Genome-wide investigation of aromatic acid transporters in *Corynebacterium glutamicum*

Muhammad Tausif Chaudhry,^{1,2} Yan Huang,^{1,2} Xi-Hui Shen,¹
Ansgar Poetsch,³ Cheng-Ying Jiang¹ and Shuang-Jiang Liu¹

Correspondence
Shuang-Jiang Liu
liusj@sun.im.ac.cn

¹State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, 100080, China

²Graduate University of Chinese Academy of Sciences, Beijing, 100049, China

³Lehrstuhl für Biochemie der Pflanzen, Ruhr Universität, Bochum, Germany

Genome-wide data mining indicated that six genes (*ncgl1031*, *ncgl2302*, *ncgl2325*, *ncgl2326*, *ncgl2922* and *ncgl2953*) encoding putative transport proteins are involved in uptake of various aromatic compounds that are further degraded through the β -ketoadipate, gentisate and resorcinol pathways in *Corynebacterium glutamicum*. The gentisate (GenK/NCgl2922) and vanillate (VanK/NCgl2302) transporters have been identified previously. In this study, physiological functions of the remaining four putative transporters as well as the vanillate transporter (VanK/NCgl2302) were examined by genetic disruption/complementation and uptake assays. Results indicated that *ncgl1031* encodes PcaK for 4-hydroxybenzoate and protocatechuate transport, and *ncgl2302* encodes VanK for vanillate transport. Genetic studies and uptake assays indicated that both *ncgl2325/benK* and *ncgl2326/benE* are involved in benzoate transport in *C. glutamicum*. When growth rates were compared for two benzoate transporter mutants, *benK* and *benE*, a high growth rate was observed for the *benE* mutant. Sequence alignments revealed that PcaK, VanK, BenK and GenK belong to the major facilitator superfamily (MFS). Modelling of secondary structures based on previously characterized MFS members revealed that NCgl1031, NCgl2302, NCgl2325 and NCgl2922 are typical 12 helix transmembrane proteins but NCgl2326 contains only 11 α -helices. Thus the functionally identified NCgl2326 belongs to a novel type of benzoate transporters. Attempts to identify the phenotype of a *hydK/ncgl2953* mutant failed, so the function of *ncgl2953* remains unclear.

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INTRODUCTION

Membrane transport in bacteria not only maintains a suitable environment inside the cell by regulating entry of essential nutrients into the cytoplasm and extrusion of deleterious substances from the cell, but also enhances communication between cell and environment (Mitchell, 1967). Membrane proteins are the main players in this transport. Sequencing data from several prokaryotic and eukaryotic genomes have revealed that about 30 % of all the integral membrane proteins are involved in transport across biological membranes (Paulsen *et al.*, 2000).

Among the various categories of transporters classified (Ren *et al.*, 2004), the major facilitator superfamily (MFS; Pao *et al.*, 1998; Saier *et al.*, 1999) is the largest (54 families), best known and functionally the most diverse superfamily of the electrochemical potential-driven transporter subclass. Recently, the structures and functions of three MFS

transporters have been studied in detail (Hirai *et al.*, 2002; Abramson *et al.*, 2003; Huang *et al.*, 2003). The aromatic acid:H⁺ symporter (AAHS) family of the MFS presently contains six members, namely PcaK of *Pseudomonas putida* (Nichols & Harwood, 1997), TfdK of *Ralstonia eutropha* (Leveau *et al.*, 1998), BenK, VanK and MucK of *Acinetobacter* sp. ADP1 (Collier *et al.*, 1997; Williams & Shaw, 1997; D'Argenio *et al.*, 1999) and MhpT of *Escherichia coli*, which are all from Gram-negative bacteria.

Corynebacterium glutamicum is a Gram-positive, non-pathogenic, non-motile, aerobic, coryneform bacterium with a high G + C content that belongs to the actinomycetes subphylum (Stackebrandt *et al.*, 1997). Since its isolation (Kinoshita *et al.*, 1957), *C. glutamicum* has been extensively employed for industrial scale production of amino acids (L-glutamate, L-lysine, etc.), vitamins (D-pantothenic acid, etc.) and nucleotides, and is among the most important microorganisms for industrial biotechnology. The accessibility of genome data for *C. glutamicum* (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003) has greatly stimulated studies that use *C. glutamicum* as a model microorganism in genetic

Abbreviations: AAHS, aromatic acid:H⁺ symporter; MFS, major facilitator superfamily.

and physiological research. Recent studies have demonstrated that *C. glutamicum* can degrade various aromatic compounds (Shen *et al.*, 2004, 2005a; Shen & Liu, 2005), and a novel mycothiol-dependent gentisate pathway has been identified (Feng *et al.*, 2006). Although transport systems for amino acids (Simic *et al.*, 2001; Kennerknecht *et al.*, 2002; Eggeling & Sahm, 2003; Ren *et al.*, 2004) and sugars (Dominguez & Lindley, 1996; Dominguez *et al.*, 1998; Gourdon *et al.*, 2003) have been well characterized in *C. glutamicum*, knowledge of aromatic compound transport is very limited. In this study, the entire genome of *C. glutamicum* ATCC 13032 was searched for aromatic acid transporter genes and six putative genes encoding transporters were identified through genetic disruption/complementation and uptake assays for various aromatic compounds.

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 in Luria-Bertani (LB) broth on a rotary shaker (150 r.p.m.) or on LB plates containing 1.5% (w/v) agar, at 37 °C. *C. glutamicum* strains were routinely cultivated in LB broth or BHIS (brain heart infusion broth supplemented with 50 mM sorbitol). To test their ability to grow on various aromatic compounds, wild-type *C. glutamicum* and mutants were inoculated into minimal medium (MM, pH 9.0; Konopka, 1993). Yeast extract (0.1 g per litre) was added to meet the vitamin requirements of bacterial strains. Benzoate, protocatechuate and vanillate were autoclaved in MM broth. Stock solutions of gentisate (100 mM), resorcinol (200 mM), 3-hydroxybenzoate (100 mM) and 4-hydroxybenzoate (100 mM) were sterilized by filtration through 0.2 µm pore size filters and were added to MM at final concentrations of 2 mM. All the *C. glutamicum* strains were grown with rotary shaking at 150 r.p.m. at 30 °C. Growth was monitored by measuring turbidity at 600 nm (OD₆₀₀). For selection of mutants, in accordance with the vector used, antibiotics were added at the following concentrations: ampicillin, 100 µg ml⁻¹ for *E. coli*; chloramphenicol, 20 µg ml⁻¹ for *E. coli* and 10 µg ml⁻¹ for *C. glutamicum*; kanamycin, 50 µg ml⁻¹ for *E. coli* and 25 µg ml⁻¹ for *C. glutamicum*; nalidixic acid, 50 µg ml⁻¹ for *C. glutamicum*.

DNA manipulation and cloning of transporter genes. Genomic DNA of *C. glutamicum* was isolated by the method of Tauch *et al.* (1995). DNA manipulation, plasmid isolation and agarose gel electrophoresis were routinely carried out using standard methods (Sambrook *et al.*, 1989). Restriction endonucleases, ligase and DNA polymerase were used according to the manufacturer's instructions. Vectors were electroporated into *E. coli* and *C. glutamicum* by the methods of Tauch *et al.* (2002). Primers used in this work for amplification of intact and disrupted genes are listed in Table 2. To facilitate cloning, both forward and reverse primers were flanked with cleavage sites for restriction endonucleases. PCR consisted of 30 cycles of denaturation at 95 °C for 1 min, annealing at 42 °C for 1 min and extension at 72 °C for 1.5 min followed by a final extension at 72 °C for 10 min. Amplified DNA was analysed by electrophoresis using 1% agarose, purified by extraction from the gel, restricted with the corresponding endonucleases to obtain the disrupted gene, and finally ligated with pGEM-T Easy vector (Promega).

Disruption of transporter genes in *C. glutamicum*. For gene disruption, plasmids pK18mobsacBΔncgl1031, pK18mobsacBΔncgl2302,

pK18mobsacBΔncgl2325, pK18mobsacBΔncgl2326 and pK18mobsacBΔncgl2953 were constructed. All pK18mobsacB derivatives were constructed by ligation of the appropriate disrupted gene (restricted from pGEM-T Easy) with pK18mobsacB. Plasmids were electroporated into *C. glutamicum* RES167 competent cells. Integration of introduced plasmids into the chromosome by first crossover was selected on BHIS plates containing kanamycin (25 mg ml⁻¹) and nalidixic acid (50 mg ml⁻¹). These antibiotic-resistant cells were grown overnight in LB medium and spread on LB plates containing 10% (w/v) sucrose. The second crossover of chromosomal DNA led to kanamycin-sensitive (Km^S) cells that were tested for their ability to grow in MM supplemented with aromatic compounds (benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, gentisate, protocatechuate, resorcinol and vanillate) as sole carbon and energy sources. Mutant strains that were unable to grow on a particular aromatic acid were tested for gene deletion by PCR amplification using the same primers as used for amplification of intact genes. The knockout mutants were designated RES167Δncgl1031, RES167Δncgl2302, RES167Δncgl2325, RES167Δncgl2326 and RES167Δncgl2953. A double knockout mutant, RES167Δncgl(2325-2326), was also constructed by disrupting both ncgl2325 and ncgl2326. The deletion of target genes in pK18mobsacB derivatives and in *C. glutamicum* mutants was also confirmed by DNA sequencing.

Genetic complementation. For genetic complementation, ncgl2031, ncgl2302, ncgl2325, ncgl2922 and ncgl2953 were amplified by PCR. The native ribosome-binding site (RBS) was replaced with the consensus RBS sequence identified by Amador *et al.* (1999). The endonuclease-digested PCR product was ligated into *E. coli*-*C. glutamicum* shuttle expression vector pXMJ19 (Jakoby *et al.*, 1999) restricted with the appropriate endonucleases. These complementation plasmids were introduced into *C. glutamicum* RES167 mutants by electroporation to produce complemented strains.

Determination of growth rates of two mutants for benzoate transport at different benzoate concentrations. In order to differentiate the function of the two benzoate transporters, mutants RES167Δncgl2325 and RES167Δncgl2326 were inoculated in MM (pH 9.0) containing different benzoate concentrations (1–5 mM). Triplicate cultures were incubated using a rotary shaker (150 r.p.m.) at 30 °C. Growth was measured by the increase in OD₆₀₀ and growth rates for the two mutants at different benzoate concentrations were determined.

Assay for uptake of aromatic acids by resting cells. *C. glutamicum* RES167 and the mutant strains were grown in 100 ml MM (pH 9.0) supplemented with 40 mM sodium acetate to an OD₆₀₀ of 2.0. Cultures were centrifuged at 8000 r.p.m. for 5 min at 4 °C and washed twice with 50 mM phosphate buffer (pH 8.0). Cells were resuspended in the same buffer and incubated at 30 °C for 30 min to exhaust all endocellular carbon reserves. Cells were again centrifuged, washed and resuspended (at OD₆₀₀ 2.0) in 50 ml phosphate buffer (pH 8.0) containing 2 mM aromatic acid as substrate. Uptake of aromatic acids, as indicated by the decrease in their concentrations in the supernatant, was analysed by HPLC in a 1050 Hewlett Packard chromatograph equipped with a reverse-phase RP-18 column (4.6 mm × 240 mm × 0.5 mm) and a photodiode array detector. Standards were prepared in phosphate buffer (pH 8.0). Aqueous solvent contained 20% (v/v) methanol in 100 mM ammonium acetate buffer (pH 4.2). Benzoate, 4-hydroxybenzoate and vanillate were typically eluted and monitored with this system at 7.9 min and 225 nm, at 6.0 min and 250 nm, and at 7.1 min and 253 nm, respectively.

Sequence data analysis. The nucleotide sequence of *C. glutamicum* ATCC 13032 genome was obtained from GenBank (accession no. NC003450; Ikeda & Nakagawa, 2003). Sequence comparisons and protein sequence similarity searches were performed using

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)*	Source or reference
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> (F' <i>proAB lac^rΔM15 Tn10</i> (Tet ^r))	Stratagene (cat. no. 200228)
JM109	<i>e14-(mcrA⁻) recA1 endA1 gyrA96 thi-1 hsdR17 (r_k⁻ m_k⁺) supE44 relA1 D(lac-proAB)</i> (F' <i>traD36 proAB lac^rΔM15</i>)	Stratagene (cat. no. 200235)
<i>C. glutamicum</i>		
RES167	Restriction-deficient mutant of ATCC 13032, Δ(<i>cglIM—cglIIR—cglIIR</i>).	Tauch <i>et al.</i> (2002)
RES167Δ <i>ncgl1031</i>	Fragment of DNA encoding amino acids 22–126 of <i>ncgl1031</i> deleted	This study
RES167Δ <i>ncgl2302</i>	Fragment of DNA encoding amino acids 76–179 of <i>ncgl12302</i> deleted	This study
RES167Δ <i>ncgl2325</i>	Fragment of DNA encoding amino acids 239–356 of <i>ncgl2325</i> deleted	This study
RES167Δ <i>ncgl2326</i>	Fragment of DNA encoding amino acids 215–408 of <i>ncgl2326</i> deleted	This study
RES167Δ <i>ncgl</i> (2325–2326)	Fragments of DNA encoding amino acids 239–356 of <i>ncgl2325</i> and 215–407 of <i>ncgl2326</i> deleted	This study
RES167Δ <i>ncgl2922</i>	Fragment of DNA encoding amino acids 111–302 of <i>ncgl2922</i> deleted	Shen <i>et al.</i> (2005b)
RES167Δ <i>ncgl2953</i>	Fragment of DNA encoding amino acids 174–382 of <i>ncgl2953</i> deleted	This study
RES167Δ <i>ncgl1031</i> /pXMJ19- <i>ncgl1031</i>	RES167Δ <i>ncgl1031</i> containing pXMJ19- <i>ncgl1031</i>	This study
RES167Δ <i>ncgl2302</i> /pXMJ19- <i>ncgl2302</i>	RES167Δ <i>ncgl2302</i> containing pXMJ19- <i>ncgl2302</i>	This study
RES167Δ <i>ncgl</i> (2325–2326)/pXMJ19- <i>ncgl2325</i>	Double-knockout strain RES167Δ <i>ncgl</i> (2325–2326) containing pXMJ19- <i>ncgl2325</i>	This study
RES167Δ <i>ncgl</i> (2325–2326)/pXMJ19- <i>ncgl2326</i>	Double-knockout strain RES167Δ <i>ncgl</i> (2325–2326) containing pXMJ19- <i>ncgl2326</i>	This study
Plasmids		
pGEMT-easy	Cloning of PCR products, Amp ^r	Promega (cat. no. A1360)
pK18 <i>mobsacB</i>	Mobilizable cloning vector, for selecting double crossover in <i>C. glutamicum</i> , Km ^r	Schäfer <i>et al.</i> (1994)
pK18 <i>mobsacB</i> Δ <i>ncgl1031</i>	pK18 <i>mobsacB</i> carrying Δ <i>ncgl1031</i> (refer to RES167Δ <i>ncgl1031</i>)	This study
pK18 <i>mobsacB</i> Δ <i>ncgl2302</i>	pK18 <i>mobsacB</i> carrying Δ <i>ncgl2302</i> (refer to RES167Δ <i>ncgl2302</i>)	This study
pK18 <i>mobsacB</i> Δ <i>ncgl2325</i>	pK18 <i>mobsacB</i> carrying Δ <i>ncgl2325</i> (refer to RES167Δ <i>ncgl2325</i>)	This study
pK18 <i>mobsacB</i> Δ <i>ncgl2326</i>	pK18 <i>mobsacB</i> carrying Δ <i>ncgl2326</i> (refer to RES167Δ <i>ncgl2326</i>)	This study
pK18 <i>mobsacB</i> Δ <i>ncgl2953</i>	pK18 <i>mobsacB</i> carrying Δ <i>ncgl2953</i> (refer to RES167Δ <i>ncgl2953</i>)	This study
pXMJ19	<i>E. coli</i> – <i>C. glutamicum</i> shuttle vector (Cam ^r <i>Ptac lac^qpBL1 oriV_{C.g.}</i> pK18 <i>oriV_{E.c.}</i>)	Jakoby <i>et al.</i> (1999)
pXMJ19- <i>ncgl1031</i>	pXMJ19 carrying PCR amplified <i>ncgl1031</i> (for complementation of Δ <i>ncgl1031</i>)	This study
pXMJ19- <i>ncgl2302</i>	pXMJ19 carrying PCR amplified <i>ncgl2302</i> (for complementation of Δ <i>ncgl2302</i>)	This study
pXMJ19- <i>ncgl2325</i>	pXMJ19 carrying PCR amplified <i>ncgl2325</i> (for complementation of Δ <i>ncgl2325</i>)	This study
pXMJ19- <i>ncgl2326</i>	pXMJ19 carrying PCR amplified <i>ncgl2326</i> (for complementation of Δ <i>ncgl2326</i>)	This study

*Abbreviations: *E.c.*, *E. coli*; *C.g.*, *C. glutamicum*; Amp, ampicillin; Cam, chloramphenicol; Km, kanamycin.

BLAST programs at the BLAST server of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). Pairwise and multiple sequence alignments were made with the CLUSTAL W program (Thompson *et al.*, 1994).

Secondary structure modelling and search for conserved domains and residues. Five topology prediction methods, MEMSAT (Jones *et al.*, 1994), HMMTOP (Tusnády & Simon,

2001), TMHMM (Krogh *et al.*, 2001; Kyte–Doolittle (Kyte & Doolittle, 1982; Fariselli *et al.*, 2005) and HTMR (Fariselli *et al.*, 2005) were employed to predict topology of aromatic acid transporters in *C. glutamicum* and the three well characterized MFS transporters. The domains conserved in MFS transporters (Jessen-Marshall *et al.*, 1995; Pao *et al.*, 1998) and charged amino acid residues conserved in the AAHS family (Ditty & Harwood, 1999) were located on the basis of predicted secondary structures.

Table 2. Primers

Underlined sites indicate endonuclease restriction sites added to primers for cloning. The ribosome-binding sites are given in bold.

Name	Sequence (5'–3') (restriction enzyme)	Notes
1031F	GATTCTAGACAACCGGAGCTAAGGG (<i>Xba</i> I)	To construct pK18 <i>mobsacB</i> Δ <i>ncgl1031</i>
1031R	ACAGAATTCCGAAGGCGTAGATGAGTA (<i>Eco</i> RI)	
1031Fc	CTAGTCTAGAAAGGAGGACAACCGTGTCAACGACCACCCCA (<i>Xba</i> I)	To construct pXMJ19- <i>ncgl1031</i>
1031Rc	CGGAATTCACGACCTTTTTCAGTGTT (<i>Eco</i> RI)	
2302F	TCCCCCGGGCAGCACGCAGCCTTGA (<i>Sma</i> I)	To construct pK18 <i>mobsacB</i> Δ <i>ncgl2302</i>
2302R	CGGAATTCGCGATACGGTCTTCCTCA (<i>Eco</i> RI)	
2302Fc	CTAGTCTAGAAAGGAGGACAACCATGGATATCCGCCAAACA (<i>Xba</i> I)	To construct pXMJ19- <i>ncgl2302</i>
2302Rc	CGGAATTCGCGATACGGTCTTCCTCA (<i>Eco</i> RI)	
2325F	TCCCCCGGGTTGGTGACCGCCTTGG (<i>Sma</i> I)	To construct pK18 <i>mobsacB</i> Δ <i>ncgl2325</i>
2325R	ACGCGTCGACGCTAGTGAAATGCCGAAA (<i>Sal</i> I)	
2325Fc	CTAGTCTAGAAAGGAGGACAACCATGGCTTCCCAAATTCT (<i>Xba</i> I)	To construct pXMJ19- <i>ncgl2325</i>
2325Rc	CGGAATTCGCTAGTGAAATGCCGAAA (<i>Eco</i> RI)	
2326F	TCCCCCGGGCATTTCAGTACGATTT (<i>Sma</i> I)	To construct pK18 <i>mobsacB</i> Δ <i>ncgl2326</i>
2326R	ACGCGTCGACATGGCAATTTACGACACC (<i>Sal</i> I)	
2326Fc	CGCGGATCCAAAGGAGGACAACCATGGCAAGCTCAGTATTATT (<i>Bam</i> HI)	To construct pXMJ19- <i>ncgl2326</i>
2326Rc	AAAGAGCTCATGGCAATTTACGACACC (<i>Sac</i> I)	
2953F	GATTCTAGAAAAGGAGGACACATATGACGGACATCAAGGC (<i>Xba</i> I)	To construct pK18 <i>mobsacB</i> Δ <i>ncgl2953</i>
2953R	CGGAATTCGGTGGTTTGTAGGTG (<i>Eco</i> RI)	

RESULTS

Genome data mining and identification of *C. glutamicum* genes involved in aromatic acid transport

According to the publicly available genome sequences of *C. glutamicum*, coding regions for putative membrane proteins represent 19.7–22 % (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003) of the total coding sequences. Using BLAST searches based on sequence similarities as listed in Table 3, six genes (*ncgl1031*, *ncgl2302*, *ncgl2325*, *ncgl2326*, *ncgl2922* and *ncgl2953*) were identified as encoding putative transport proteins for aromatic acids. The physical map and genetic organization are shown in Fig. 1. Three genes, *ncgl2302*, *ncgl2325* and *ncgl2326*, are located in the β -ketoadipate metabolic island, which constitutes almost 1 % of the *C. glutamicum* genome and contains all the genes necessary for catabolism of aromatic compounds via the β -ketoadipate pathway. In contrast to the location of all necessary genes for the protocatechuate branch of this pathway in the β -ketoadipate metabolic island, the putative transporter gene, *ncgl1031*, is located elsewhere in the genome. The *ncgl2922* gene is located in another genetic cluster, and this gene has been previously shown to be necessary for 3-hydroxybenzoate and gentisate assimilation (Shen *et al.*, 2005b). The *ncgl2953* gene is located in one of the two genetic clusters (*ncgl1110–ncgl1113* and *ncgl2950–ncgl2953*) that have recently been determined to be involved in resorcinol and hydroxyquinol degradation (Huang *et al.*, 2006).

The *ncgl1031* (*pcaK*) gene is involved in transport of both 4-hydroxybenzoate and protocatechuate

BLAST search results indicated that NCgl1031 had 31 % and 30 % similarity to 4-hydroxybenzoate and protocatechuate transporters (PcaK) of *Chromobacterium violaceum* ATCC 12472 and *Burkholderia pseudomallei*, respectively. To investigate its function, the *ncgl1031* gene was disrupted in *C. glutamicum*. The resulting mutant, RES167Δ*ncgl1031*, had lost the ability to grow with 4-hydroxybenzoate and protocatechuate (Fig. 2a) but retained the ability to grow with benzoate and other substituted benzoates. The prevention of mutant growth in the absence of the transporter may possibly be related to the high pH to which the cells were cultivated. When this mutant was complemented with pXMJ19-*ncgl1031*, the ability to grow on 4-hydroxybenzoate and protocatechuate was restored (Fig. 2a). This clearly indicated that *ncgl1031* is essential for 4-hydroxybenzoate and protocatechuate assimilation in *C. glutamicum*. Furthermore, assays with resting cells indicated that uptake of 4-hydroxybenzoate was active in wild-type RES167 but not in mutant RES167Δ*ncgl1031*. Within the first 10 min of culture the concentration of 4-hydroxybenzoate was halved with wild-type RES167, whereas no significant change in 4-hydroxybenzoate was observed with RES167Δ*ncgl1031* (Fig. 3a). Thus, it is concluded that NCgl1031 is a transporter for 4-hydroxybenzoate and protocatechuate in *C. glutamicum*.

Table 3. BLAST search results regarding function of genes involved in transport of aromatic acids in *C. glutamicum*

Gene/ accession no.	Position in genome sequence (nt)	Annotated function of protein	Identified function of protein	Calculated mol. mass (Da) (no. of aa)	Protein with homologous sequence (organism; accession no.)	Sequence identity (%)
<i>ncgl1031</i> NP600304	1123531–1124826	4-Hydroxybenzoate transporter	4-Hydroxybenzoate and protocatechuate transporter (PcaK)	46008.25 (431)	PcaK (<i>Chromobacterium violaceum</i> ATCC 12472; NP900663)	121/381 (31 %)
					PcaK (<i>Burkholderia pseudomallei</i> K96243; YP109336)	119/384 (30 %)
<i>ncgl2302</i> NP601586	2527223–2528563	Permease of MFS	Vanillate transporter (VanK)	46673.86 (446)	VanK (<i>P. putida</i> ; AAL27558)	185/411 (45 %)
					VanK (<i>Acinetobacter</i> sp. ADP1; YP045696)	176/411 (42 %)
<i>ncgl2325</i> NP601609	2552606–2553946	Benzoate transporter	Benzoate transporter (BenK)	46290.43 (446)	Benzoate transport protein (<i>Rhodococcus</i> sp. 19070; AAK58907)	219/377 (58 %)
					Benzoate transporter, MFS superfamily (<i>Rhodococcus</i> sp. RHA1; YP702351)	211/378 (55 %)
<i>ncgl2326</i> NP601610	2554027–2555271	Putative benzoate membrane transport protein	Benzoate transporter (BenE)	42896.80 (414)	BenE (<i>Rhodococcus</i> sp. RHA1; YP705462)	205/396 (51 %)
					Benzoate membrane transport protein (<i>Moorella thermoacetica</i> ATCC 39073; ZP00575807)	128/378 (33 %)
<i>ncgl2922</i> NP602219	3227749–3229083	Putative benzoate transporter	Gentisate transporter (GenK)	46803.55 (444)	BenK (<i>P. putida</i> ; AAF63452)	146/401 (36 %)
					Benzoate transport protein (<i>Pseudomonas</i> sp. ND6; NP943150)	151/421 (35 %)
<i>ncgl2953</i> NP602250	3258559–3260085	Permease of MFS	Unknown	53704.60 (508)	Permease of MFS (<i>Brevibacterium linens</i> BL2; ZP00381254)	196/472 (41 %)

The *ncgl2302* (*vanK*) gene is involved in transport of vanillate

The protein encoded by *ncgl2302* had 45 % and 42 % similarity with the vanillate transport protein (VanK) of *P. putida* and *Acinetobacter* sp. ADP1, respectively. Mutant strain RES167 Δ *ncgl2302* had lost the ability to grow with vanillate (Fig. 2b) without effects on growth on benzoate, 3- and 4-hydroxybenzoate, protocatechuate, gentisate, or resorcinol. After complementation with pXMJ19-*ncgl2302*, the ability to grow on vanillate was restored (Fig. 1b). Uptake of vanillate by resting cells of wild-type strain RES167 was active, but was inactive in mutant RES167 Δ *ncgl2302* (Fig. 3b). Combining these results, we concluded that *ncgl2302* encoded vanillate transporter in *C. glutamicum*.

Both *ncgl2325* (*benK*) and *ncgl2326* (*benE*) are involved in transport of benzoate

NCgl2325 and NCgl2326 are active in uptake of benzoate. Both *ncgl2325* and *ncgl2326* are putative benzoate transporters. NCgl2325 shows 58 % and 55 % similarity with putative benzoate transporters identified from the genomic annotations of *Rhodococcus* sp. strain 19070 (AAK58907) and *Rhodococcus* sp. RHA1

(YP702351), respectively. Homologues of NCgl2326 were identified from genomes of *Rhodococcus* sp. RHA1 (YP705462) and *Moorella thermoacetica* ATCC 39073 (ZP00575807), but the function of these homologues had not been experimentally characterized. We found that individual disruption of *ncgl2325* and *ncgl2326* did not result in any phenotypic variation from wild-type in assimilation of aromatic compounds, including benzoate. However, the double-knockout mutant RES167 Δ *ncgl*(2325–2326) lost the ability to grow on benzoate (Fig. 1c, d) but there was no effect on growth on 3- or 4-hydroxybenzoate, protocatechuate, gentisate, resorcinol or vanillate. These results indicated that both *ncgl2325* and *ncgl2326* are involved in benzoate assimilation and in the absence of either, the other is functional. The involvement of *ncgl2325* and *ncgl2326* in benzoate assimilation was further confirmed by complementation experiments. When either one of these genes was introduced into the double-knockout mutant using pXMJ19 the ability of the mutants to grow on benzoate was restored (Fig. 1c, d). Results for uptake of benzoate from resting cell assays indicated that there was no uptake by the double knockout mutant RES167 Δ *ncgl*(2325–2326); the single knockout mutants RES167 Δ *ncgl2325* and RES167 Δ *ncgl2326* retained the ability to take up benzoate,

Fig. 1. Physical map and genetic organization of *C. glutamicum* genes involved in aromatic acid transport in the β -ketoadipate pathway, gentisate pathway and hydroxyquinol pathway (for details see text). Other genes relevant to these pathways are also shown. Double triangles represent portion of genes enzymically deleted. Restriction sites are indicated as follows: B, *Bam*HI; E, *Eco*RI; M, *Mfe*I; P1, *Pvu*I; P2, *Pvu*II; Sa, *Sa*I; Sd, *Sdu*I; Sm, *Sma*I; X, *Xba*I.

and the uptake rates for benzoate in RES167 Δ *ncg12326* were higher than in RES167 Δ *ncg12325* (Fig. 3c). It was also observed that the growth rate of RES167 Δ *ncg12326* (0.123 h^{-1}) was higher than that of RES167 Δ *ncg12325* (0.100 h^{-1}).

NCgl2326 (BenE) represents a novel type of benzoate transporter. Sequence alignments and secondary structure prediction revealed that NCgl2325 (BenK), NCgl1031 (PcaK) and NCgl2032 (VanK) all had the typical 12 transmembrane α -helices, and had conserved domains at loops 2–3 and 8–9 and conserved charged amino acid residues, characteristic of MFS transporters (Jessen-Marshall *et al.*, 1995; Pao *et al.*, 1998; Ditty & Harwood, 1999). Strikingly, the transporter encoded by *ncg12326* was predicted to have 11 transmembrane α -helices, a structure entirely different from transporters of the MFS, and did not contain any of the above-mentioned conserved features. Since both BenE and BenK are benzoate transporters, alignment of the currently known BenE and BenK sequences (including putative and functionally

identified) was performed and results indicated that they formed two clearly separate clusters (Fig. 4). NCgl2326 (BenE) is the first functionally characterized benzoate transporter of its type and was predicted to have 11 helices.

The *ncg12953* gene is not involved in resorcinol and hydroxyquinol transport and its function is still unknown

As *ncg12953* was located in one of the two genetic clusters encoding the hydroxyquinol pathway in *C. glutamicum* (Shen *et al.*, 2005a), it was assumed to be involved in transport of related aromatic compounds. Moreover, it shows 41% similarity with the permease of the MFS in *Brevibacterium linens* BL2. However, disruption of this gene did not result in any observable phenotypic change regarding assimilation of aromatic compounds as tested in this study. It is thus concluded that *ncg12953* is not involved in aromatic acid transport in the three degradation pathways mentioned earlier.

Fig. 2. Phenotypic characterization of various genetically disrupted and complemented strains of *C. glutamicum*. Data shown here are mean values from triplicates for OD₆₀₀ values. Growth curves for (a) RES167Δ*ncg1031* on 4-hydroxybenzoate (similar results were obtained with protocatechuate), (b) RES167Δ*ncg12302* on vanillate, (c) RES167Δ*ncg1(2325-2326)* on benzoate, complemented with *ncg12325*, (d) RES167Δ*ncg1(2325-2326)* on benzoate, complemented with *ncg12326*. ■, Parent strain RES167; ●, mutant strain; △, complemented strain.

DISCUSSION

Previous studies have shown that *C. glutamicum* grows on various aromatic compounds and possesses multiple pathways for aromatic compound degradation (Shen *et al.*, 2005a, b), yet how these aromatic compounds enter cells had not been determined. In this study, out of 660 predicted membrane-protein-encoding genes in *C. glutamicum* (Kalinowski *et al.*, 2003), five genes have been confirmed

Fig. 3. Resting cell assays for uptake of: (a) 4-hydroxybenzoate (○, parent strain RES167; ■, RES167Δ*ncg1031*); (b) vanillate (○, parent strain RES167; ■, RES167Δ*ncg12302*); (c) benzoate [○, parent strain RES167; △, RES167Δ*ncg12325*; ▲, RES167Δ*ncg12326*; ■, RES167Δ*ncg1(2325-2326)*]. Error bars represent standard deviation from the mean of three samples.

to code for aromatic acid transporters. As demonstrated in this study, vanillate is transported via VanK, and VanK in *C. glutamicum* (encoded by *ncg12302*) was found to be exclusively responsible for vanillate transport, and was devoid of any involvement in transport of protocatechuate or 4-hydroxybenzoate. This is different from *Acinetobacter* sp. strain ADP1, in which VanK was reported to be also involved in 4-hydroxybenzoate and protocatechuate transport (D'Argenio *et al.*, 1999). While working on nitrogen metabolism, Merkens *et al.* (2005) reported that *vanK* mutation in *C. glutamicum* led to decreased growth on protocatechuate and thus proposed the occurrence of an additional protocatechuate transporter. We indeed found that NCgl1301 (PcaK) was responsible for protocatechuate and 4-hydroxybenzoate transport in *C. glutamicum*. However, our results were significantly different from those of Merkens *et al.* (2005). A similar transporter was

Fig. 4. Phylogenetic relationship of the BenE family of benzoate transporters. Homologues were selected from results of a BLASTP search of GenBank. The phylogenetic tree was constructed using the neighbour joining method (Saitou & Nei, 1987) and multiple sequence alignment was done using CLUSTAL W (Thompson *et al.*, 1994). The scale bar indicates percentage divergence (distance). The accession numbers (from top to bottom) represent the following species: *Shewanella baltica* OS195, *Xanthomonas campestris* pv. *campestris* str. ATCC 33913, *Burkholderia mallei* ATCC 23344, *P. putida* KT2440, *P. fluorescens* PfO-1, *Acinetobacter* sp. ADP1, *Bordetella parapertussis* 12822, *Moorella thermoacetica* ATCC 39073, *C. glutamicum* ATCC 13032, *Rhodococcus* sp. RHA1. BenK of *C. glutamicum* ATCC 13032 was used as the outgroup.

identified in *P. putida* (Nichols & Harwood, 1997); expression of *pcaK* was repressed by benzoate and preferential degradation of benzoate over 4-hydroxybenzoate was observed (Nichols & Harwood, 1995).

Two modes have been reported for benzoate transport in bacteria. The first is by simple or facilitated diffusion (Harwood & Gibson, 1986), and the second by specific membrane transporters (Miguez *et al.*, 1995; Collier *et al.*, 1997). In *C. glutamicum* this transport is through membrane transporters. BenK and BenE, have been identified in *C. glutamicum*. It is of most interest that the predicted 11 helix transmembrane protein, NCgl2526 (BenE), is active in benzoate transport. Homologues of BenE were also identified in *Agrobacterium tumefaciens*, *Acinetobacter* sp. strain ADP1, *Rhodococcus* sp. RHA1 and *P. putida*. In all of these cases, sequence similarities between BenK and BenE are very low, i.e. 15.0% identity for *C. glutamicum*, 15.5% for *Ag. tumefaciens* strain C58 (accession numbers NP355471 and NP532938), 15.0% for *Acinetobacter* sp. strain ADP1 (YP046120 and YP046126), 14.4% for *Rhodococcus* sp. RHA1 (YP702351 and YP705462) and 14.0% for *P. putida* KT2440 (NP745309 and NP745311).

Further work is needed on characterization of the structure–function relationship of this novel type of benzoate transporter (BenE) and on comparison of benzoate uptake kinetics between BenK and BenE.

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