Genetic and biochemical identification of the chorismate mutase from *Corynebacterium glutamicum*

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Chorismate mutase (CM) catalyses the rearrangement of chorismate to prephenate and is also the first and the key enzyme that diverges the shikimate pathway to either tryptophan (Trp) or phenylalanine (Phe) and tyrosine (Tyr). *Corynebacterium glutamicum* is one of the most important amino acid producers for the fermentation industry and has been widely investigated. However, the gene(s) encoding CM has not been experimentally identified in *C. glutamicum*. In this study, the *ncgI0819* gene, which was annotated as 'conserved hypothetical protein' in the *C. glutamicum* genome, was genetically characterized to be essential for growth in minimal medium, and a mutant deleted of *ncgI0819* was a Phe and Tyr auxotroph. Genetic cloning and expression of *ncgI0819* in *Escherichia coli* resulted in the formation of a new protein (NCgl0819) having CM activity. It was concluded that *ncgI0819* encoded the CM of *C. glutamicum* (CM0819). CM0819 was demonstrated to be a homodimer and is a new member of the monofunctional CMs of the AroQ structural class. The CM0819 activity was not affected by Phe, Tyr or Trp. Two 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthases (DS0950 and DS2098, formerly NCgl0950 and NCgl2098) had been previously identified from *C. glutamicum*. CM0819 significantly stimulated DAHP synthase (DS2098) activity. Physical interaction between CM0819 and DS2098 was observed. When CM0819 was present, DS2098 activity was subject to allosteric inhibition by chorismate and prephenate. Conserved hypothetical proteins homologous to CM0819 were identified in all known *Corynebacterium* genomes, suggesting a universal occurrence of CM0819-like CMs in the genus *Corynebacterium*.

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INTRODUCTION

Chorismate mutases (CMs, EC 5.4.99.5) are of interest for bioindustry and medicine, because the enzyme is the target to improve production of aromatic amino acids such as L-phenylalanine (Phe) and L-tyrosine (Tyr) (Chavez-Bejar *et al.*, 2008; Yakandawala *et al.*, 2008) as well as being a putative new drug target against deadly diseases such as tuberculosis (Qamra *et al.*, 2006; Schneider *et al.*, 2008). CMs catalyse the conversion of chorismate to prephenate, a reaction also known as the Claisen rearrangement, and the only naturally occurring Claisen reaction in the primary metabolism of living organisms (Krappmann *et al.*, 1999; Sugimoto & Shio, 1980a). This conversion is a key step in the biosynthetic pathway of Phe and Tyr in archaea, bacteria, fungi and plants (Helmstaedt *et al.*, 2004).

Examples of CMs that have been characterized are the EcCM from *Escherichia coli* (Hudson *et al.*, 1984), the MjCM from *Methanococcus jannaschii* (MacBeath *et al.*, 1998), the ScCM from *Saccharomyces cerevisiae* (Ball *et al.*, 1986), the HpCM from *Hansenula polymorpha* (Krappmann *et al.*, 2000), the MtbCM from *Mycobacterium tuberculosis* (Prakash *et al.*, 2005), the AtCM from *Arabidopsis thaliana* (Eberhard *et al.*, 1993), the BsCM from *Bacillus subtilis* (Gray *et al.*, 1990), and the TtCM from *Thermus thermophilus* (Helmstaedt *et al.*, 2004). Although those CMs are structurally conserved (Lee *et al.*, 1995; Chook *et al.*, 1993; Strater *et al.*, 1997), their amino acid sequences are much less similar. For example, the secreted MtbCM (encoded by Rv1885c) shares only 10–20% identity with EcCM and ScCM (Qamra *et al.*, 2006). Thus, identification of CM genes based on sequence homology from genomes is problematic. Prokaryotic CMs are grouped into either the AroQ class or the AroH class based on their crystal structure information (Chook *et al.*, 1994; Strater *et al.*, 1997; Calhoun *et al.*, 2001). The

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Abbreviations: CD, circular dichroism; CM, chorismate mutase; DAHP, 3-deoxy-D-arabino-heptulosonate 7-phosphate.

AroQ class CMs are dimeric and folded as a helix bundle. The AroH class is trimeric and folded as a pseudo- α/β -barrel (Helmstaedt *et al.*, 2001). The AroQ class CMs are further divided into cytoplasmic and monofunctional CMs that are not subject to allosteric regulation [e.g. Rv0948c from *Mycobacterium tuberculosis* (Schneider *et al.*, 2008)], periplasmic/secreted and monofunctional CMs [e.g. Rv1885c from *M. tuberculosis* (Prakash *et al.*, 2005)], and multiple functional fusion CMs [e.g. chorismate mutase/prephenate dehydrogenase from *E. coli* (Hudson *et al.*, 1984)].

Corynebacterium glutamicum is commercially used for the production of amino acids, including the aromatic amino acids Phe and Tyr (Ikeda, 2005). Because of low sequence similarity among the CMs, CM was not annotated in the initial genome sequence of *C. glutamicum* (Ikeda & Nakagawa, 2003). Later on, Ikeda (2005, 2006) reported *ncgl0819*, which had been annotated as a 'conserved hypothetical protein', to be a putative CM gene. But experimental identification of this putative CM gene was not carried out. In this study, we verified experimentally that *ncgl0819* is the sole gene encoding an active CM in *C. glutamicum* (CM0819). CM0819 was shown to belong to the cytoplasmic and monofunctional CMs of the AroQ class and is not subject to feedback inhibition by aromatic amino acids (Phe, Trp, Tyr). Two 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthases (DS0950 and DS2098, formerly NCgl0950 and NCgl2098) had been previously identified from *C. glutamicum*. CM0819 interacted with DS2098 from *C. glutamicum* and this interaction resulted in allosteric regulation of the DAHP synthase activity.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically on a rotary shaker (150 r.p.m.) at 37 °C in Luria-Bertani (LB) broth or on LB plates with 1.5 % (w/v) agar. *C. glutamicum* strains were routinely grown at 30 °C in LB medium or in mineral salts medium (Konopka, 1993), which was adjusted to pH 7.5 and supplemented with biotin (100 $\mu\text{g l}^{-1}$) and thiamine (500 $\mu\text{g l}^{-1}$) instead of yeast extract, on a rotary shaker (150 r.p.m.). When needed, glucose and aromatic amino acids (Phe, Tyr, Trp) were added as carbon and energy sources at final concentrations of 2 mM and 0.2 mM, respectively. Cellular growth was monitored by measuring the turbidity at 600 nm using a spectrophotometer. For generation of mutants and maintenance of *C. glutamicum*, BHIS (brain heart broth with 0.5 M sorbitol) medium was used. Antibiotics were used at the following concentrations: kanamycin, 50 $\mu\text{g ml}^{-1}$ for *E. coli* and 25 $\mu\text{g ml}^{-1}$ for *C. glutamicum*; ampicillin, 100 $\mu\text{g ml}^{-1}$ for *E. coli*; chloramphenicol, 20 $\mu\text{g ml}^{-1}$ for *E. coli* and 10 $\mu\text{g ml}^{-1}$ for *C. glutamicum*; nalidixic acid, 100 $\mu\text{g ml}^{-1}$ for *C. glutamicum*.

DNA and protein sequences analysis. The genome sequence of *C. glutamicum* ATCC 13032 (accession no. 003450) was retrieved from GenBank. Sequence comparisons and database searches were carried out using the BLAST service of the NCBI website (<http://www.ncbi.nlm.nih.gov>). The protein secondary structure prediction

was carried out with online protein structure prediction servers, namely YASPIN (<http://ibi.vu.nl/programs/>), SABLE (<http://sable.-cchmc.org/>), PORTER (<http://distill.ucd.ie/porter/>), GARNIER (<http://vm-bioinfo.toulouse.inra.fr/emboss/emboss.cgi/garnier>), QL server (<http://abs.cit.nih.gov/ql1>) and PROF (<http://www.predictprotein.org/>).

DNA extraction and manipulation. The total genomic DNA of *C. glutamicum* was isolated according to the procedure of Tauch *et al.* (1995). DNA restriction enzyme digestion, plasmid isolation and agarose gel electrophoresis were carried out as described by Sambrook *et al.* (1989). *E. coli* and *C. glutamicum* were transformed by electroporation according to the method of Tauch *et al.* (2002).

Amplification of DNA fragments with PCR and construction of plasmids. PCRs were performed by using *Pfu* DNA polymerase or *Taq* DNA polymerase (Transgen). PCR products were purified using an agarose gel DNA fragment recovery kit (TIANGEN). Cloning of PCR fragments was performed with the pEASY-T1 cloning vector systems (Transgen). Various plasmids (Table 1) for genetic disruption and complementation in *C. glutamicum* and for expression in *E. coli* were constructed based on pK18*mobsacB*, pXMJ19, pET21a and pET28a. Primers used for amplification of the entire target gene or target fragment are listed in Table 1. Gene deletion was performed by gene splicing through an overlap extension method called Gene Soeing (Horton *et al.*, 1989). According to the genome sequence of *C. glutamicum*, fragments of about 300 bp from both sides of *ncgl0819* (homologous arms) were amplified and ligated together to generate the disrupted gene $\Delta\text{ncgl0819}$. Then $\Delta\text{ncgl0819}$ was fused into pK18*mobsacB* to generate plasmid pK18*mobsacB*- $\Delta\text{ncgl0819}$. For complementation, pXMJ19-*ncgl0819* was constructed by insertion of PCR-amplified intact gene *ncgl0819* into pXMJ19 (Jakoby *et al.*, 1999). A plasmid for heterologous expression of the target gene was created by ligation of the PCR-amplified gene into pET28a and pET21a.

Genetic disruption and complementation in *C. glutamicum*. The pK18*mobsacB* derivative was transformed into *C. glutamicum* RES167 by electroporation (Tauch *et al.*, 2002) to replace the target gene *ncgl0819* with $\Delta\text{ncgl0819}$ by homologous recombination. Screening for the first and second recombination events, as well as confirmation of the chromosomal deletion, was performed as described previously (Schafer *et al.*, 1994). The resulting strain was designated *C. glutamicum* RES167 $\Delta\text{ncgl0819}$ (Table 1). The deletion of the target gene *ncgl0819* in the pK18*mobsacB* derivative and in the *C. glutamicum* mutant was verified by PCR amplification and DNA sequencing. Complementary plasmid pXMJ19-*ncgl0819* was introduced into *C. glutamicum* RES167 $\Delta\text{ncgl0819}$ by electroporation. Expression of *ncgl0819* in *C. glutamicum* was induced by the direct addition of IPTG to the culture, to a final concentration of 0.6 mM.

Heterologous expression in *E. coli*, preparation of cellular lysate, and protein purification. Plasmids pET28-*ncgl0950*, pET28-*ncgl2098*, pET28-*ncgl0819* and pET21-*ncgl2098* were transformed into *E. coli* BL21(DE3) by electroporation for heterologous expression of the corresponding genes (*ncgl0950*, *ncgl2098*, *ncgl0819*). Synthesis of recombinant proteins in *E. coli* BL21(DE3) cells was initiated by the addition of IPTG (0.6 mM), and cultivation was continued for an additional 10 h at 14 °C. Cells were harvested by centrifugation at 10 000 g and resuspended in 0.05 M Tris/HCl buffer, pH 7.5, containing 1 mM DTT (Ikeda & Nakagawa, 2003). For DS0950 and DS2098, cells were washed twice with 0.2 % KCl before resuspension. Then cells were disrupted by sonification in an ice-water bath. Cellular lysate was centrifuged at 20 000 g for 30 min at 4 °C and the supernatant was used for protein purification. The hexahistidine cascade of pET28a was fused to the target genes at the 5' end during plasmid construction. Then the recombinant proteins were purified

Table 1. Bacterial strains, plasmids and primers used

Strain/plasmid/primer	Relevant characteristics/sequences	Source/reference/notes
<i>E. coli</i>		
XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F'[proAB⁺ lacI^q lacZΔM15 Tn10(Tet^R)]</i>	Stratagene (Cat. No. 200249)
BL21(DE3)	<i>hsdS gal (λ.cIs857 ind-1 Sam7 nin-5 lacUV5-T7 gene 1)</i>	Novagen (Cat. No. 69450-3)
<i>C. glutamicum</i>		
RES167	Restriction-deficient mutant of ATCC 13032, Δ(<i>cglIM-cglIR-cglIIR</i>)	Gift from University of Bielefeld
RES167Δ <i>ncgl0819</i>	DNA fragment encoding amino acids 1–96 of <i>ncgl0819</i> deleted	This study
Plasmids		
pK18 <i>mobsacB</i>	Mobilizable vector, allows for selection of double crossover in <i>C. glutamicum</i>	Schafer <i>et al.</i> (1994)
pK18 <i>mobsacB</i> -Δ <i>ncgl0819</i>	Carrying <i>ncgl0819</i> deletion (refer to RES167Δ <i>ncgl0819</i>)	This study
pXMJ19	Shuttle vector (Cam ^r , <i>Ptac</i> , <i>lacI^q</i> , pBL1 <i>oriV_{C. glu}</i> pK18 <i>oriV_{E. coli}</i>)	Jakoby <i>et al.</i> (1999)
pXMJ19- <i>ncgl0819</i>	Carrying <i>ncgl0819</i> (to generate complementation for <i>ncgl0819</i>)	This study
pET28	Expression vector with N-terminal His ₆ affinity tag	Novagen
pET28- <i>ncgl0950</i>	pET28a derivative for expression of <i>ncgl0950</i>	Liu <i>et al.</i> (2008)
pET28- <i>ncgl2098</i>	pET28a derivative for expression of <i>ncgl2098</i>	Liu <i>et al.</i> (2008)
pET28- <i>ncgl0819</i>	pET28a derivative for expression of <i>ncgl0819</i>	This study
pET21	Expression vector with no His ₆ affinity tag	Novagen
pET21- <i>ncgl2098</i>	pET21a derivative for expression of <i>ncgl2098</i>	This study
Primers*		
0819a	<u>GACGGATCCGACACGCTCACGCATCTC</u> (<i>Bam</i> HI)	To generate pK18 <i>mobsacB</i> -Δ <i>ncgl0819</i>
0819b	<i>CTTATCCGAGTTTTCCGC</i> GCACACTATTCTGCCACG	
0819c	GCGGAAAACCTCGGATAAG	
0819d	<u>CGAATTCTAGACCTGTGACGATGGC</u> (<i>Eco</i> RI)	
0819F	<u>GACGGATCCAAAGGAGGAGACCATATGACTAATGCAGGTGAC</u> (<i>Bam</i> HI/ <i>Nde</i> I)	
0819R	<u>CGAATTCACCTTATCCGAGTTTCCG</u> (<i>Eco</i> RI)	To generate pXMJ19- <i>ncgl0819</i> and pET28- <i>ncgl0819</i>

*Restriction enzyme sites are underlined. Ribosome-binding sites are in bold type. Letters in italic denote the annealing regions for Gene Soeing PCR.

with the His-Bind protein purification kit (Novagen) according to the manufacturer's instructions.

Interaction between CM0819 and DS2098. In order to determine whether CM0819 and DS2098 interact with each other, recombinant *E. coli* cellular lysates containing CM0819 or DS2098 were sequentially loaded onto a His-Bind protein purification column (Novagen). The *E. coli* BL21(DE3)/pET28-*ncgl0819* cellular lysate was first loaded, and the column was then washed to remove residue proteins. CM0819 expressed in *E. coli* BL21(DE3)/pET28-*ncgl0819* has a His-tag, so it bound to the resin and remained within the column after washing with buffer. The BL21(DE3)/pET21-*ncgl2098* cellular lysate was then loaded. DS2098 had no His-tag, so it was not able to bind with resin and would be easily washed out with buffer. However, if CM0819 interacted with DS2098 and the two formed a complex, this DS2098 would remain within the column. Only when CM0819 was eluted with eluant was DS2098 eluted simultaneously with CM0819. As controls, in parallel, cellular lysates of BL21(DE3)/pET28-*ncgl0819*, BL21(DE3)/pET21-*ncgl2098*, or BL21(DE3)/pET28-*ncgl2919* (carrying fumarylpyruvate hydrolase) (Shen *et al.*, 2005) were loaded onto the same His-Bind protein purification columns.

Assay of CM. CM activity was assayed following the procedure of Barrie and Graham (Davidson & Hudson, 1987). The assay mixture contained 50 mM Tris/HCl buffer, pH 7.5 (containing 1 mM DTT), and 1 mM chorismic acid in a total volume of 0.2 ml. The mixture

was incubated at 37 °C for 5 min and then enzyme was added to initiate the reaction; 10 min later the reaction was stopped by addition of 0.4 ml 1 M HCl. After a further 10 min at 37 °C to convert prephenate to phenylpyruvate, 0.8 ml 2.5 M NaOH was added and the absorbance at 320 nm of the final solution was measured immediately. A blank correction was made using a solution treated as above, except that the enzyme was added after the NaOH. One unit of activity of CM is defined as the amount of enzyme that catalyses the conversion of 1 μmol chorismate to prephenate per min at 37 °C; the absorption coefficient of phenylpyruvate at 320 nm (ϵ_{320}) was determined as $17.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Davidson & Hudson, 1987). Aromatic amino acids (1 mM) were added to the mixture when feedback inhibition of the enzyme was tested.

K_m , V_{max} and k_{cat} values were obtained by fitting the kinetic data directly to the equation $v = V_{max} \times S / (K_m + S)$, where v is the initial velocity, V_{max} is the maximal initial velocity, K_m is the Michaelis constant, and S is the variable substrate concentration.

Assay of DAHP synthase. DAHP synthase activity was determined according to Siehl (1997). The assay mixture contained 50 mM Tris/HCl buffer, pH 7.5, 5 mM phosphoenolpyruvate, 2 mM E4P (erythrose 4-phosphate) and enzyme in a volume of 75 μl. The mixture was incubated at 30 °C for 10 min. The rate of reaction was proportional to the enzyme concentration and was linear with time. The reaction was initiated by the addition of E4P or enzyme and

terminated by the addition of 400 µl 10 % (w/v) TCA. Then 100 µl 25 mM NaIO₄ in 62.5 mM H₂SO₄ was added to the mixture and incubated at 37 °C for 30 min, after which the reaction was stopped by addition of 100 µl 2 % (w/v) Na₂SO₃ in 0.5 M HCl, and the tubes were mixed before adding 1 ml of 0.36 % (w/v) thiobarbituric acid. Then the tubes were incubated at 100 °C for 10 min and the colour of the mixture turned to pink. The tubes were allowed to cool to room temperature and the absorbance at 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 catalyses the synthesis of 1 µmol DAHP per min at 30 °C. For calculation, an absorption coefficient of DAHP at 549 nm (ϵ_{549}) of $4.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ was adopted (Schoener & Herrmann, 1976).

Inhibition to DAHP synthase activity by chorismate or prephenate was determined by inclusion of chorismate or prephenate in the assay mixtures. Parallel experiments without enzymes in the assay mixture were run as control.

SDS-PAGE, determinations of molecular mass of the purified enzymes and protein estimation. SDS-PAGE was conducted with 5 % stacking gels and 12 % resolving gel and was run with a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad) according to the manufacturer's instructions. After electrophoresis, the protein bands were visualized by Coomassie brilliant blue staining. Apparent molecular mass was estimated according to the relative mobility of protein markers (Transgen). The native molecular mass of the enzyme was 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. Molecular mass was calculated according to the elution volumes and calibrated with a standard molecular mass kit. Protein concentration was determined according to the method of Bradford (1976), with BSA as the standard.

Circular dichroism (CD) spectrometry. The CD spectrum of the recombinant protein CM0819 was recorded at a wavelength of 200–240 nm stepwise (1 nm), with four accumulations at each step. The spectral baseline was corrected by subtracting the control. Ellipticity (mdeg) was plotted as a function of wavelength (nm). The percentage of helicity for secondary structure determination was calculated using the K2D2 software (<http://www.ogic.ca/projects/k2d2/>).

RESULTS

Bioinformatic analysis

The gene *ncgl0819* is located at base pairs 910852–911157 on the *C. glutamicum* genome, and encodes a hypothetical protein of 101 amino acid residues with a theoretical molecular mass of 11.3 kDa. A BLAST search with CM0819 revealed a range of homologous ORFs that were all annotated as 'hypothetical proteins' identified in other bacterial genomes: These hypothetical proteins include cgR_0968 (locus_tag of the encoding gene; identity 100 %) of *C. glutamicum* strain R, CE0929 (91 %) of *Corynebacterium efficiens* YS-314, DIP0838 (81 %) of *Corynebacterium diphtheriae* NCTC 13129, CU0528 (72 %) of *Corynebacterium urealyticum* DSM 7109, jk1565 (68 %) of *Corynebacterium jeikeium* K411, MMAR_4555 (54 %) of *Mycobacterium marinum* M, RHA1_ro05569 (55 %) of *Rhodococcus* sp. RHA1, and nfa49960 (57 %) of

Nocardia farcinica IFM 10152. CM0819 also showed sequence similarity to functionally identified CMs, the highest similarity being to the recently identified cytoplasmic MtbCM (encoded by Rv0948c, identity 57 %) (Schneider *et al.*, 2008), and to previously identified EcCM from *E. coli* (27 %), MjCM from *M. jannaschii* (10.7 %), ScCM from *S. cerevisiae* (9.4 %), HpCM from *H. polymorpha* (7.9 %), and AtCM3 from *A. thaliana* (7.4 %). An alignment of the deduced CMs and the structurally characterized *E. coli* CM revealed a range of conserved amino acid residues (data not shown). Phylogenetic analysis based on the deduced amino acid sequences of these hypothetical proteins and the AroQ CMs characterized so far demonstrated that the functionally identified or the putative CMs from high-G + C Gram-positive bacteria clustered together, and two groups of CMs within this cluster, the mycobacteria-group CMs and the corynebacteria-group CMs, could be easily identified (Fig. 1).

The mutant RES167Δ*ncgl0819* is auxotrophic for Phe and Tyr

In order to observe the phenotype of a *ncgl0819* mutant and to identify the function of *ncgl0819*, the mutant strain RES167Δ*ncgl0819* was constructed by disruption of *ncgl0819* in *C. glutamicum* RES167 (see Methods). Phenotypic characterization indicated that mutant RES167Δ*ncgl0819* had lost the ability to grow on minimal medium. The complementation of mutant RES167Δ*ncgl0819* with intact *ncgl0819* or supplementation with three aromatic amino acids (Phe + Trp + Tyr, each 0.2 mM) restored its growth. Supplementation with Phe, Trp or Tyr individually, or with the combination of (Phe + Trp) or (Trp + Tyr) was not able to restore the growth of RES167Δ*ncgl0819* in minimal medium. However, the combination of (Phe + Tyr) did restore the growth of the mutant in minimal medium (Table 2, Fig. 2). These results clearly indicated that *ncgl0819* is essential for the growth of *C. glutamicum* in minimal medium, and suggested that this gene is involved in the biosynthesis of Phe and Tyr (but not Trp) in *C. glutamicum*.

ncgl0819 encodes a monofunctional CM, CM0819

The entire *ncgl0819* gene was PCR amplified and cloned into *E. coli* BL21(DE3) via plasmid pET28-*ncgl0819*. Upon induction with IPTG, the *E. coli* BL21(DE3)/pET28-*ncgl0819* cells promptly synthesized new proteins that corresponded to the theoretical molecular mass of CM0819 (Fig. 3, lane 2). This protein was purified (Fig. 3, lanes 3 and 4), and its enzyme activity was determined. The enzymic assay showed that the native CM0819 had strong CM activity, but no DAHP synthase activity. Thus, it was concluded that *ncgl0819* encodes a monofunctional CM in *C. glutamicum*.

The purified CM0819 protein behaved as a single band of ~12 kDa in SDS-PAGE. When analysed by gel-filtration

Fig. 1. Phylogenetic analysis of functionally identified (showed in bold type) and putative (annotated as hypothetical proteins) CMs. The phylogenetic tree was constructed by the neighbour-joining method with Kimura's two-parameter calculation model in MEGA version 3.1 (Kumar *et al.*, 2004). AroQ, monofunctional CM; *AroQ, periplasmic CM. A bullet indicates a fusion joint. AroQ-PheA indicates an AroQ domain fused at its carboxyl terminus with the amino-terminal portion of a PheA domain.

chromatography, CM0819 was eluted as a molecule of 23.5 kDa. This suggested that CM0819 is a homodimer.

The DAHP synthases from *C. glutamicum* do not show CM activity, but CM0819 stimulates DAHP synthase activity

Previously, a two-component CM (components A and B) was reported for *C. glutamicum* subsp. *flavum* [formerly *Brevibacterium flavum* (Liebl 2005)]. Component A had a molecular mass of 250 kDa and component B was 25 kDa. The elution patterns of component A coincided with DAHP synthase activity, and both components A and B were essential for CM activity (Shiio & Sugimoto, 1979). In the present study, CM0819 alone showed sharp CM activity, but no DAHP synthase activity (Table 3). Our previous work had identified two DAHP synthases in *C. glutamicum*, encoded by *ncgl0950* and *ncgl2098* (Liu *et al.*, 2008). Enzymic assays carried out in the current study showed that neither of these two DAHP synthases showed any CM activity (Table 3). However, CM0819 from *C. glutamicum* significantly stimulated DAHP synthase activity: DS0950 activity was increased by 39.4 % and DS2098 by 253.3 %. Stimulation of CM activity by either of the two DAHP synthases was not observed (Table 3).

CM0819 and DS2098 interact with each other

The fact that CM0819 stimulated DS2098 DAHP synthase activity suggested a possible interaction between these two proteins. Our results from affinity chromatography confirmed this (Fig. 4): pre-loading of CM0819 onto the His-Bind protein purification column resulted in subsequent binding of DS2098 (which did not have a His-tag and thus did not bind on itself) onto the column. This suggested that CM0819 and DS2098 might form a complex and that their function might be regulated.

It was previously reported that *B. subtilis* had a bifunctional DAHP synthase-CM and that the DAHP synthase activity was subject to feedback inhibition by chorismate (Jensen &

Nester, 1965). We examined the effects of chorismate and prephenate on the two DAHP synthases (DS2098 and DS0950) from *C. glutamicum*. The results indicated that DS2098 activity was not affected by chorismate or prephenate when DS2098 was alone. However, in the presence of CM0819, the DAHP synthase activity of DS2098 was inhibited by chorismate (at 1 mM) by 45.7 % and by prephenate by 24.9 % (at 1 mM) and 41.0 % (at 2 mM). In contrast to DS2098, DS0950 activity was inhibited by chorismate (at 1 mM) by 36.1 % and by prephenate (at 1 mM) by 17.3 %, when it was alone. When DS0950 and CM0819 were together in the mixture, the DAHP synthase activity of DS0950 was inhibited by chorismate (at 1 mM) by 43.2 % and by prephenate (at 1 mM) by 18.1 %, similar to the inhibition seen when DS0950 was alone.

CM0819 activity is not regulated by Phe, Tyr and Trp, but is affected by metal ions

The feedback regulation effect of Phe, Tyr and Trp on CM0819 activity was determined for each individual amino acid. There was no observable feedback regulation of CM activity at concentrations of 0.01–10 mM of any of Phe, Trp or Tyr. This is similar to MtbCM from *Mycobacterium tuberculosis*, which was not subject to feedback regulation by Phe, Trp or Tyr (Kim *et al.*, 2006).

Effects of various divalent metal ions on CM0819 activity were studied. Mg^{2+} and EDTA at concentrations of 0.1–2 mM did not affect CM0819 activity. Mn^{2+} , Co^{2+} and Ni^{2+} , each at 2 mM, decreased CM0819 activity by 38.9 %, 52.4 % and 41.8 %, respectively.

The kinetic constants of the CM0819 were determined. The K_m for chorismate was determined to be 2.83 ± 0.53 mM, and V_{max} was 2.75 ± 0.52 $\mu\text{mol min}^{-1} \text{mg}^{-1}$. The molar catalytic activity (k_{cat}) was calculated to be 1.05 ± 0.19 s^{-1} .

CM0819 is an α -helix-predominant protein

The CD spectrum of the CM0819 was recorded on a JASON spectropolarimeter (Fig. 5). The data were analysed

Table 2. Phenotypes of mutant RES167 Δ *ncgl0819* grown in minimal medium with addition of different aromatic amino acids (each at final concentration of 0.2 mM)

	None	Phe	Tyr	Trp	Phe + Tyr	Phe + Trp	Tyr + Trp	Phe + Tyr + Trp
RES167	+	+	+	+	+	+	+	+
RES167 Δ <i>ncgl0819</i>	–	–	–	–	+	–	–	+
RES167 Δ <i>ncgl0819</i> /pXMJ19- <i>ncgl0819</i>	+	+	+	+	+	+	+	+

Fig. 2. Growth curves of *C. glutamicum* strains. To observe the phenotype after gene disruption or complementation, strains were cultivated in minimal medium with 2 mM glucose as the only carbon and energy source. ◆, RES167; ▲, RES167 Δ *ncg10819*; △, RES167 Δ *ncg10819* complemented with *ncg10819*; ◇, RES167 Δ *ncg10819* in minimal medium with Phe and Tyr (each 0.2 mM). Data are means $\pm$ SD from three parallel cultures.

using the K2D2 software, and the results revealed that CM0819 had 67.5 % α -helices and 3.2 % β -sheets, indicating a predominantly helical structure for the enzyme. Similarly, the CMs from yeast and *E. coli* were reported also to be helix-predominant proteins (Lee *et al.*, 1995; Strater *et al.*, 1997).

DISCUSSION

In this study, the gene (*ncg10819*) encoding chorismate mutase (CM0819) from *C. glutamicum* was identified and the CM0819 enzyme was characterized. To our knowledge, this is the first functionally identified CM gene of the genus *Corynebacterium*. Highly conserved hypothetical proteins

Fig. 3. Cloning and expression of CM0819 in *E. coli*. Lanes: M, molecular mass markers; 1, *E. coli* BL21(DE3)/pET28-*ncg10819*, not induced by IPTG; 2, *E. coli* BL21(DE3)/pET28-*ncg10819*, induced by IPTG; 3, purification of CM0819, after His-tag chromatography; 4, purification of CM0819, after gel filtration.

Table 3. Determination of CM activity and interactions between CM0819 and DAHP synthases from *C. glutamicum* RES167

DAHP synthases are encoded by *ncg10950* and *ncg12098* (Liu *et al.*, 2008). Data are means $\pm$ SD from three parallel determinations.

	Relative CM activity (%)	Relative DAHP synthase activity (%)
CM0819	100* $\pm$ 9.0	None
DS0950	None	100† $\pm$ 12.2
DS0950 + CM0819	102.1 $\pm$ 7.2	139.4 $\pm$ 25.9
DS2098	None	100‡ $\pm$ 35.3
DS2098 + CM0819	105.9 $\pm$ 7.0	353.3 $\pm$ 33.3

*100 % CM0819 activity is defined as 0.845 units mg^{-1} .

†100 % DS0950 activity is defined as 0.034 units mg^{-1} .

‡100 % DS2098 activity is defined as 0.032 units mg^{-1} . Purified enzymes were used in the assay.

Fig. 4. SDS-PAGE of proteins eluted from columns of His-tag chromatography, showing the interaction between CM0819 and DS2098. M, molecular mass markers. 1, His-tag labelled CM0819, eluted from a His-tag binding chromatography column loaded with BL21(DE3)/pET28-*ncg10819* cellular lysate. 2, Eluate from column loaded with BL21(DE3)/pET21-*ncg12098* cellular lysate (containing DS2098 without His-tag), indicating no binding of DS2098. 3, Interaction of CM0819 with DS2098. Both proteins were simultaneously eluted from a His-tag binding chromatography column, which was first loaded with BL21(DE3)/pET28-*ncg10819* cellular lysate and then with BL21(DE3)/pET21-*ncg12098* cellular lysate. 4, His-tagged NCgl2919 (fumarylpyruvate hydrolase), eluted from a His-tag binding chromatography column loaded with BL21(DE3)/pET28-*ncg12919* cellular lysate. 5, A His-tag binding chromatography column was first loaded with BL21(DE3)/pET28-*ncg12919* cellular lysate and then loaded with BL21(DE3)/pET21-*ncg12098* cellular lysate. Only NCgl2919 was eluted, indicating that NCgl2919 did not interact with DS2098.

Fig. 5. CD spectrum of CM0819, suggesting a predominantly α -helical secondary structure of the protein. The percentage of helicity for secondary-structure determination was calculated using the K2D2 software. The spectrum confirms a highly helical structure of the recombinant protein with signature peaks at 207 and 222 nm.

homologous to CM0819 were identified in all currently known *Corynebacterium* genomes. We deduced that those hypothetical proteins would function as CMs and are involved in the central shikimate pathway in *Corynebacterium* species. According to the sequence-similarity analysis, CM0819 is more closely related to the cytoplasmic MtbCM (Rv0948c) than to the secreted MtbCM (Rv1885c) in *Mycobacterium tuberculosis*. Considering that *ncgl0819* was necessary for growth of *C. glutamicum* in minimal medium, we conclude that *ncgl0819* is the sole CM gene in *C. glutamicum*.

Our results indicate that CM0819 is a homodimer, and its secondary structure is rich in α -helices. Thus, it is proposed that CM0819 is a new member of the monofunctional AroQ class of CMs. Further, this CM0819 stimulates DAHP synthase activity in *C. glutamicum*, a new observation that has not been reported before. It is proposed that CM0819 interacts with DS2098 and the two proteins possibly form a complex. This hypothesis is supported by the previous observation that CM and DAHP synthase activities associated with each other and were isolated as a protein complex from *C. glutamicum* (Sugimoto & Shiio, 1980b).

CM catalyses the committed step in Phe and Tyr biosynthesis (Prakash *et al.*, 2005). The CMs of the AroQ class originating from various hosts are subject to different types of regulation. EcCM exists as a fusion protein in *E. coli* and is subject to allosteric inhibition only by pathway-specific inhibitors (Prakash *et al.*, 2005). The ScCM of *S. cerevisiae* and AnCM of *Aspergillus nidulans* are inhibited by Tyr and Phe and are stimulated by Trp (Krappmann *et al.*, 1999; Schmidheini *et al.*, 1989). Similar to MtbCM (Kim *et al.*, 2006), CM0819 in *C. glutamicum* is not subject to any regulation by aromatic amino acids. But CM0819 together with chorismate and prephenate regulates DS2098 activity via allosteric inhibition, similar to what was

observed for the bifunctional CM from *B. subtilis* (Jensen & Nester, 1965). However, CM0819 and DS2098 from *C. glutamicum* differ from the bifunctional CM from *B. subtilis* in that the former was more sensitive to chorismate regulation (this study) and the latter was more sensitive to prephenate regulation (Jensen & Nester, 1965). Previously, Gosset *et al.* (2001) reported that DAHP synthase from *Xanthomonas campestris* was subject to allosteric inhibition by chorismate and was slightly inhibited by Trp. We found that DS2098 alone was not subject to inhibition by chorismate (this study) and was slightly inhibited by Trp (Liu *et al.*, 2008). When DS2098 and CM0819 occurred together, allosteric inhibition of the DAHP synthase activity was observed, a new observation that has not been reported before.

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