

Conserved residues in the aromatic acid/H⁺ symporter family are important for benzoate uptake by NCgl2325 in *Corynebacterium glutamicum*

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ABSTRACT

Corynebacterium glutamicum is a model organism for genetic and physiological studies in Gram-positive bacteria. NCgl2325 in *C. glutamicum*, a transporter belonging to the aromatic acid/H⁺ symporter family, has previously been reported to be involved in benzoate assimilation. Here, we showed that this transporter, fused with GFP, was associated with the cell membrane in *Escherichia coli* and *C. glutamicum*. Uptake assays with [¹⁴C]-labeled benzoate demonstrated that NCgl2325 transported benzoate into the cells at a V_{\max} of 0.19 ± 0.01 nmol/min/mg of dry weight, and the K_m value was determined to be 1.11 ± 0.24 μ M. Among the competing substrates tested, hydroxyl-substituted benzoates resulted in significant inhibition (>50%) of benzoate uptake. Site-directed mutagenesis of conserved residues in the hydrophilic cytoplasmic loops (Gly-80, Asp-84 and Asp-312) and the hydrophobic transmembrane regions (Asp-35, Arg-119, Glu-139 and Arg-386) resulted in loss of benzoate transport activity. This is the first study to investigate the molecular basis of benzoate transport.

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1. Introduction

Various microorganisms are able to utilize a series of aromatic acids as their sole source of carbon and energy. The uptake of aromatic compounds into the cell is the first step in their catabolism in bacteria. Transport of these substrates across the cytoplasmic membrane has been found to be facilitated by members of the aromatic acid/H⁺ symporter (AAHS) family within the major facilitator superfamily (MFS). Members of the MFS generally exhibit 12 transmembrane (TM) segments, and conserved motifs are observed in both the cytoplasmic loops between the second and third membrane-spanning segments (2–3 loop) and between the eighth and ninth membrane-spanning segments (8–9 loop) (Pao et al., 1998). The functionally identified members of the AAHS family include PcaK (4-hydroxybenzoate and protocatechuate transporter) from *Pseudomonas putida* PRS2000 (Nichols and Harwood, 1997), BenK (benzoate transporter) from *Acinetobacter* sp. strain ADP1 (Collier et al., 1997)

and TfdK (2,4-dichlorophenoxyacetate transporter) from *Ralstonia eutropha* JMP134 (Leveau et al., 1998). The function of these transporters was confirmed by uptake assays using the relevant [¹⁴C]-labeled substrates, and the mechanism of 4-hydroxybenzoate transport by PcaK was further investigated with important residues in the conserved motifs being confirmed.

Corynebacterium glutamicum, a Gram-positive bacterium with a high GC content, has been extensively employed in the production of amino acids (Shimizu and Hirasawa, 2007; Wittmann and Becker, 2007). Since its genome was sequenced (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003), *C. glutamicum* has become a model organism for genetic and physiological studies in Gram-positive bacteria (Shen et al., 2005b; Feng et al., 2006). Recently, this strain was also found to be capable of growing on a variety of aromatic acids including benzoate (Shen et al., 2005a), and the product encoded by open reading frame *ncgl2325* was demonstrated to be involved in benzoate transport by gene knockout and complementation experiments (Chaudhry et al., 2007). To date, little has been reported regarding the molecular mechanism of benzoate transport. Here, we further confirmed the role of NCgl2325 in benzoate transport in *C. glutamicum* using a [¹⁴C]-labeled substrate and identified conserved residues important in benzoate uptake by this transporter. The inhibition of benzoate uptake by benzoate derivatives was also investigated.

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

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2. Materials and methods

2.1. Strains and plasmids

The bacterial strains, plasmids and primers used in this study are listed in Table 1.

2.2. Chemicals, media and growth conditions

Escherichia coli strains were grown in lysogeny broth (LB) at 37 °C. *C. glutamicum* RES167 strains were also grown in LB, but at 30 °C. When necessary, antibiotics were added at the following concentrations: kanamycin, 50 µg/ml for *E. coli*; chloramphenicol, 20 µg/ml for *E. coli* and 10 µg/ml for *C. glutamicum*. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium for induction when appropriate. As a tracer, [ring-UL-¹⁴C] benzoate acid (70 mCi/mmol) was used (American Radiolabeled Chemicals, Inc. ARC, St. Louis, MO, USA).

2.3. DNA manipulation

Plasmid DNA extraction and DNA gel extraction kits (OMEGA BIO-TEK Inc., Doraville, GA, USA), restriction endonucleases and *Pyrobest*TM DNA polymerase (TaKaRa Biotechnology Co. Ltd., Dalian, China) and T4 DNA ligase (New England Biolabs, Inc., Beverly, MA, USA) were routinely used. All procedures were performed in accordance with the manufacturer's instructions. *E. coli* strains were transformed according to standard procedures (Sambrook et al., 1989). Variants of the *E. coli*–*C. glutamicum* shuttle expression vector pXMJ19 (Jakoby et al., 1999) were electroporated into *C. glutamicum* as previously described (Tauch et al., 2002). Nucleotide sequences were determined by Invitrogen Biotechnology Co. Ltd. (Shanghai, China). To ensure that PCR products were compatible with a variety of different cloning vectors, several pairs of primers (Table 2) were designed to amplify fragments of the *ncgl2325* gene encoding the benzoate transporter. The PCR-amplified fragments were digested with restriction endonucleases before being inserted into similarly digested vectors. All inserts were sequenced prior to characterization studies to ensure that no mutations had been incorporated during the PCR.

2.4. Site-directed mutagenesis of NCgl2325

The desired mutants of NCgl2325 were obtained by overlap-extension PCR (Pogulis et al., 1996). The outer amplification primers were *ncgl2325* Fc-*Xba*I and *ncgl2325* Rc-*Eco*RI. The inner primers were designed to incorporate one codon change. The overlap-extension PCR products were digested with endonucleases before ligating into the similarly digested vector pXMJ19. The sequences of all of the mutated *ncgl2325* gene constructs were verified by DNA sequencing to ensure that only the desired mutations have occurred.

2.5. Uptake assays

Benzoate transport was measured in *C. glutamicum* RES167 Δ *ncgl* (2325–2326) expressing wild-type and mutant forms of NCgl2325. Cells were grown in LB, induced with IPTG and harvested by centrifugation. After being washed, they were resuspended in Tris–HCl buffer (50 mM, pH 8.0) to an OD₆₀₀ of 1–3 and were kept on ice. Before the uptake assay, cells were incubated for 3 min at 30 °C with 10 mM glucose for energization (Youn et al., 2008). The assays were initiated by the addition of 450 µl of the cell suspension to 150 µl of Tris–HCl buffer containing 142 µM [¹⁴C] benzoate. Samples (100 µl) were taken at timed intervals and filtered through Nucleopore polycarbonate membranes (0.22 µm pore size; Xinya, Shanghai, China). The filters were immediately washed with 2 ml of 0.1 M LiCl. The amount of substrate accumulated in the cells on the filters was determined in a scintillation counter (1450 MicroBeta TriLux, PerkinElmer Life Sciences, Boston, MA, USA). The biomass concentration was calculated from the OD₆₀₀ values and the correlation factor was taken as 0.25 g cells (dry weight, DW)/litre for an OD₆₀₀ of 1 (Youn et al., 2009). Apparent *K*_m and *V*_{max} values were obtained by measuring the uptake of [¹⁴C] benzoate in triplicate at 1 min with nine substrate concentrations ranging from 0.14 to 3.5 µM. Data were fitted with the Michaelis–Menten equation using the least-squares method (Cleland, 1967).

2.6. Localization of green fluorescence proteins by confocal microscopy

E. coli BL21 (DE3) [pGFPe], *E. coli* BL21 (DE3) [pGFPe-*ncgl2325*], *C. glutamicum* RES167 [pXMJ19-*gfp*] and *C. glutamicum* RES167

Table 1
Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics ^a	Reference or source
Strains		
<i>Escherichia coli</i> DH5α	<i>supE44 DlacY169</i> (f80 <i>lacZ</i> DM15) <i>hsdR17 recA1 endA1 gyrA96 thi–1 relA1</i>	Novagen, USA
<i>Escherichia coli</i> BL21 (DE3)	<i>hsdS gal</i> (λclts857 <i>ind–I Sam7 nin–5 lacUV5–T7</i> gene 1)	Novagen, USA
<i>Corynebacterium glutamicum</i> RES167	Restriction-deficient mutant of <i>Corynebacterium glutamicum</i> ATCC 13032, Δ(<i>cgIIIM–cgIIIR</i>)	Tauch et al. (2002)
<i>Corynebacterium glutamicum</i> RES167 Δ <i>ncgl</i> (2325–2326)	Fragments of DNA encoding residues 239–356 of NCgl2325 and 215–407 of NCgl2326 deleted	Chaudhry et al. (2007)
Plasmids		
pGFPe	Km ^r , ColE1 replicon, derivative of pWaldo	Rapp et al. (2004)
pGFPe- <i>ncgl2325</i>	1338 bp PCR-amplified fragment containing <i>ncgl2325</i> inserted into pGFPe at <i>Xho</i> I and <i>Eco</i> RI restriction sites	This study
pXMJ19	<i>E. coli</i> – <i>C. glutamicum</i> shuttle vector (Cam ^r <i>Ptac lac</i> ^d pBL1 <i>oriV</i> _{C.g.} pK18 <i>oriV</i> _{E.c.})	Jakoby et al. (1999)
pXMJ19- <i>ncgl2325</i>	1341 bp PCR-amplified fragment containing <i>ncgl2325</i> inserted into pXMJ19 at <i>Xba</i> I and <i>Eco</i> RI restriction sites	This study
pXMJ19- <i>ncgl2325–gfp</i>	2145 bp PCR-amplified fragment containing <i>ncgl2325</i> and <i>gfp</i> inserted into pXMJ19 at <i>Xba</i> I and <i>Sma</i> I restriction sites	This study
pXMJ19- <i>gfp</i>	789 bp PCR-amplified fragment containing <i>gfp</i> inserted into pXMJ19 at <i>Xba</i> I and <i>Sma</i> I restriction sites	This study

^a Km^r, kanamycin resistant; Cam^r, chloramphenicol resistant; C.g., *Corynebacterium glutamicum*; E.c., *Escherichia coli*.

Table 2
Primers used in this study.

Name	Sequence (5'–3') (restriction enzyme)	Notes
<i>ncgl2325</i> F for pGFPe- <i>XhoI</i>	CGCCTCGAGATGGCTTCCCAAAATTTCT (<i>XhoI</i>)	Amplifying gene <i>ncgl2325</i> from <i>Corynebacterium glutamicum</i> RES167 genome
<i>ncgl2325</i> R for pGFPe- <i>EcoRI</i>	TATGAATTCGACACGTGCAAGTGGTTCCTG (<i>EcoRI</i>)	
<i>ncgl2325</i> Fc- <i>XbaI</i>	CTAGTCTAGAAAAGGAGGACAACCATGGCTTCCC AAATTTCT (<i>XbaI</i>)	Amplifying gene <i>ncgl2325</i> from <i>Corynebacterium glutamicum</i> RES167 genome
<i>ncgl2325</i> Rc- <i>EcoRI</i>	CGGAATTCGCTAGTCAAATGCCGA AA (<i>EcoRI</i>)	
<i>ncgl2325-gfp</i> for pXMJ19 F <i>XbaI</i>	CTAGTCTAGAAAAGGAGGACAACCATGGCTTCCC AAATTTCT (<i>XbaI</i>)	Amplifying gene <i>ncgl2325-gfp</i> from pGFPe- <i>ncgl2325</i>
<i>ncgl2325-gfp</i> for pXMJ19 R <i>SmaI</i>	ATACCCGGGTCAGTGGTGGTGGTGGTGGTG (<i>SmaI</i>)	
<i>gfp</i> for pXMJ19 F <i>XbaI</i>	TCCTCTAGAAAAGGAGGACAACCATGGTAAACCTGTACTTCCAGGGT (<i>XbaI</i>)	Amplifying gene <i>gfp</i> from pGFPe- <i>ncgl2325</i>
<i>gfp</i> for pXMJ19 R <i>SmaI</i>	ATACCCGGGTCAGTGGTGGTGGTGGTGGTG (<i>SmaI</i>)	

Restriction sites in the sequences of the primers are underlined. Ribosome binding sites are boldfaced.

[pXMJ19-*ncgl2325/gfp*] cells were incubated in LB with the appropriate antibiotics. When the OD₆₀₀ of the cultures reached 0.5, 1 and 0.6 mM of IPTG was added to the *E. coli* and *C. glutamicum* cultures respectively. The former were incubated for another 4 h and the latter were incubated overnight. The cells were harvested, washed and maintained in 10 mM phosphate buffer (pH 7.4), which was mixed with agarose (0.3%) to prevent cells moving around, for imaging under a confocal microscope with a 488 nm excitation filter and a 520 nm emission filter (Xu et al., 2006). The imaging experiments were performed using a confocal microscope (Leica TCS SP2, Leica Microsystems, Mannheim, Germany) equipped with a cooled CCD camera.

3. Results

3.1. NCgl2325 is an inner membrane protein

NCgl2325 from *C. glutamicum* shares 28.9% identity with BenK from *Acinetobacter* sp. strain ADP1. Its membrane topology, as predicted using TMHMM (Krogh et al., 2001), indicated that NCgl2325 contains 12 α -helix TM spanners (Fig. 1), that is a typical feature of the MFS members. To confirm its cellular location, NCgl2325 was tagged with GFP and then expressed both in *E. coli* and *C. glutamicum*. An amino acid linker between these two domains ensured that they folded correctly and functioned individually. Under confocal microscopy, GFP in the negative controls of strains *E. coli* BL21 (DE3) [pGFPe] and *C. glutamicum* RES167

[pXMJ19-*gfp*] without NCgl2325, was distributed in the cytoplasm (A-2 and B-2 in Fig. 2). By contrast, the fusion protein in strains *E. coli* BL21 (DE3) [pGFPe-*ncgl2325*] and *C. glutamicum* RES167 [pXMJ19-*ncgl2325/gfp*] with NCgl2325, was associated with the membrane of cells (A-1 and B-1 in Fig. 2), consistent with the bioinformatics prediction. These findings indicated that NCgl2325 is an inner membrane binding protein, as GFP fluoresces only when located in the cytoplasm, not in the periplasmic space (Feilmeier et al., 2000; Drew et al., 2002).

3.2. Benzoate transport kinetics of NCgl2325

The kinetics of benzoate transport by NCgl2325 were measured using *C. glutamicum* RES167 Δ *ncgl* (2325–2326) as the expression host, as this strain is deficient in benzoate utilization (Chaudhry et al., 2007). As shown in Fig. 3, the time courses of benzoate transport demonstrated that cells of *C. glutamicum* RES167 Δ *ncgl* (2325–2326) [pXMJ19-*ncgl2325*] expressing wild-type NCgl2325 (indicated as wild-type) could accumulate [¹⁴C]-labeled benzoate, demonstrating that NCgl2325-mediated benzoate transport in *C. glutamicum*. The V_{max} of benzoate transport was 0.19 ± 0.01 nmol/min/mg of dry weight and the K_m was 1.11 ± 0.24 μ M. By contrast, no detectable accumulation of benzoate was observed in cells harboring only the pXMJ19 vector (indicated as vector).

The inhibition of benzoate uptake was also determined using different benzoate derivatives as substrates and their impacts on NCgl2325 benzoate transport varied as shown in Fig. 4. When mono- or di-hydroxylated benzoates (salicylate, 3-hydroxybenzoate, 4-hydroxybenzoate, gentisate and protocatechuate) were present, benzoate transport was significantly inhibited, retaining only 50% or less of the original activity. Mono-nitro-substituted benzoates (2-nitrobenzoate, 3-nitrobenzoate and 4-nitrobenzoate) showed less inhibition of benzoate transport than the hydroxylated benzoates, and 3,5-dinitrobenzoate and catechol (the intermediate of benzoate degradation in this strain (Shen et al., 2005a)) had virtually no impact on its transport activity.

3.3. Identification of critical residues in NCgl2325 for benzoate transport

Three conserved residues of NCgl2325 located in cytoplasmic loops (Gly-80, Asp-84 and Asp-312) and four conserved charged amino acids localized in hydrophobic TM regions (Asp-35, Arg-119, Glu-139 and Arg-386) were selected for site-directed mutagenesis, as highlighted in Fig. 1. The glycine residue was changed to a valine (G80V) in 2–3 loop, and the two aspartic acids Asp-84 and Asp-312 were changed to asparagines (D84N in 2–3 loop and D312N in 8–9 loop). Furthermore, charged residues in the TM segments were replaced by uncharged residues: Asp-35 in the DGXD motif of TM1 was substituted with alanine (D35A), Arg-119 and Glu-139 in the

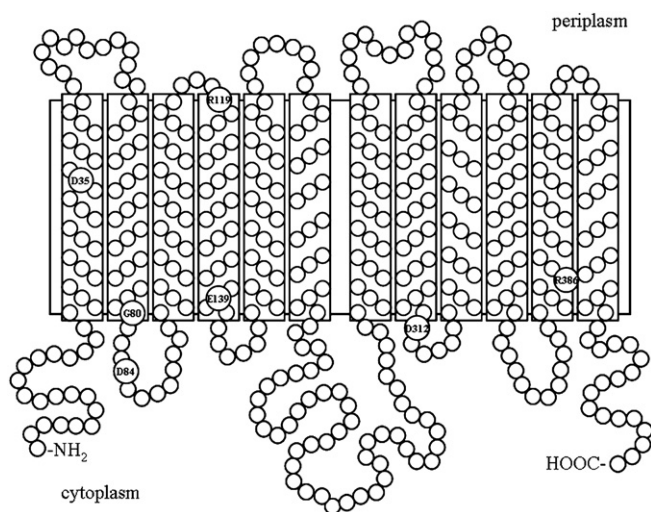


Fig. 1. Transmembrane segments prediction for NCgl2325. Hydrophobic transmembrane segments are enclosed in boxes. Amino acids within the consensus transporter sequences for site-directed mutagenesis are marked.

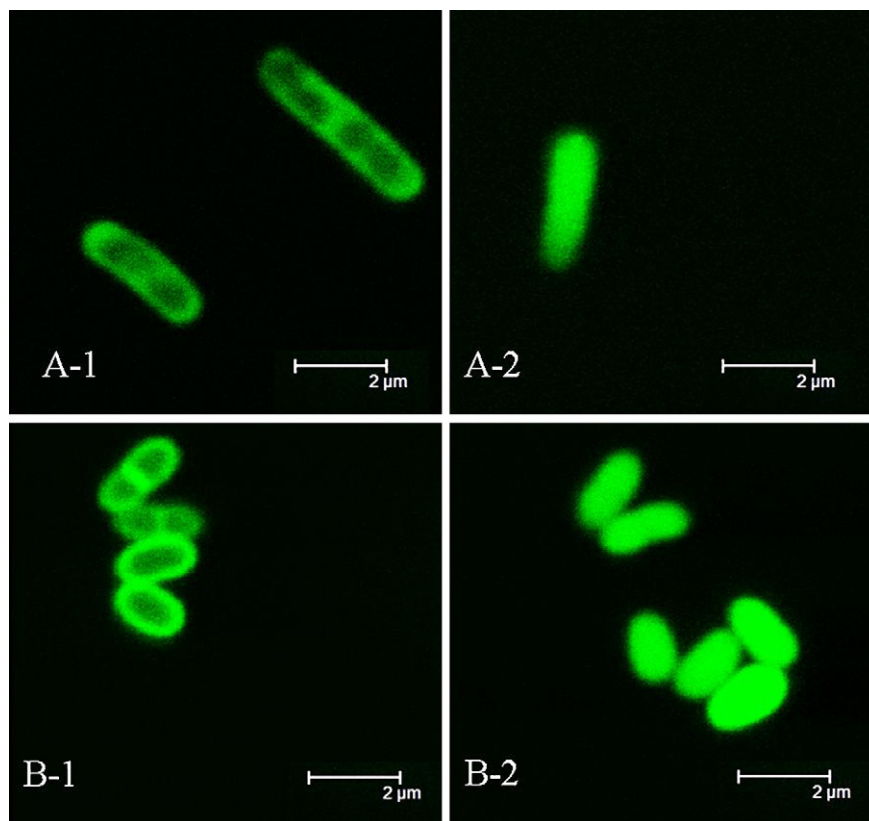


Fig. 2. Localization of NCgl2325-GFP in *E. coli* BL21 and *C. glutamicum*, visualized by confocal microscopy. A-1 and B-1, NCgl2325-GFP expressed in *E. coli* BL21 and *C. glutamicum*, respectively, showing that NCgl2325 is associated with the membrane. A-2 and B-2, *E. coli* and *C. glutamicum* expressing GFP only, as negative controls.

fourth TM helix (TM4) were substituted with alanine residues (R119A and E139A), and Arg-386 in TM11 was also substituted with alanine (R386A).

Each mutant NCgl2325 protein was expressed in *C. glutamicum* RES167 $\Delta ncgI$ (2325–2326) using vector pXMJ19. The uptake assays showed that neither the mutants with altered conserved residues in the cytoplasmic loops nor the mutants with uncharged residues

inserted into the hydrophobic regions could transport [14 C]-labeled benzoate as seen in the wild-type (Fig. 3). These findings suggested that these residues in NCgl2325 are critical for benzoate transport activity.

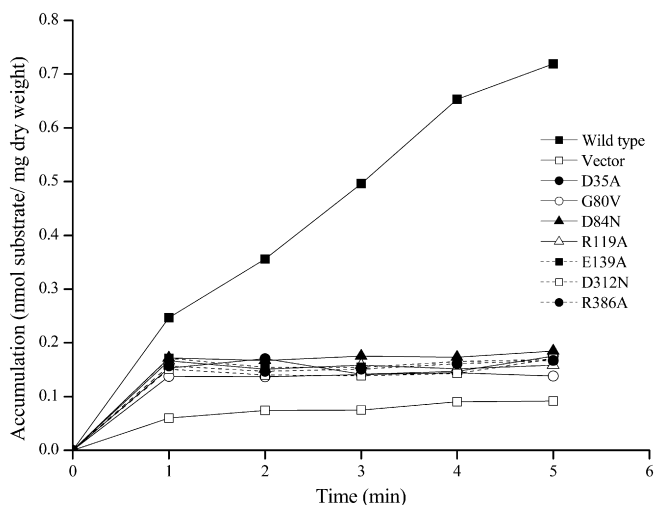


Fig. 3. Accumulation of [14 C]-labeled benzoate by *C. glutamicum* RES167 $\Delta ncgI$ (2325–2326) expressing wild-type and mutant NCgl2325 constructs using vector pXMJ19. Experiments were repeated at least three times and a representative time course is presented.

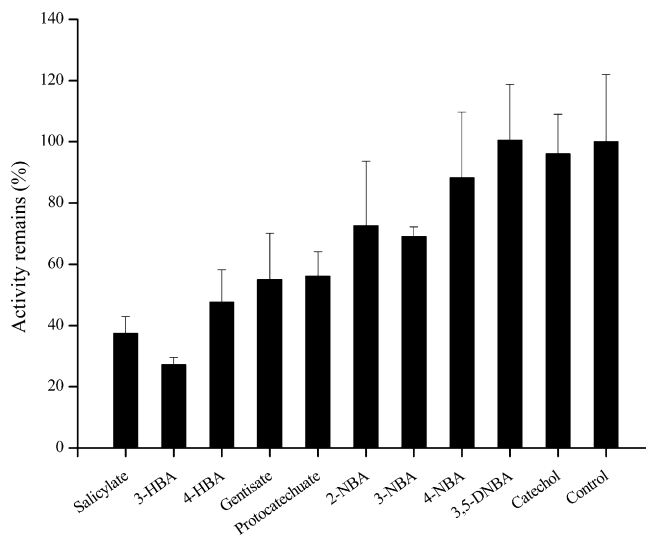


Fig. 4. Inhibition of NCgl2325-mediated [14 C]-labeled benzoate uptake in *C. glutamicum* RES167. The concentrations of benzoate and competing substrates were 5 and 100 μ M, respectively. The accumulation rate of benzoate (without competing substrates) was 0.09 ± 0.02 nmol/min/mg of dry weight, which was defined as 100% activity. Inhibition was determined by comparing the rate of benzoate uptake in the absence and presence of competing substrate. Values are the averages from three experiments \pm standard deviations. (Abbreviations: 3/4-HBA, 3/4-hydroxybenzoate; 2/3/4-NBA, 2/3/4-nitrobenzoate; 3,5-DNBA, 3,5-dinitrobenzoate).

4. Discussion

The presence of 12 membrane-spanning α -helices in the benzoate transporter NCgl2325 from *C. glutamicum* is a characteristic feature of MFS members. These α -helices have been shown to be packed to form the perimeter of a pore through which the substrate crosses the cell membrane in several cases (Guan and Kaback, 2006; Law et al., 2008). NCgl2325 contains a GAVGDLRGR motif and its partial repletion DRIGAK, two characteristic features of MFS members in the 2–3 loop [GXXXD(R/K)XGR(R/K)] and the 8–9 loop respectively. Conserved residues (Gly-80 and Asp-84) in the 2–3 loop of NCgl2325 have been shown to be important for substrate transport activity in the current study, as reported in TetA (tetracycline antiporter of *E. coli*) (Yamaguchi et al., 1992b) and LacY (lactose permease of *E. coli*) (Jessen-Marshall et al., 1995, 1997) in which the corresponding residues were changed. By contrast, Asp-312 in the 8–9 loop of NCgl2325 is only conserved in the AAHS family and was shown to be required for benzoate uptake. This finding was similar to that reported for the Asp-323 residue of PcaK with a confirmed role in 4-HBA transport activity (Ditty and Harwood, 1999). Therefore, this negatively charged residue may be important for aromatic acid transport in the transporters of AAHS family, regardless of whether they are from Gram-positive or negative organisms.

In addition to the conserved residues investigated in the hydrophilic cytoplasmic loops, certain charged residues in the hydrophobic TM regions have previously been shown to be critical for the transport activities of MFS members, despite the fact that they are not conserved. For example, the aspartate residues located in the TM helices of TetA were demonstrated to be required for the exchange of tetracycline and an H^+ (Yamaguchi et al., 1992a; Someya et al., 1995). In LacY, pairs of charged residues (a negatively charged side chain paired with a positively charged side chain) were found to be involved in substrate translocation, H^+ translocation or helix packing (Frillingos et al., 1998). The benzoate transport activity of NCgl2325 was virtually lost in the current study when residues Asp-35 in TM1, Arg-119 in TM4 and Arg-386 in TM11 were replaced by uncharged alanine. The aspartate and arginine residues in these hydrophobic TM regions are likely to be paired for benzoate transport activity. These charged residues are also conserved in PcaK (belonging to the AAHS family) and have been found to be necessary for 4-HBA transport in Gram-negative *Pseudomonas* spp. (Ditty and Harwood, 2002). Notably, a glutamic acid (Glu-126) of LacY localized in the cytoplasm, but next to residues in TM4, was shown to form a charged pair with an arginine (Arg-144) in TM5, which was crucial for substrate binding (Venkatesan and Kaback, 1998). In our study, Glu-139 located in TM4 of NCgl2325 appeared close to the interface of the membrane and cytoplasm and substitution of this negatively charged residue with an uncharged alanine impaired benzoate transport activity. Thus, it is possible that this glutamate residue is also part of a charged pair, although no positively charged residues are present in TM5.

In conclusion, this is the first study to investigate the molecular basis of benzoate transport. Our findings provide a better understanding of the relationship between the structure and function of aromatic acids transporters.

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References

- Chaudhry, M.T., Huang, Y., Shen, X.H., Poetsch, A., Jiang, C.Y., Liu, S.J., 2007. Genome-wide investigation of aromatic acid transporters in *Corynebacterium glutamicum*. *Microbiology* 153, 857–865.
- Cleland, W.W., 1967. The statistical analysis of enzyme kinetic data. *Advances in Enzymology and Related Areas of Molecular Biology* 29, 1–32.
- Collier, L.S., Nichols, N.N., Neidle, E.L., 1997. *benK* encodes a hydrophobic permease-like protein involved in benzoate degradation by *Acinetobacter* sp. strain ADP1. *Journal of Bacteriology* 179, 5943–5946.
- Ditty, J.L., Harwood, C.S., 1999. Conserved cytoplasmic loops are important for both the transport and chemotaxis functions of PcaK, a protein from *Pseudomonas putida* with 12 membrane-spanning regions. *Journal of Bacteriology* 181, 5068–5074.
- Ditty, J.L., Harwood, C.S., 2002. Charged amino acids conserved in the aromatic acid/ H^+ symporter family of permeases are required for 4-hydroxybenzoate transport by PcaK from *Pseudomonas putida*. *Journal of Bacteriology* 184, 1444–1448.
- Drew, D., Sjostrand, D., Nilsson, J., Urbig, T., Chin, C.N., de Gier, J.W., von Heijne, G., 2002. Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA/GFP fusion analysis. *Proceedings of the National Academy of Sciences of the United States of America* 99, 2690–2695.
- Feilmeier, B.J., Iseminger, G., Schroeder, D., Webber, H., Phillips, G.J., 2000. Green fluorescent protein functions as a reporter for protein localization in *Escherichia coli*. *Journal of Bacteriology* 182, 4068–4076.
- Feng, J., Che, Y., Milse, J., Yin, Y.J., Liu, L., Ruckert, C., Shen, X.H., Qi, S.W., Kalinowski, J., Liu, S.J., 2006. The gene *ncgl2918* encodes a novel maleylpyruvate isomerase that needs mycothiol as cofactor and links mycothiol biosynthesis and gentisate assimilation in *Corynebacterium glutamicum*. *The Journal of Biological Chemistry* 281, 10778–10785.
- Frillingos, S., Sahin-Toth, M., Wu, J., Kaback, H.R., 1998. Cys-scanning mutagenesis: a novel approach to structure function relationships in polytopic membrane proteins. *The FASEB Journal* 12, 1281–1299.
- Guan, L., Kaback, H.R., 2006. Lessons from lactose permease. *Annual Review of Biophysics and Biomolecular Structure* 35, 67–91.
- Ikeda, M., Nakagawa, S., 2003. The *Corynebacterium glutamicum* genome: features and impacts on biotechnological processes. *Applied Microbiology and Biotechnology* 62, 99–109.
- Jakoby, M., Nguoto-Nkili, C.E., Burkovski, A., 1999. Construction and application of new *Corynebacterium glutamicum* vectors. *Biotechnology Techniques* 13, 437–441.
- Jessen-Marshall, A.E., Parker, N.J., Brooker, R.J., 1997. Suppressor analysis of mutations in the loop 2–3 motif of lactose permease: evidence that glycine-64 is an important residue for conformational changes. *Journal of Bacteriology* 179, 2616–2622.
- Jessen-Marshall, A.E., Paul, N.J., Brooker, R.J., 1995. The conserved motif, GXXX(D/E)(R/K)XG(X)(R/K)(R/K), in hydrophilic loop 2/3 of the lactose permease. *The Journal of Biological Chemistry* 270, 16251–16257.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., Dusch, N., Eggeling, L., Eikmanns, B.J., Gaigalat, L., Goesmann, A., Hartmann, M., Huthmacher, K., Kramer, R., Linke, B., McHardy, A.C., Meyer, F., Mockel, B., Pfeifferle, W., Puhler, A., Rey, D.A., Ruckert, C., Rupp, O., Sahm, H., Wendisch, V.F., Wiegrabe, I., Tauch, A., 2003. The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *Journal of Biotechnology* 104, 5–25.
- Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E.L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *Journal of Molecular Biology* 305, 567–580.
- Law, C.J., Maloney, P.C., Wang, D.N., 2008. Ins and outs of major facilitator superfamily antiporters. *Annual Review of Microbiology* 62, 289–305.
- Leveau, J.H., Zehnder, A.J., van der Meer, J.R., 1998. The *tfdK* gene product facilitates uptake of 2,4-dichlorophenoxyacetate by *Ralstonia eutropha* JMP134(pJP4). *Journal of Bacteriology* 180, 2237–2243.
- Nichols, N.N., Harwood, C.S., 1997. PcaK, a high-affinity permease for the aromatic compounds 4-hydroxybenzoate and protocatechuate from *Pseudomonas putida*. *Journal of Bacteriology* 179, 5056–5061.
- Pao, S.S., Paulsen, I.T., Saier Jr., M.H., 1998. Major facilitator superfamily. *Microbiology and Molecular Biology Reviews* 62, 1–34.
- Pogulis, R.J., Vallejo, A.N., Pease, L.R., 1996. In vitro recombination and mutagenesis by overlap extension PCR. *Methods in Molecular Biology* 57, 167–176.
- Rapp, M., Drew, D., Daley, D.O., Nilsson, J., Carvalho, T., Melen, K., De Gier, J.W., von Heijne, G., 2004. Experimentally based topology models for *E. coli* inner membrane proteins. *Protein Science* 13, 937–945.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (Eds.), 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Shen, X.H., Huang, Y., Liu, S.J., 2005a. Genomic analysis and identification of catabolic pathways for aromatic compounds in *Corynebacterium glutamicum*. *Microbes and Environments* 20, 160–167.
- Shen, X.H., Jiang, C.Y., Huang, Y., Liu, Z.P., Liu, S.J., 2005b. Functional identification of novel genes involved in the glutathione-independent gentisate pathway in *Corynebacterium glutamicum*. *Applied and Environmental Microbiology* 71, 3442–3452.
- Shimizu, H., Hirasawa, T., 2007. Production of glutamate and glutamate related amino acids: molecular mechanism analysis and metabolic engineering. In: Wendisch, V.F. (Ed.), *Amino acid biosynthesis: Pathways, regulation, and metabolic engineering*. Springer, Heidelberg, Germany, pp. 1–38.

- Someya, Y., Niwa, A., Sawai, T., Yamaguchi, A., 1995. Site-specificity of the second-site suppressor mutation of the Asp-285-Asn mutant of metal-tetracycline/H⁺ antiporter of *Escherichia coli* and the effects of amino acid substitutions at the first and second sites. *Biochemistry* 34, 7–12.
- Tauch, A., Kirchner, O., Löffler, B., Gotker, S., Pühler, A., Kalinowski, J., 2002. Efficient transformation of *Corynebacterium glutamicum* with a mini-replicon derived from the *Corynebacterium glutamicum* plasmid pGA1. *Current Microbiology* 45, 362–367.
- Venkatesan, P., Kaback, H.R., 1998. The substrate-binding site in the lactose permease of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 95, 9802–9807.
- Wittmann, C., Becker, J., 2007. The L-lysine story: from metabolic pathways to industrial production. In: Wendisch, V.F. (Ed.), *Amino acid biosynthesis: Pathways, regulation, and metabolic engineering*. Springer, Heidelberg, Germany, pp. 39–70.
- Xu, Y., Yan, D.Z., Zhou, N.Y., 2006. Heterologous expression and localization of gentisate transporter Ncg12922 from *Corynebacterium glutamicum* ATCC 13032. *Biochemical and Biophysical Research Communications* 346, 555–561.
- Yamaguchi, A., Akasaka, T., Ono, N., Someya, Y., Nakatani, M., Sawai, T., 1992a. Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by transposon Tn10. Roles of the aspartyl residues located in the putative transmembrane helices. *The Journal of Biological Chemistry* 267, 7490–7498.
- Yamaguchi, A., Someya, Y., Sawai, T., 1992b. Metal-tetracycline/H⁺ antiporter of *Escherichia coli* encoded by transposon Tn10. The role of a conserved sequence motif, GXXXXRXGRR, in a putative cytoplasmic loop between helices 2 and 3. *The Journal of Biological Chemistry* 267, 19155–19162.
- Youn, J.W., Jolkver, E., Kramer, R., Marin, K., Wendisch, V.F., 2008. Identification and characterization of the dicarboxylate uptake system Dcct in *Corynebacterium glutamicum*. *Journal of Bacteriology* 190, 6458–6466.
- Youn, J.W., Jolkver, E., Kramer, R., Marin, K., Wendisch, V.F., 2009. Characterization of the dicarboxylate transporter DctA in *Corynebacterium glutamicum*. *Journal of Bacteriology* 191, 5480–5488.