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The *ncgl1108* (*PhePCg*) gene encodes a new L-Phe transporter in *Corynebacterium glutamicum*

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Abstract *Corynebacterium glutamicum* played a central role in the establishment of fermentative production of amino acids, and it is a model for genetic and physiological studies. The general aromatic amino acid transporter, $AroP_{Cg}$, was the sole functionally identified aromatic amino acid transporter from *C. glutamicum*. In this study, the *ncgl1108* (named as $pheP_{Cg}$), which is located upstream of the genetic cluster (*ncgl1110* ~ *ncgl1113*) for resorcinol catabolism, was identified as a new L-Phe specific transporter from *C. glutamicum* RES167. The disruption of $pheP_{Cg}$ resulted in RES167Δ*ncgl1108*, and this mutant showed decreased growth on L-Phe (as nitrogen source) but not on L-Tyr or L-Trp. Uptake assays with unlabeled and ^{14}C-labeled L-Phe and L-Tyr indicated that the mutants RES167Δ*ncgl1108* showed significant reduction in L-Phe uptake than RES167. Expression of $pheP_{Cg}$ in RES167Δ*ncgl1108*/pGXKZ1 or RES167Δ(*ncgl1108-aroP*$_{Cg}$)/pGXKZ1 restored their ability to uptake for L-Phe and growth on L-Phe. The uptake of L-Phe was not inhibited by nine amino acids but by L-Tyr. The K_m and V_{max} values of RES167Δ(*ncgl1108-aroP*$_{Cg}$)/pGXKZ1 for L-Phe were determined to be 10.4±1.5 μM and 1.2±0.1 nmol min^{-1} (mg DW)$^{-1}$, respectively, which are different from K_m and V_{max} values of RES167Δ(*ncgl1108-aroP*$_{Cg}$) for L-Phe [4.0±0.4 μM and 0.6±0.1 nmol min^{-1} (mg DW)$^{-1}$]. In conclusion, this $PheP_{Cg}$ is a new L-Phe transporter in *C. glutamicum*.

Keywords Aromatic amino acid transporter · *Corynebacterium glutamicum* · $PheP_{Cg}$ · $AroP_{Cg}$ · L-Phenylalanine

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Introduction

Since its isolation, *Corynebacterium glutamicum* has been playing a central role in the developments of new knowledge and technology for various amino acid productions (Burkovski 2008; Eggeling and Bott 2005; Jetten et al. 1994; Kinoshita et al. 1957). Stimulated by the accessibility of the *C. glutamicum* genome (Ikeda and Nakagawa 2003; Kalinowski et al. 2003), this bacterium has also been used as a model for Gram-positive actinobacteria to understand microbial metabolism of aromatic compounds in our lab. A novel mycothiol-dependent gentisate catabolic pathway (Feng et al. 2006) and a link between aromatic degradation and gluconeogenesis for cell growth (Qi et al. 2007) were discovered. Regulations of

aromatic metabolism in this strain were also investigated recently, and a novel atypical Lux family regulator was identified (Zhao et al. 2010). Those studies invoked the idea that aromatic compounds, such as derivatives of lignin, are potential substrates for production of amino acids. Recently, Lee et al. (2010) demonstrated that phenol was converted to glutamate and proline by *C. glutamicum*.

The robust ability of *C. glutamicum* to grow on a variety of aromatic compounds (Shen et al. 2004, 2005) relies on its multiple transporters for uptake of aromatic compounds. Genome data mining and experimental results confirmed that *C. glutamicum* had five transporters, i.e., the BenE/BenK, PcaK, VanK, and GenK, which were respectively responsible for the uptake of benzoate, protocatechuate, vaniliate, and gentisate (Chaudhry et al. 2007). A putative transporter (NCgl2953) located at downstream of resorcinol degradative genetic cluster (*ncgl2950–ncgl2952*) was proved to be a *myo*-inositol transporter (IolT2) (Krings et al. 2006), and it was not involved in resorcinol transport. Another putative transporter gene (*ncgl1108*) was located at the upstream of the regulator-encoding gene (*ncgl1110*) for resorcinol degradation (Huang et al. 2006). This invoked our interest to investigate the function of *ncgl1108* in *C. glutamicum*. In this study, gene disruption/complementation and ^{14}C-labeled aromatic amino acid uptake assays were carried out to identify the function of this putative transporter gene. It turned out that the gene *ncgl1108* was involved in the uptake of L-Phe but not in resorcinol uptake or degradation.

Materials and methods

Bacterial strains, growth conditions, and plasmids The bacterial strains and plasmids used in this study are listed in Table 1. All *Escherichia coli* strains were grown in Luria–Bertani (LB) broth aerobically on a rotary shaker (200 rpm) at 37 °C or on LB plates with 1.2% (*w*/*v*) agar. *C. glutamicum* strains were routinely grown at 30 °C on a rotary shaker (200 rpm) in LB broth. To evaluate the growth of *C. glutamicum* strains on resorcinol and various aromatic amino acids, minimal medium (Konopka 1993) was supplemented with 2 mM resorcinol, L-Phe, L-Trp, or 1.5 mM L-Tyr as carbon or nitrogen source. Cell growth was monitored by measuring the turbidity at a wavelength of 600 nm (OD_{600}). Antibiotics were used at the following concentrations: kanamycin, 50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for *C. glutamicum*; ampicillin, 100 μg ml^{-1} for *E. coli*; chloramphenicol, 20 μg ml^{-1} for *E. coli* and 10 μg ml^{-1} for *C. glutamicum*.

DNA extraction and manipulation The total genomic DNA of *C. glutamicum* was isolated according to Tauch et al. (1995). DNA restriction enzyme digestion, plasmid isolation, and agarose gel electrophoresis were carried out as described previously (Sambrook et al. 1989). Plasmids were transformed into *E. coli* and *C. glutamicum* by electroporation (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 (Takara, Japan). The PCR products were purified by using agarose gel DNA fragment recovery kit (Sangon, China). Cloning of PCR fragments was performed with the pMD19-T simple cloning vector system (Takara, Japan). Five plasmids for genetic disruption (pGXKZ4 and pGXKZ5), complementation (pGXKZ1), and gene expression (pGXKZ2 and pGXKZ3) in *E. coli* and *C. glutamicum* were constructed with pK18*mobsacB* or pXMJ19 (Table 1). The primers used for amplification of the intact or disrupted target gene fragments are listed in Table 1. For gene expression and genetic complementation, the pGXKZ1 was constructed by the insertion of the PCR-amplified intact gene, *ncgl1108*, into pXMJ19. The pGXKZ2 was constructed by insertion of the PCR-amplified *gfp* from pAcGFP into pXMJ19 and was used as a reference for cellular localization of NCgl1108. The pGXKZ3 was constructed by consecutively cloning of *ncgl1108* (stop codon was deleted) and the *Sal*I/*Eco*RI *gfp* fragment from pAcGFP into pXMJ19. In vitro disruption of *ncgl1108* or $aroP_{Cg}$ was performed by removal of its partial region through restriction enzyme digestion. The full lengths of the intact *ncgl1108* and $aroP_{Cg}$ were 1,407 and 1,392 bp, respectively. pGXKZ4 was constructed by cloning the disrupted *ncgl1108* (the fragment from 255 to 971 bp was removed with *Sty*I digestion) into pK18*mobsacB*. pGXKZ5 was constructed by cloning the disrupted $aroP_{Cg}$ (the fragment from 448 to 1057 bp was removed with *Sca*I digestion) into pK18*mobsacB*.

Genetic disruption and complementation in C. glutamicum The pK18*mobsacB* derivatives were transformed into *C. glutamicum* RES167 by electroporation (Tauch et al. 2002). 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 strains were designated *C. glutamicum* RES167Δ*ncgl1108*, RES167Δ$aroP_{Cg}$, and RES167Δ*(ncgl1108-*$aroP_{Cg}$*)* (Table 1). The deletion of the target genes in pK18*mobsacB* derivatives and in *C. glutamicum* mutants was verified by PCR amplification and DNA sequencing. The gene expression in *C. glutamicum* was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to culture media.

Assays for aromatic amino acid transport Uptake assays with unlabeled L-Phe, L-Tyr, and L-Trp. *C. glutamicum*

Table 1 Bacterial strains, plasmids, and primers used in this study

Strain/plasmid/primer	Relevant characteristics/sequences	Notes
E. coli		
DH5α	*supE44 DlacY169 (u80 lacZ DM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
C. glutamicum		
RES167	Restriction-deficient mutant of ATCC13032; Δ*cglIM* Δ*cglIR* Δ*cglIIR*	University of Bielefeld
RES167Δ$aroP_{Cg}$	DNA fragment encoding amino acids 149–353 of $aroP_{Cg}$ deleted	This study
RES167Δ*ncgl1108*	DNA fragment encoding amino acids 85–324 of *ncgl1108* deleted	This study
RES167Δ(*ncgl1108-*$aroP_{Cg}$)	DNA fragments encoding amino acids 85–324 of *ncgl1108* and 149–353 of $aroP_{Cg}$ deleted	This study
Plasmids		
pAcGFP1	GFP expression plasmid; Amp^r	Clontech
pXMJ19	*E. coli-C. glutamicum* shuttle vector; Cam^r P*tac lacI*^{q} pBL1 $oriV_{Cg}$ pK18 $oriV_{Ec}$	Jakoby et al. (1999)
pGXKZ1	pXMJ19 carrying PCR amplified *ncgl1108*; to generate *ncgl1108* expression or complementation for Δ*ncgl1108*	This study
pGXKZ2	pXMJ19 carrying PCR amplified *gfp*; to generate *gfp* intracellular expression	This study
pGXKZ3	pXMJ19 carrying PCR amplified *ncgl1108* and *Sal*I/*Eco*RI *gfp* fragments from pAcGFP1	This study
pK18*mobsacB*	Mobilizable vector, allows for selection of double crossover in *C. glutamicum*	Schafer et al. (1994)
pGXKZ4	pK18*mobsacB* carrying Δ*ncgl1108*; refer to RES167Δ*ncgl1108*	This study
pGXKZ5	pK18*mobsacB* carrying Δ$aroP_{Cg}$; refer to RES167Δ$aroP_{Cg}$	This study
Primers		
1108F	ATACTGCAG**AAAGGAGGA**CAACCATGAATGCCTCCCCTGCC (*Pst*I)	To generate pGXKZ1 and pGXKZ3
1108R	CGTGAATTCAGGCAGCATCTCCTCCAT (*Eco*RI)	
GFPf	ATACTGCAG**AAAGGAGGA**CAACCATGGTGAGCAAGGGC (*Pst*I)	To generate pGXKZ2
GFPr	CGCGAATTCTCACTTGTACAGCTCA (*Eco*RI)	
1108GFPr2	ATTATCTAGAGTCGACATAGGAGGAGGATCGCGTCGCGGATCTAG (*Xba*I, *Sal*I)	To generate pGXKZ3
1108Fk2	TACTAGCATGCACCGGTCTGTGCTAGACCA (*Sph*I)	To generate pGXKZ4
1108Rk2	GCATAGTCGACATTCGGCGATGGCAATTGT (*Sal*I)	
1062Fk	AATGCATGCTGAGTTCCGGTGTGGT (*Sph*I)	To generate pGXKZ5
1062Rk	ATACCCGGGAACCACATAGTCGACCAT (*Sma*I)	

Restriction enzyme sites are underlined. Ribosome binding sites are boldfaced

RES167 cells were grown in LB medium. Cells at exponential phase were harvested and washed with ammonium-free minimal medium CGXII containing 0.1 M glucose and supplemented with 0.2 mg l^{-1} thiamine (Keilhauer et al. 1993). The harvested cells were resuspended (cell density OD_{600} of 8–10) in this CGXII medium containing 1 mM L-Phe, L-Tyr, or L-Trp and were incubated at 30 °C. At the indicated intervals, portions of the reaction mixture were withdrawn, filtered, and analyzed with HPLC (Yang et al. 2003). Uptake assay with ^{14}C-labeled L-Phe, L-Tyr, or L-Trp. *C. glutamicum* RES167 and its mutants were cultivated and harvested according to the above described procedures. Cells were washed twice with 0.1 M Tris phosphate buffer (pH 6.8) and resuspended in the same buffer. The uptake of aromatic amino acids was measured by ^{14}C-labeling liquid scintillation counting (Ikeda and Katsumata 1994) with the following modifications. The reaction mixture (1 ml) contained 100 μmol Tris phosphate (pH 6.8), 1 μmol $MgSO_4$, 10 μmol glucose, 100 μg chloramphenicol, and 0.1 ml of the cell suspension (approximately 0.2 mg dry cells). The reaction was started by the addition of L-[^{14}C(U)]-Phe, L-[side chain-3-^{14}C]-Trp (PerkinElmer, Inc., USA), or L-[^{14}C(U)]-Tyr (ARC, Inc., USA). At the indicated intervals, 50 μL of the reaction mixture was withdrawn, vacuum-filtered using nitrocellulose filters with a pore size of 0.22 μm, and immediately washed two times with 2 ml portions of cold 0.1 M LiCl. The filters containing cells were put into 2.0 ml centrifuge tubes filled with scintillation liquid. Radioactivity was determined by a PerkinElmer MicroBeta Liquid Scintillation counter. In order to obtain the uptake kinetics, 1–50 μM of ^{14}C-L-Phe was applied, and the uptake rates for the first 60 s were determined. The uptake kinetics and activity was expressed as nanomoles of amino acid taken up per milligram of dry cell weight.

For export assay, the cells were cultivated and harvested as above described, but suspended (cell density OD_{600} of 2.0) in ammonium-free CGXII containing 1 mM tri-peptide (Phe–Phe–Phe, Tyr–Tyr–Tyr, or Trp–Trp–Trp; Sangon Biotech, Shanghai). This cell suspension was incubated at

30 °C for 2 h. Then, the cells were harvested and washed with ammonium-free CGXII solution. Cells were again suspended (cell density OD_{600} was 8–10) in ammonium-free CGXII containing 1 mM tri-peptide. The cells were incubated at 30 °C. At the indicated intervals, portions of the reaction mixture were withdrawn and filtered. The extracellular aromatic amino acid concentration was quantified by HPLC (Yang et al. 2003). The intracellular aromatic amino acid concentration was determined according to the procedures described by Simic et al. (2001).

Data analysis and statistics The data obtained from uptake assays were analyzed with Microsoft Office Excel 2007. The differences of uptake (amounts) between wild type and mutants were expressed as averages of all determinations at the time period specified in this study.

Cellular localization of NCgl1108-GFP fusion proteins with confocal microscopy The localization of NCgl1108-GFP was conducted according to Xu et al. (2006). Specifically, plasmid pGXKZ2 and pGXKZ3 were transformed into competent *E. coli* DH5α and *C. glutamicum* RES167 by electroporation. The recombination strains were incubated overnight in LB broth. When the culture OD_{600} reached approximately 0.5, IPTG was added to a final concentration of 0.1 mM. Cells were harvested and washed twice and suspended in 0.9% sodium chloride. This cell suspension was mixed with agarose (final concentration of 0.24%). Samples of the cell–agarose mixture were imaged under confocal microscope with excitation filter 475 nm and emission filter 505 nm. The imaging experiments were performed using a Leica TCS SP2 laser scanning spectral confocal microscope equipped with a cooled CCD camera.

Results

Bioinformatic analyses of ncgl1108 and its translational product (NCgl1108) The gene *ncgl1108* was located at upstream of the previously characterized resorcinol gene cluster (*ncgl1110–ncgl1113*) (Huang et al. 2006). It encodes a hypothetical protein of 468 amino acid residues with a calculated molecular mass of 50.2 kDa. BLAST-P searches showed that the NCgl1108 had 51% sequence identity to the $ProY_{St}$ (L-Pro-specific permease) of *Salmonella typhimurium* (Liao et al. 1997). In addition, NCgl1108 showed 36% identity to the $AroP_{Cg}$ (general aromatic amino acid transporter) of *C. glutamicum* (Wehrmann et al. 1995). Other proteins that showed significant identities to NCgl1108 were $AroP_{Ec}$ (Chye et al. 1986), and $PheP_{Ec}$ (L-Phe-specific transporter, 41%; Pi et al. 1991). NCgl1108 was predicted to be a membrane protein with 12 transmembrane helices, and alignment of NCgl1108 to its analogous transporters revealed that it possessed the signature sequences of the AAT family of APC superfamily (Jack et al. 2000). Previously, Marin and Krämer (2007) predicted that NCgl1108 (Cgl1155 or Cg1305) coded for an APC-type carrier of unknown substrates, and this *ncgl1108* was annotated later as a putative proline permease (http://www.membranetransport.org). Our analyses suggested that *ncgl1108* was possibly involved in resorcinol, L-Pro, L-Tyr, L-Phe, and/or L-Trp transport.

Genetic disruption of ncgl1108 affected the growth of C. glutamicum on L-Phe, but not on resorcinol, L-Pro, L-Tyr, and L-Trp In order to investigate its function, *ncgl1108* was disrupted in *C. glutamicum* RES167, resulting in the mutant RES167Δ*ncgl1108* (Table 1). The RES167Δ*ncgl1108* and RES167 were cultivated in LB and minimal media with resorcinol as carbon source, and no phenotypic differences were observed. This result ruled out our hypothesis that *ncgl1108* was involved in resorcinol metabolism although it neighbored the resorcinol gene cluster.

Genome data mining with KEGG pathway tool showed that *C. glutamicum* had incomplete metabolic pathways for L-Pro, L-Phe, L-Tyr, or L-Trp, indicating that *C. glutamicum* RES167 was not able to grow on them as carbon source. Our experiments confirmed this genome-mining result: *C. glutamicum* did not grow on L-Pro, L-Phe, L-Tyr, or L-Trp as carbon source. However, we found that those amino acids could support the growth of RES167 when they were served as sole nitrogen sources, although the biomass accumulation was not high (Fig. 1a–d). Genetic disruption of *ncgl1108* did not affect the growth of RES167Δ*ncgl1108* on L-Tyr (Fig. 1a), L-Pro (Fig. 1b), or L-Trp (Fig. 1c), but impaired its growth on L-Phe (Fig. 1d). Genetic complementation of *ncgl1108* in RES167Δ*ncgl1108*/pGXKZ1 restored its growth on L-Phe (Fig. 1d).

Disruption and hyperexpression of ncgl1108 significantly affected the uptake of L-Phe by C. glutamicum cells Combining the bioinformatic analyses and the above experimental results, it was deduced that the gene *ncgl1108* encoded a putative L-Phe transporter. Uptake assays for L-Phe, L-Tyr, L-Pro, or L-Trp by wild RES167 and mutant RES167Δ*ncgl1108* were conducted. The results showed that the disruption of $pheP_{Cg}$ resulted in differences for L-Tyr, L-Pro, or L-Trp uptake between RES167 and RES167Δ*ncgl1108* (Fig. 2; white and gray columns). Statistical analysis showed that uptakes of L-Phe, L-Tyr, L-Pro, and L-Trp by RES167Δ*ncgl1108* decreased by 18.2±4.9%, 0.6±2.7%, 6.8±10.4%, 6.0±6.3%, respectively, when compared to RES167. In order to characterize the effect of NCgl1108 on L-Phe uptake further, the *ncgl1108* was hyperexpressed with multicopy pGXZ1 in RES167

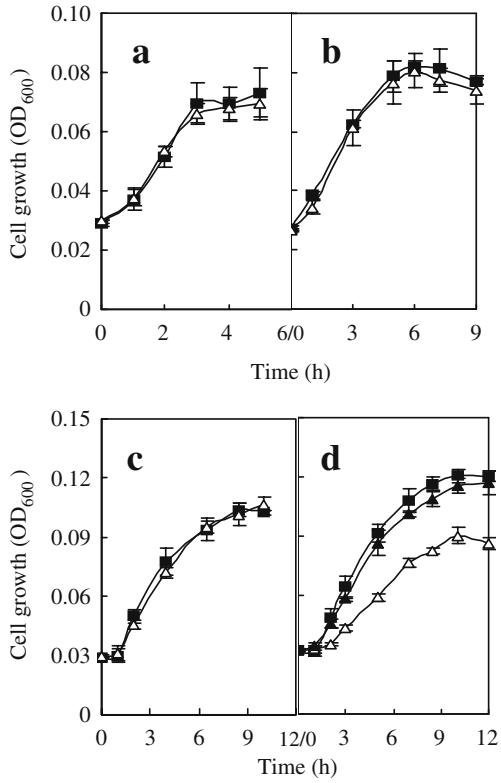

Fig. 1 Growth of *C. glutamicum* RES167 (*filled square*), RES167Δ*ncgl1108* (*empty triangle*), and RES167Δ*ncgl1108*/pGXKZ1 (*filled triangle*) on minimal medium supplemented with L-Tyr (**a**), L-Pro (**b**), L-Trp (**c**), or L-Phe (**d**) as sole nitrogen source

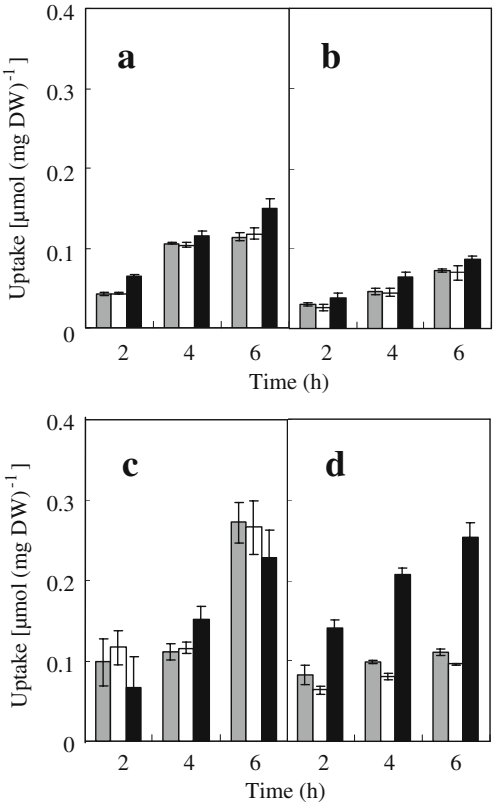

Fig. 2 Uptake of L-Tyr (**a**), L-Trp (**b**), L-Pro (**c**), or L-Phe (**d**) by *C. glutamicum* RES167 (*gray column*), RES167Δ*ncgl1108* (*white column*), and RES167/pGXKZ1 (*black column*)

cells. Compared to RES167 and mutant RES167Δ*ncgl1108*, this hyperexpression of *ncgl1108* in RES167/pGXKZ1 resulted in significant increase (104.0±29% in average) of L-Phe uptake (Fig. 2d; black columns). The effects of hyperexpression of *ncgl1108* in RES167/pGXKZ1 on L-Tyr, L-Trp, or L-Pro uptake were also observed, but not so significant. Based on these results, it is concluded that *ncgl1198* encodes an L-Phe transporter and is named as $pheP_{Cg}$.

To determine if $PheP_{Cg}$ functioned as an exporter for L-Phe, L-Tyr, or L-Trp, export experiments with tri-peptides (Phe–Phe–Phe, Tyr–Tyr–Tyr, or Trp–Trp–Trp) were carried out. The results revealed that the bulk concentrations of L-Phe, L-Tyr, and L-Trp in experiments with RES167/pGXKZ1 were not higher than that with RES167, indicating that the $PheP_{Cg}$ did not have export function (Fig. 3a–c). It is noteworthy that the bulk concentrations of L-Phe in experiment with RES167/pGXKZ1 were even lower than that with RES167 (Fig. 3a). Further studies showed that the intracellular L-Phe levels in RES167/pGXKZ1 and in RES167 were higher than that in RES167Δ*ncgl1108* (Fig. 3d), indicating the accumulation of L-Phe caused by the occurrence of $PheP_{Cg}$ in RES167/pGXKZ1 and RES167 cells. These results supported that $PheP_{Cg}$ functioned as an importer for L-Phe.

Construction of the double mutant RES167Δ(ncgl1108-$aroP_{Cg}$) and determination of uptake kinetics for aromatic amino acids The previously identified $AroP_{Cg}$ uptakes all three aromatic amino acids in *C. glutamicum* (Wehrmann et al. 1995). In order to eliminate the effect of $AroP_{Cg}$ on aromatic amino acid uptake assay and to estimate the uptake kinetics of $PheP_{Cg}$, we constructed a double mutant, RES167Δ(*ncgl1108-*$aroP_{Cg}$), by further disruption of $aroP_{Cg}$ in RES167Δ*ncgl1108* in this study (Table 1). Difference in growth in LB medium among RES167Δ(*ncgl1108-*$aroP_{Cg}$), RES167Δ*ncgl1108*, and RES167 was not observed.

The uptake of ^{14}C-labeled L-Phe or L-Tyr was determined with wild recombinant strains and mutants. The uptake of ^{14}C-labeled L-Phe by mutants RES167Δ*ncgl1108* and RES167Δ(*ncgl1108-*$aroP_{Cg}$) was significantly lower compared to the RES167 (Fig. 4a). Statistical analysis of these data revealed that the uptakes of L-Phe by mutants RES167Δ*ncgl1108* and RES167Δ(*ncgl1108-*$aroP_{Cg}$) decreased by 13.2±1.9% and 39.8±1.8%, respectively. Hyperexpression of $PheP_{Cg}$ in RES167Δ(*ncgl1108-*

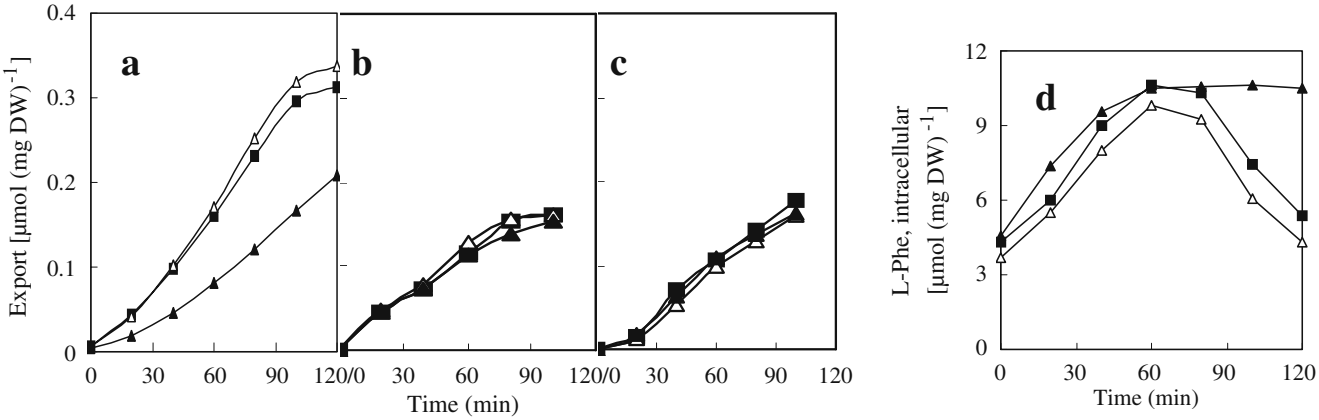

Fig. 3 Export assays for L-Phe (**a**), L-Tyr (**b**), or L-Trp (**c**), and intracellular concentration for LPhe (**d**) by *C. glutamicum* RES167 (*filled square*), RES167Δ*ncgl1108* (*empty triangle*) and RES167Δ*ncgl1108*/pGXKZ1 (*filled triangle*) in minimal medium containing 1 mM of Phe–Phe–Phe (**a**, **d**), Tyr–Tyr–Tyr (**b**) or Trp-Trp-Trp (**c**)

aroP$_{Cg}$)/pGXKZ1 resulted in 47.6±6.8% increase of L-Phe uptake. The effect of PheP$_{Cg}$ disruption on ^{14}C-labeled L-Tyr uptake was much less significant (Fig. 4b). Compared to RES167, the uptake for L-Tyr by mutant RES167Δ*ncgl1108* decreased by 7.8±3.1%. Determination of L-Phe uptake kinetics of RES167Δ(*ncgl1108-aroP*$_{Cg}$)/pGXKZ1 (Fig. 4c) showed that its K_m and V_{max} values were 10.4±1.5 μM and 1.2±0.1 nmol min^{-1} (mg DW)$^{-1}$, respectively. The K_m and V_{max} values of RES167Δ(*ncgl1108-aroP*$_{Cg}$) for L-Phe were determined to be 4.0±0.4 μM and 0.6±0.1 nmol min^{-1} (mg DW)$^{-1}$, respectively. The higher V_{max} value of RES167Δ (*ncgl1108-aroP*$_{Cg}$)/pGXKZ1 than that of RES167Δ (*ncgl1108-aroP*$_{Cg}$) clearly indicated that PheP$_{Cg}$ was active and functional in RES167Δ(*ncgl1108-aroP*$_{Cg}$)/pGXKZ1 at the conditions tested in this study. The kinetic analysis also revealed that additional L-Phe transporter(s) besides *aroP*$_{Cg}$ and *PheP*$_{Cg}$ still occurs in the double mutant RES167Δ (*ncgl1108-aroP*$_{Cg}$).

The substrate specificity of PheP$_{Cg}$ in *C. glutamicum* RES167Δ(*ncgl1108-aroP*)/pGXKZ1 was examined with ^{14}C-labeled L-Phe in the presences of 20-fold unlabeled various amino acids (Table 2). Results indicated that PheP$_{Cg}$ was specific to L-Phe, and its transport activity for L-Phe was not affected by all tested amino acids, except for L-Tyr (Table 2). The uptake of ^{14}C-labeled L-Phe was strongly inhibited by L-Tyr was surprising. We deduced that the inhibition by L-Tyr was due to the structural similarity between L-Phe and L-Tyr.

PheP$_{Cg}$ *was localized at cellular membrane* In order to identify the cellular localization of PheP$_{Cg}$, a fusion protein was engineered from the PheP$_{Cg}$ and GFP. For the purpose

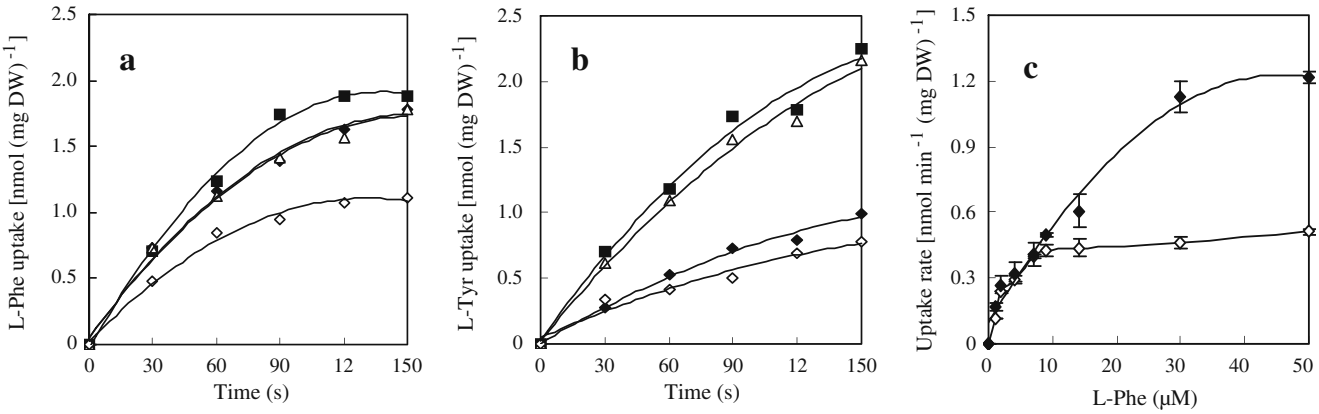

Fig. 4 Uptake of ^{14}C-labeled L-Phe (**a**) and L-Tyr (**b**) by *C. glutamicum* RES167 (*filled square*), RES167Δ*ncgl1108* (*empty triangle*), RES167Δ(*ncgl1108-aroPCg*) (*empty diamond*), and RES167Δ(*ncgl1108-aroP*)/pGXKZ1 (*filled diamond*), and determination of K_m and V_{max} for L-Phe (**c**) by *C. glutamicum* RES167Δ(*ncgl1108-aroPCg*)/pGXKZ1. The initial concentrations of ^{14}C-labeled L-Phe was 50 μM (**a**, **b**) and 1–50 μM (**c**). The K_m and V_{max} values of *C. glutamicum* RES167Δ(*ncgl1108-aroPCg*)/pGXKZ1 and *C. glutamicum* RES167Δ(*ncgl1108-aroPCg*) for L-Phe were obtained by use of the experimental data shown in **c** and by conversion of those data into Lineweaver–Burk plots

Table 2 Effects of various amino acids on L-Phe uptake in *C. glutamicum* RES167Δ(*ncgl1108-aroP*)/pGXKZ1

Competitors	L-[^{14}C]-Phe relative uptake rate (%)	Competitors	L-[^{14}C]-Phe relative uptake rate (%)
None	100.0±9.4	L-Ala	102.4±10.9
L-Phe	25.4±6.8	L-Leu	100.7±13.8
L-Tyr	34.0±3.2	L-Met	94.0±2.2
L-Trp	103.5±8.0	L-His	116.7±20.5
L-Pro	101.7±7.1	L-Glu	105.6±2.4
γ-Aminobutyrate	112.3±5.9	L-Lys	113.3±8.7

The concentration of L-[^{14}C]-Phe was 10 μM. The L-Phe uptake rate in the absence of competitors was determined to be 0.40±0.04 nmol min^{-1} (mg DW)$^{-1}$, and this was calculated as 100%. The concentration of each competitor was 200 μM. Data are averages from three parallel determinations and the standard deviations are provided

to ensure correct folding of peptides, a 16-amino acid-long linker was installed between the PheP$_{Cg}$ and GFP peptides, so that each of them was still folded correctly and functioned individually. The fusion protein PheP$_{Cg}$-GFP was synthesized under induction with IPTG in cells of *C. glutamicum* RES167/pGXKZ3. Confocal microscopy clearly showed that the fusion protein PheP$_{Cg}$-GFP was located at the cellular periphery membrane part of *C. glutamicum* RES167/pGXKZ3 (Fig. 5).

Discussion

The transport of aromatic acids into cells is the first step for bacterial metabolism of these compounds. In *C. glutamicum*, the transporter genes involving in aromatic compound metabolism such as *genK* and *benK/benE* often associate with the degradative gene clusters (Chaudhry et al. 2007). Although the gene *pheP*$_{Cg}$ (*ncgl1108*) is located immediately upstream of the resorcinol degradative gene cluster, our results demonstrated that this gene was not involved in resorcinol degradation. Instead, *pheP*$_{Cg}$ encodes an L-Phe specific transporter in *C. glutamicum*.

So far, as we know, PheP$_{Cg}$ is the first L-Phe specific transporter identified from *C. glutamicum*, and it represents the first functionally identified L-Phe specific transporter from Gram-positive bacteria. Early studies suggested that L-Phe and L-Tyr were transported in *Bacillus subtilis* by a common system (D'Ambrosio et al. 1973); however, any L-Phe specific transporter has not been identified. In *E. coli*, three L-Phe transport systems were identified, i.e., the general aromatic amino acid transporter AroP$_{Ec}$ that transports all three aromatic amino acids (Chye et al. 1986; Honore and Cole 1990), the L-Phe specific transporter PheP$_{Ec}$ that is similar to PheP$_{Cg}$ and they share 36% identity of amino acid sequence (Pi et al. 1991), and the branched-chain amino acid transport system LIV-I/LS system that functions as L-Phe transport (Koyanagi et al. 2004). Compared to other L-Phe transport systems, the RES167/PheP$_{Cg}$ cells have moderate affinity to L-Phe: It has higher affinity than that of the *E. coli*/LIV-I$_{Ec}$ (K_m= 19 μM; Koyanagi et al. 2004) and *Neurospora crassa*/PheP$_{Nc}$ (K_m=100 μM; DeBusk and DeBusk 1965), but much lower affinity when compared to that of the *E. coli*/AroP$_{Ec}$ (K_m=0.47 μM; Brown 1970) and *E. coli*/PheP$_{Ec}$ (K_m=2 μM; Brown 1970; Cosgriff et al. 2000). We observed that the occurrence of unlabeled L-Tyr significantly decreased the uptake of ^{14}C-labeled L-Phe by PheP$_{Cg}$. As determined in this study, uptake of L-Tyr by PheP$_{Cg}$ was not obvious in this study. This substrate spectrum of PheP$_{Cg}$ is clearly different from the previously identified AroP$_{Cg}$ from *C. glutamicum* (Wehrmann et al. 1995).

Based on our observation, the PheP$_{Cg}$ is physiologically active and plays a role in uptake of L-Phe by *C. glutamicum* under the conditions examined in this study. Disruption of *pheP*$_{Cg}$ resulted in significant decreases of L-Phe uptake. Phenotypically, this disruption of *pheP*$_{Cg}$ reduced the growth of *C. glutamicum* when L-Phe served as sole nitrogen source. Exploitation of the *C. glutamicum* genome

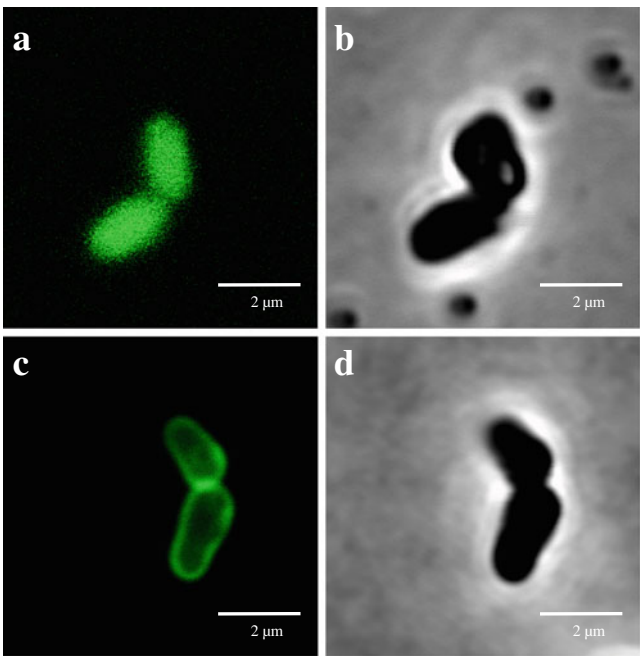

Fig. 5 Confocal microscopy of *C. glutamicum* RES167/pGXKZ2 (control) and *C. glutamicum* RES167/pGXKZ3. Cells were cultivated as described in "Materials and methods", and were induced with IPTG. Fusion protein of PheP*Cg*-GFP in *C. glutamicum* was visualized by its fluorescence. *C. glutamicum* RES167/pGXKZ2 under fluorescence (**a**) and visible light (**b**) and *C. glutamicum* RES167/pGXKZ3 under fluorescence (**c**) and visible light (**d**)

with KEGG pathway tools revealed that this bacterium is possibly able to assimilate L-Phe as nitrogen source and supports our observation that *C. glutamicum* grew on L-Phe as nitrogen source. Two candidate genes, *ncgl0215* and *ncgl2020*, which encode putative L-Phe /L-Tyr aminotransferases and are possibly involved in deamination of L-Phe, were identified. It is proposed that the uptake of L-Phe by $PheP_{Cg}$ increased the intracellular L-Phe concentration and subsequently invoked the activation of the putative aminotransferases in *C. glutamicum*. This physiological adaptation enables *C. glutamicum* growing on the L-Phe as sole nitrogen source. However, the growth was very limited when L-Phe served as sole nitrogen sources. Accumulation of phenylpyruvate, a deduced metabolite from L-Phe deamnination, was observed (data not shown).

It is deduced that other transport system(s), besides $PheP_{Cg}$ and $AroP_{Cg}$, for L-Phe or other aromatic amino acids occur in *C. glutamicum*. This hypothesis is based on the observation that disruption of both $PheP_{Cg}$ and $AroP_{Cg}$ did not result in complete loss of L-Phe and other aromatic amino acid uptake. Genome-wide searches according to gene/amino acid sequence similarity revealed other putative aromatic amino acid transport genes, including *ncgl0453* and *ncgl0464*. Functions of those putative transporter genes are currently under investigation.

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