES167 Δ NCgl2098/pXMJ19-NCgl0950	—	—	—	—	—	+	—
RES167 Δ NCgl(0950 + 2098)/pXMJ19-NCgl0950	—	—	—	—	—	+	—
RES167 Δ NCgl(0950 + 2098)/pXMJ19-NCgl2098	+	+	+	+	+	+	+

^a Aromatic amino acids were added each at a final concentration of 0.2 mM.

FIG. 2. Growth curves of wild-type *C. glutamicum* RES167 and *C. glutamicum* RES167 Δ NCgl2098 mutants. To observe the phenotypes after gene disruption or complementation, strains were cultivated in minimal medium with 10 mM glucose as the only source of carbon and energy. Symbols: $\blacklozenge$, RES167; $\blacktriangle$, RES167 Δ NCgl2098; $\diamond$, RES167 Δ NCgl2098 supplemented with NCgl2098. Data are averages $\pm$ standard deviations of the results from three parallel cultures. OD₆₀₀, optical density at 600 nm.

Kinetic analysis of NCgl0950 and NCgl2098 DAHP synthases. DAHP synthase catalyzed the condensation reaction of PEP and E4P. Kinetic analyses of both the NCgl0950 and NCgl2098 DAHP synthase were performed with different concentrations of PEP or E4P when the concentration of the other substrate was relatively saturated at 2 mM for E4P or 5 mM for PEP. The kinetic parameters were calculated by using the double-reciprocal plot method (see Fig. S1 in the supplemental material). The apparent K_m values of the NCgl0950 DAHP synthase (0.16 mM for PEP and 0.29 mM for E4P) were much lower than those of the NCgl2098 DAHP synthase (8.52 mM for PEP and 2.17 mM for E4P), indicating that the NCgl0950 DAHP synthase had high affinities to PEP and E4P. However, the k_{cat} values of the NCgl2098 DAHP synthase were higher than those of the NCgl0950 DAHP synthase (Table 3), indicating that the catalytic efficiency of the NCgl2098 DAHP synthase was higher than that of the NCgl0950 DAHP synthase. The k_{cat}/K_m ratios reflect substrate specificity, and the NCgl0950 DAHP synthase was more specific for PEP, while the NCgl2098 DAHP synthase was more specific for E4P.

Effects of amino acids and metal ions on NCgl0950 DAHP synthase. NCgl0950 DAHP synthase was strongly inhibited by low concentrations of Tyr; 0.03 mM of Tyr resulted in more than an 80% loss of its activity (Fig. 3). Furthermore, Tyr-linked inhibition was competitive to PEP, and the K_i was determined to be 4.76 ± 0.24 μ M (mean $\pm$ standard deviation). Tyr was noncompetitive to E4P, and the K_i was determined to be 14.4 ± 0.03 μ M (see Fig. S1 in the supplemental material). Trp or Phe at 2 mM yielded 29.1 to 38.8% inhibition (Fig. 3),

FIG. 3. Feedback inhibition of NCgl0950 and NCgl2098 DAHP synthases from *C. glutamicum* RES167 by aromatic amino acids (Phe, Tyr, and Trp). Symbols: $\blacklozenge$, NCgl0950 DAHP synthase inhibited by Phe; $\blacksquare$, NCgl0950 DAHP synthase inhibited by Tyr; $\blacktriangle$, NCgl0950 DAHP synthase inhibited by Trp; $\diamond$, NCgl2098 DAHP synthase inhibited by Phe; $\square$, NCgl2098 DAHP synthase inhibited by Tyr; $\triangle$, NCgl2098 DAHP synthase inhibited by Trp. DAHP synthase activities without the addition of aromatic amino acids were determined to be 0.0081 ± 0.0012 and 0.089 ± 0.009 μ mol mg^{-1} min^{-1} , respectively. These average activities were calculated as 100%. Data are averages of the results from three parallel determinations.

and there was no significant synergistic inhibition by Phe plus Tyr or Phe plus Tyr plus Trp.

EDTA significantly inhibited NCgl0950 DAHP synthase activity. Mn^{2+} and Co^{2+} stimulated NCgl0950 DAHP synthase activity. In contrast, Mg^{2+} and Ni^{2+} were not stimulatory (Table 4).

Effects of amino acids and metal ions on NCgl2098 DAHP synthase. NCgl2098 DAHP synthase activity was not as sensitive to Tyr, Trp, and Phe feedback inhibition as was NCgl0950 DAHP synthase activity (Fig. 3). High concentrations (e.g., 5 mM) resulted in only a 10 to 15% decrease of DAHP synthase activity. Synergistic inhibition by Tyr, Trp, and Phe was not observed.

EDTA significantly inhibited NCgl2098 DAHP synthase activity. In contrast to the results for NCgl0950 DAHP synthase, only Mn^{2+} was strongly stimulatory to NCgl2098 DAHP synthase activity (Table 4).

Transcriptional analysis of NCgl0950 in RES167 cells. Based on the above results, it is clear that NCgl0950 encodes an active DAHP synthase in recombinant *E. coli*, but not in *C. glutamicum*. RT-PCR tests were conducted to reveal if NCgl0950 was transcribed. RT-PCR yielded a DNA fragment (Fig. 4) the sequence of which was the same as that of NCgl0950, thus indicating that NCgl0950 was transcribed in *C. glutamicum*. Two pairs of primers (Table 1) were used for

TABLE 3. Kinetic parameters of the NCgl0950 and NCgl2098 DAHP synthases from *C. glutamicum* RES167^a

Gene	Substrate	K_m (mM)	$V_{\max}$ (μ mol min^{-1} mg^{-1})	K_i (μ M)	k_{cat} (s^{-1}) ^b	k_{cat}/K_m (s^{-1} mM^{-1})
NCgl0950	PEP	0.16 ± 0.03	0.52 ± 0.03	4.76 ± 0.24	0.35	2.18
	E4P	0.29 ± 0.03	0.46 ± 0.02	14.4 ± 0.03	0.30	1.04
NCgl2098	PEP	8.52 ± 0.81	1.98 ± 0.20	ND	1.65	0.19
	E4P	2.17 ± 0.63	1.37 ± 0.43	ND	1.14	0.53

^a Experiments were done such that the concentration of one substrate (PEP or E4P) was varied while the concentration of the other substrate (E4P or PEP) was at a relatively saturated level (i.e., 5 mM for PEP and 2 mM for E4P). Data are averages from three independent determinations. Standard errors are provided for K_m , $V_{\max}$, and K_i . The k_{cat} and k_{cat}/K_m values were calculated from the averages of K_m and $V_{\max}$. ND, not determined.

^b Calculation of k_{cat} values was based on the subunit molecular mass of each enzyme.

TABLE 4. Effects of EDTA and divalent metal cations on NCgl0950 and NCgl2098 DAHP synthase activities^a

Additive (2 mM)		Relative activity (%) of DAHP synthase of:	
EDTA	Metal ions	NCgl0950	NCgl2098
—	—	100	100
+	—	22.7 ± 12.9	49.1 ± 11.3
—	Mg ²⁺	101.5 ± 11.0	131.3 ± 60.8
—	Mn ²⁺	357.5 ± 26.4	1,443.3 ± 419.1
—	Ni ²⁺	58.9 ± 8.1	98.8 ± 44.7
—	Co ²⁺	190.6 ± 21.6	131.8 ± 42.5

^a Metal ions were in the form of chloride salts. For EDTA assays, enzymes were incubated with EDTA for 15 min. The NCgl0950 and NCgl2098 DAHP synthase activities without the addition of EDTA or any metal cations were determined to be 0.032 ± 0.01 and $0.015 \pm 0.04 \mu\text{mol mg}^{-1} \text{min}^{-1}$, respectively. These average activities were calculated as 100%. Data are averages of three parallel determinations. Standard errors are provided. +, present; —, absent.

RT-PCR and yielded the same results (the results with one pair are shown).

DISCUSSION

Two DAHP synthases were previously detected in *C. glutamicum* subsp. *lactofermentum* (12). DNA fragments pAR1 (15.3 kb) and pAR2 (6.8 kb) were cloned into *E. coli* and yielded DAHP synthase activities. Unfortunately, neither the DNA nor the amino acid sequences of the DAHP synthases were reported (12). The results of this study demonstrated that *C. glutamicum* ATCC 13032 contains two DAHP synthase genes, namely NCgl0950 and NCgl2098, that encode the NCgl0950 and NCgl2098 DAHP synthases, respectively. NCgl0950 was cloned and expressed in *E. coli*, and the purified NCgl0950 DAHP synthase was subject to strong feedback inhibition by Tyr and, to a lesser extent, by Phe and Trp. Synergistic inhibition by the three aromatic amino acids was not apparent. Based on these observations, the NCgl0950 DAHP synthase is similar to the DAHP synthase from *C. glutamicum* subsp. *lactofermentum* (17). However, genetic disruption of NCgl0950 did not result in any observable changes of phenotype and DAHP synthase activity in *C. glutamicum*. Furthermore, NCgl0950 was not able to restore the phenotype of RES167ΔNCgl2098. pAR1 from *C. glutamicum* subsp. *lactofermentum* was not able to complement a DAHP synthase-deficient mutant of *E. coli* (12). Our results demonstrated that NCgl0950 was transcribed and that a putative Shine-Dalgarno sequence (5'-AAGG-3') occurred upstream of NCgl0950. The translational product of NCgl0950, the NCgl0950 DAHP synthase, was not at detectable levels in RES167 cells. These collective findings suggest that NCgl0950 was not involved in either the biosynthesis of Phe, Trp, and Tyr or the shikimate pathway in *C. glutamicum* RES167.

NCgl2098 functioned differently than NCgl0950 and encoded a DAHP synthase that was dissimilar to the NCgl0950 DAHP synthase. The results of genetic disruption and complementation experiments confirmed that NCgl2098 was necessary for the growth of *C. glutamicum* in mineral medium and that DAHP synthase activity in RES167 cells was linked to NCgl2098. The results from gene disruption and the enzymatic activity assays suggested that NCgl2098 encodes active DAHP

synthase and is involved in the biosynthesis of Phe, Trp, and Tyr, as well as the shikimate pathway in *C. glutamicum*. NCgl2098 was cloned and expressed in *E. coli* cells, and the purified NCgl2098 DAHP synthase was, surprisingly, not subject to feedback inhibition by Tyr and Phe, which is inconsistent with the commonly accepted properties for the NCgl2098 DAHP synthase of *C. glutamicum* (9, 10). Scientists in the late 1970s and early 1980s worked on the biosynthesis of aromatic amino acids with *Brevibacterium flavum* [currently *Corynebacterium glutamicum* subsp. *flavum* (18)] and discovered that a DAHP synthase was subject to feedback inhibition by Tyr and Phe separately and by Tyr and Phe synergistically (21). However, it is not known if that DAHP synthase was the NCgl0950 or NCgl2098 DAHP synthase. Indeed, at that time, it was not known that *C. glutamicum* contained two DAHP synthase genes and could theoretically produce two distinct DAHP synthases. Nonetheless, DAHP activity assays in these historical studies used crude ammonium sulfate precipitation fractions as enzyme preparations, and it is therefore very likely that these crude protein preparations contained both the NCgl0950 and NCgl2098 DAHP synthase. In a subsequent report, purified NCgl2098 product-like DAHP synthase (the DAHP synthase-chorismate mutase component A) had an estimated molecular mass of 250 kDa and was comprised of four identical subunits of 55 kDa (23). The potential feedback inhibition by aromatic amino acids of the activity of this NCgl2098 product-like DAHP synthase was not resolved, and recent reports take it for granted that the NCgl2098 DAHP synthase of *C. glutamicum* ATCC 13032 is subject to feedback inhibition by Tyr and Phe (9, 10). In the present study, the NCgl2098 DAHP synthase activity of *C. glutamicum* RES167 was not inhibited by Tyr and/or Phe. Trp exerted a slight inhibition of NCgl2098 synthase activity. These results do not agree with those reported for the DAHP synthase of *C. glutamicum* subsp. *flavum* (21, 23). The discovery that the NCgl2098 DAHP synthase from *C. glutamicum* is not sensitive to feedback inhibition by Tyr and Phe is potentially useful for improving productivity of the aromatic amino acids, as well as for the production of aromatic compounds, such as shikimic acid. The DAHP synthase of *B. subtilis* is insensitive to Phe, Trp, and Tyr and is regulated by the shikimate pathway intermediates prephenate and chorismate (28). Whether the NCgl2098 DAHP synthase of *C. glu-*

FIG. 4. Detection of NCgl0950 transcription by RT-PCR. Lanes: M, DNA ladder (1,000, 750, 500, and 250 bp); 1, PCR with cDNAs as template; 2, PCR with genome of RES167 as template; 3, negative control.

tamicum is regulated by prephenate and chorismate is not known.

The NCgl2098 DAHP synthase from *C. glutamicum* RES167 was different from the previously purified NCgl2098 product-like DAHP synthase of *C. glutamicum* subsp. *flavum* in that the former was strongly stimulated by Mn^{2+} and, to a lesser extent, by Co^{2+} , whereas the latter had been reported to be stimulated similarly by both Mn^{2+} and Co^{2+} (23). Interaction of the NCgl2098 product-like DAHP synthase with chorismate mutase of *C. glutamicum* subsp. *flavum* was observed (23). A putative chorismate mutase, encoded by NCgl0819, has been proposed for *C. glutamicum* ATCC 13032 (11, 15). Characterization of the chorismate mutase and interaction between the NCgl2098 DAHP synthase and the chorismate mutase in *C. glutamicum* RES167 is currently in progress.

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