APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 2007, p. 4491–4498 Vol. 73, No. 14
0099-2240/07/$08.00+0 doi:10.1128/AEM.02446-06

Mutations of the *Corynebacterium glutamicum* NCgl1221 Gene, Encoding a Mechanosensitive Channel Homolog, Induce L-Glutamic Acid Production▽

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Received 18 October 2006/Accepted 14 May 2007

Corynebacterium glutamicum **is a biotin auxotroph that secretes L-glutamic acid in response to biotin limitation; this process is employed in industrial L-glutamic acid production. Fatty acid ester surfactants and penicillin also induce L-glutamic acid secretion, even in the presence of biotin. However, the mechanism of L-glutamic acid secretion remains unclear. It was recently reported that disruption of *odhA*, encoding a subunit of the 2-oxoglutarate dehydrogenase complex, resulted in L-glutamic acid secretion without induction. In this study, we analyzed *odhA* disruptants and found that those which exhibited constitutive L-glutamic acid secretion carried additional mutations in the NCgl1221 gene, which encodes a mechanosensitive channel homolog. These NCgl1221 gene mutations lead to constitutive L-glutamic acid secretion even in the absence of *odhA* disruption and also render cells resistant to an L-glutamic acid analog, 4-fluoroglutamic acid. Disruption of the NCgl1221 gene essentially abolishes L-glutamic acid secretion, causing an increase in the intracellular L-glutamic acid pool under biotin-limiting conditions, while amplification of the wild-type NCgl1221 gene increased L-glutamate secretion, although only in response to induction. These results suggest that the NCgl1221 gene encodes an L-glutamic acid exporter. We propose that treatments that induce L-glutamic acid secretion alter membrane tension and trigger a structural transformation of the NCgl1221 protein, enabling it to export L-glutamic acid.**

L-Glutamate has a distinctive taste, known as "umami," that is neither sweet, sour, salty, nor bitter (24), and it is widely used as a flavor enhancer. About 1.8 million tons of monosodium glutamate are produced worldwide per year by fermentation using coryneform bacteria. These are rod-shaped, nonsporulating, gram-positive bacteria containing mycolic acids and are widely distributed in the natural world. A nonpathogenic species, *Corynebacterium glutamicum*, was originally isolated as an L-glutamate-producing bacterium (12, 34). Wild-type *C. glutamicum*, without breeding, releases more than 80 g/liter of L-glutamic acid under appropriate culture conditions (27).

The mechanism of L-glutamate secretion by *C. glutamicum* is unique. The presence of biotin, which is required by *C. glutamicum* for growth, inhibits L-glutamate production in the culture medium, while production is induced under biotin-limiting conditions (26) and in response to fatty acid ester surfactants (31) and penicillin (22). It is also induced by ethambutol treatment, which inhibits formation of the mycolic acid layer of the cell wall (25). Since biotin limitation and the other inducing treatments cause damage to cell surface structures of this microorganism, it has long been assumed that L-glutamate leaks through the cell membrane (32).

The *dtsR1* gene, isolated as a multicopy suppressor of a mutant hypersensitive to fatty acid ester surfactants, encodes a protein showing strong homology to the β subunit of acetyl-coenzyme A (CoA) carboxylase (10). Since biotin is a cofactor of acetyl-CoA carboxylase, biotin limitation, like treatment with fatty acid ester surfactants, might affect the biotin-enzyme complex containing DtsR1. Overexpression of *cls*, encoding cardiolipin synthase, or *acp*, encoding acyl carrier protein, also results in trigger-independent glutamate secretion (18). Penicillin inhibits cell wall biosynthesis by binding to penicillin-binding proteins, which catalyze the transglycosylation and transpeptidation of peptidoglycan. *C. glutamicum* is highly tolerant to lysis by lytic enzymes, such as egg white lysozyme, that catalyze hydrolysis of the β-1,4 glycoside bond between *N*-acetylglucosamine and *N*-acetylmuramic acid of peptidoglycan. Mutation of the *ltsA* gene, which encodes a putative glutamine-dependent amidotransferase that is involved in the formation of rigid cell wall structure, leads to production of glutamate, but the connection between cell wall biosynthesis and L-glutamate secretion remains unclear (5).

The activity of the 2-oxoglutarate dehydrogenase complex (ODHC) reportedly decreases during L-glutamate production in response to biotin limitation, fatty acid ester surfactants, and penicillin (8). Since ODHC is located at the branch point between the tricarboxylic acid (TCA) cycle and L-glutamate biosynthesis, a decrease in ODHC activity could switch the metabolic flow from the TCA cycle to L-glutamate synthesis. The ODHC generally comprises three enzymes: 2-oxoglutarate dehydrogenase (E1o), dihydrolipoamide *S*-succinyltransferase (E2o), and dihydrolipoamide dehydrogenase (E3). Disruption of *odhA*, which encodes the E1o subunit, results in L-glutamate secretion without induction (1). Metabolic linkage between acetyl-CoA carboxylase and ODHC might trigger L-glutamate secretion, since a *dtsR1* disruptant (see above) produced a significant amount of L-glutamate and exhibited reduced ODHC activity (11). Recently a novel form of ODHC activity regulation in *C. glutamicum* was reported (20): ODHC activity

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▽ Published ahead of print on 18 May 2007.

was strongly inhibited by the nonphosphorylated form of the OdhI protein, which is phosphorylated by the serine/threonine protein kinase PknG.

In the past decade, our understanding of the molecular basis of amino acid secretion has enabled the identification of the *lysE*, *thrE*, and *brnFE* genes, which encode novel carriers exporting L-lysine, L-threonine, and L-isoleucine, respectively (9, 28, 35). Although it has been suggested that secretion of L-glutamate by biotin-limited cells of *C. glutamicum* is mediated by a carrier system in the plasma membrane, no L-glutamate exporter has yet been identified (7).

Here we show that the product of a *C. glutamicum* NCgl1221 gene is homologous to mechanosensitive channels and plays an important role in L-glutamate secretion, and we propose a new model for the induction of L-glutamate secretion.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Corynebacterium glutamicum* ATCC 13869 (formerly known as *Brevibacterium lactofermentum*) was used as the wild-type strain. The L-glutamate-producing mutants 2A-1, 2A-1R, BL1 [NCgl1221(V419::IS*1207*)], BL2 [NCgl1221(A111V)], and BL3 [NCgl1221 (W15CSLW)] were constructed in this study. *Escherichia coli* JM109 (36) was used for constructing and propagating plasmids. *E. coli* and *C. glutamicum* cells were cultured at 37°C on LB medium (14) or at 31.5°C on CM2B (16), respectively. If necessary, the *C. glutamicum odhA* mutants were cultured at 31.5°C on CM-Dex (per liter, 5 g glucose, 10 g yeast extract, 10 g tryptone, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 5H_2O$, 3 g urea, 34.5 ml soybean protein hydrolysate [total nitrogen, 1.2 g], adjusted to pH 7.0 with NaOH). Plasmid pBS4S (17) was used as a nonreplicating integration vector. pBS4S was constructed by ligating the PCR-amplified *sacB* gene of *Bacillus subtilis* to pHSG299 (TAKARA BIO Inc., Shiga, Japan) cleaved with AvaII, and the SmaI recognition site in the kanamycin-resistant gene was destroyed by nucleotide substitution using crossover PCR without a change of amino acid residue. Plasmid pVK9 (17), derived from pHM1519 (16), was used to construct a genomic library.

Construction of mutants. To disrupt the *odhA* gene, an internal fragment of *odhA* was generated by PCR with the primer pair 5′-GGG GAT CCA TCG GTA TGC CAC ACC GTG GTC GCC-3′ and 5′-GGG GAT CCA CGA CTG CTT CTG CAT CTT CGT TGG-3′ (underlining indicates BamHI restriction site), using ATCC 13869 chromosomal DNA as a template. The amplified fragment was cleaved with BamHI and ligated to BamHI-cleaved pBS4S, resulting in pBS4odhAint. This plasmid, which lacks a functional replication origin in *C. glutamicum*, was introduced into ATCC 13869 by electroporation. Integration of the plasmid into the chromosomal *odhA* gene was verified by PCR.

To construct BL1 [NCgl1221(V419::IS*1207*)], a NCgl1221 gene fragment was amplified by PCR with the primer pair 5′-GGG AGC TCG ACT TTC TGG CTC CTT TAC T-3′ and 5′-GGG AGC TCG CCG ATG ACT AAT AAT GAG A-3′ (underlining indicates SacI restriction site), using the *C. glutamicum* 2A-1R chromosome as a template. The fragment was cleaved with SacI and ligated to SacI-cleaved pBS4S, yielding pBSo15832A-1.

To construct BL2 [NCgl1221(A111V)] and BL3 [NCgl1221(W15CSLW)], NCgl1221 gene fragments were amplified by PCR with the primer pair 5′-GGG AGC TCG ACT TTC TGG CTC CTT TAC T-3′ and 5′-GGG AGC TCC ACG GCA TGC CGA CCA CCG T-3′ (underlining indicates SacI restriction site), using the *C. glutamicum odhA* mutant L30-2 or A1 chromosome as a template. The fragment was digested with SacI and ligated to SacI-cleaved pBS4S, yielding pBS4S1583L30 and pBS4S1583A1, respectively.

The NCgl1221 gene disruptant pBS4ΔNCgl1221 was constructed by two successive rounds of PCR. In the first round, one-half of the fragment was amplified using the chromosomal DNA of ATCC 13869 as a template and the primer pair 5′-GGG AGC TCA CCT TTG TGG AGG AAT AGA G-3′ (underlining indicates a SacI restriction site) and 5′-GGG ACA CGT CTG TAA TCA GCG TCC TAG AGC CAA GAT TAG CGC TGA A-3′, while the other half was amplified with the primer pair 5′-TTC AGC GCT AAT CTT GGC TCT AGG ACG CTG ATT ACA GAC GTG TCC C-3′ and 5′-GGG AGC TCA GTA CTC TTC CTT GGA CAT C-3′ (underlining indicates a SacI restriction site). In the second round, the resulting products were mixed in a 1:1 molar ratio and subjected to PCR using the following primer pair: 5′-GGG AGC TCA CCT TTG TGG AGG AAT AGA G-3′ and 5′-GGG AGC TCA GTA CTC TTC CTT GGA CAT C-3′ (underlining indicates SacI restriction sites). The amplified product was digested with SacI and ligated with SacI-digested pBS4S, giving pBS4ΔNCgl1221.

Each plasmid was introduced into *C. glutamicum* ATCC 13869, and clones with the plasmid integrated into the NCgl1221 gene were selected for further use.

Enzyme preparation and assay. All steps were performed at 4°C. *C. glutamicum* cells were harvested, washed twice in 0.2% KCl, and suspended in 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-NaOH buffer, pH 7.5, containing 30% glycerol. Cells were disrupted by sonication, and the cellular debris was removed by centrifugation (15,000 × *g* for 15 min). The crude enzyme extracts were gel filtered in the same buffer on PD10 columns (GE Healthcare Bio-Science Corp., Piscataway, NJ) to remove intracellular metabolites. ODHC activity was measured at 30°C in a photometric assay by following the initial rate in absorbance of 3-acetylpyridine adenine dinucleotide at 365 nm. The standard reaction mixture contained 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-NaOH buffer, pH 7.7, 0.2 mM CoA, 0.3 mM thiamine pyrophosphate, 1 mM sodium 2-oxoglutarate, 3 mM L-cysteine, and 5 mM $MgCl_{12}$. A control experiment was performed without sodium 2-oxoglutarate.

L-Glutamate fermentation. For flask scale fermentation, we used four conditions. For L-glutamate fermentation without induction, cells were cultured in 20 ml medium GH1 [per liter, 30 g glucose, 15 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot 4\text{-}5H_2O$, 200 μg vitamin B_1 HCl, 300 μg biotin, 13.8 ml soybean protein hydrolysate (total nitrogen, 0.48 g), 50 g $CaCO_3$ (separately sterilized) adjusted to pH 8.0 with KOH] in 500-ml Sakaguchi flasks with shaking. For fatty acid ester surfactant or penicillin treatments, cells were cultured in 20 ml GH2 medium [GH1 medium but with 80 g glucose, 30 g $(NH_4)_2SO_4$, and 60 μg biotin] in 500-ml Sakaguchi flasks with shaking until the glucose was completely consumed. Aliquots (2 ml) of these cultures were inoculated into 20 ml GH2 medium. When the optical density at 620 nm (OD_{620}) reached about 20, Tween 40 or penicillin G was added to a final concentration of 5 mg/ml or 0.4 U/ml, respectively. For L-glutamate fermentation by biotin depletion, 2-ml aliquots of cell culture were inoculated into 20 ml GH3 medium (GH2 medium without biotin).

For L-glutamate fermentation in a jar fermentor, cells were grown in 300 ml GH4 medium (per liter, 60 g glucose, 1.54 g H_3PO_4, 1.45 g KOH, 0.9 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 670 μg vitamin B_1 HCl, 60 μg biotin, 0.28 g DL-methionine, 44 ml soybean protein hydrolysate [total nitrogen, 1.54 g]). For the main fermentation, 30-ml aliquots of cell culture were inoculated into 270 ml GH5 medium (per liter, 111 g glucose, 3.84 g KH_2PO_4, 1.11 g $MgSO_4 \cdot 7H_2O$, 0.011 g $FeSO_4 \cdot 7H_2O$, 256 μg vitamin B_1 HCl, 11 ml soybean protein hydrolysate [total nitrogen, 0.39 g]). Aeration was controlled by agitation to keep the dissolved oxygen level above 5%. All cells were cultured at 31.5°C. The pH was maintained at 7.2 with ammonia gas.

Extraction of intracellular metabolites. Extraction of intracellular metabolites was performed by a modification of the method previously described (29). Cells were separated from the medium by rapid vacuum filtration (Durapore HV, 0.45 μm; Millipore, Billerica, MA), followed by two washes with 10 ml MilliQ water and incubation of the filter and attached cells for 10 min in 2 ml methanol containing 5 μM morpholineethanesulfonic acid and 5 μM methionine sulfone. The extract (1.6 ml) was mixed with 1.6 ml $CHCl_3$ and 640 μl MilliQ water, and the aqueous phase was applied to a spin column to remove protein contamination (Ultrafree-MC; Millipore). The filtered extracts were analyzed using capillary electrophoresis mass spectrometry by Human Metabolome Technologies Inc. (Yamagata, Japan). The cell volume used to calculate intracellular metabolite concentrations was 1.6 μl mg^{-1} dry cell weight (DCW) (4). The DCW was calculated from the OD_{620} by the following experimentally obtained formula: DCW = 22.707 × (OD_{620})/100 (g $liter^{-1}$).

RESULTS

Disruption of *odhA* does not result in L-glutamate production. A significant decrease in ODHC activity is observed under L-glutamate-producing conditions (8), suggesting that its attenuation is a prerequisite for L-glutamate production by *C. glutamicum*. However, it has been observed that some *odhA* disruptants often produce little, if any, L-glutamate. It has been speculated that L-glutamate productivity is lost due to the genetic instability of *odhA* disruptants (1), but our results suggest the converse, that additional mutations are responsible for the L-glutamate productivity of some *odhA* disruptants.

To examine this hypothesis, we constructed *odhA* disruptants

by homologous recombination between the chromosomal *odhA* gene and a truncated *odhA* gene. The plasmid pBS4odhAint, which carries an internal fragment of the *odhA* gene of *C. glutamicum*, was introduced into wild-type *C. glutamicum* ATCC 13869 cells and kanamycin-resistant transformants selected, which should harbor the plasmid integrated into the host chromosome by homologous recombination at the *odhA* locus. The resultant *odhA*-disrupted strains had extremely unstable phenotypes, forming colonies of various sizes on CM-Dex agar plates.

We cultured several *odhA*-disrupted clones in liquid GH1 medium and analyzed the metabolites in the culture broth. Most clones produced 2-oxoglutarate, lactate, acetate, and pyruvate but not L-glutamate; however, clone 2A-1 produced high levels of L-glutamate in the presence of excess biotin. In order to determine whether 2A-1 had gained an additional mutation(s) that rendered it an L-glutamate producer, its wild-type *odhA* gene was restored by homologous recombination using pBS4odhAint. The *odhA* mutants grew very poorly on medium without sugar because their energy generation depended primarily on glycolysis. Hence, we grew cells of strain 2A-1 on CM2B plates without sugar to cure the plasmid and selected a clone that formed larger colonies. Curing of the plasmid and restoration of the wild-type *odhA* gene were confirmed by PCR and sequencing as well as measuring of ODHC activity: the *odhA*$^+$ revertant, named 2A-1R, and the wild-type strain had specific ODHC activities of 1.8 ± 0.5 and 3.7 ± 1.0 nmol min^{-1} (mg protein)$^{-1}$, respectively, while 2A-1 had no detectable ODHC activity.

The L-glutamate productivity of this *odhA*$^+$ clone was examined. As expected, 2A-1R retained the ability to produce L-glutamate in the presence of excess biotin (Fig. 1). The rates of L-glutamate production were almost the same in 2A-1 (*odhA*) and 2A-1R (*odhA*$^+$), but the maximum yield of 2A-1R was slightly lower than that of 2A-1. The L-glutamate produced by 2A-1R was assimilated after the glucose had been completely consumed. Since the ability to assimilate L-glutamate depends on ODHC activity, this indirectly confirmed restoration of ODHC activity in 2A-1R. These findings show that *odhA* disruption does not confer the ability to produce L-glutamate efficiently. Rather, 2A-1 carries an unknown mutation(s) that independently confers the ability to produce L-glutamate.

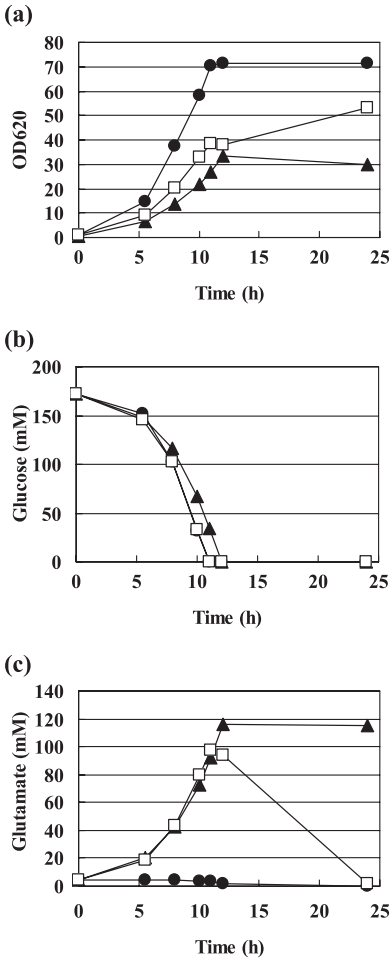

FIG. 1. Fermentation kinetics of the *odhA* mutant and revertant in the presence of excess biotin. The wild-type, *C. glutamicum* ATCC 13869, and the L-glutamic acid-producing *odhA* mutant 2A-1 and its *odhA*$^+$ derivative 2A-1R were grown with excess biotin at 31.5°C. (a) Growth; (b) residual glucose; (c) L-glutamate accumulation. Symbols: filled circles, wild-type; filled triangles, *odhA* mutant 2A-1; open squares, 2A-1R.

Identification of a mutation in strain 2A-1R inducing L-glutamate production. Growth of 2A-1R was slow on minimal medium, probably because the substrate (glucose) is used not only for growth but also for L-glutamate production. Based on this observation, we shotgun cloned a gene capable of restoring the growth of 2A-1R on minimal medium. A Sau3AI library of wild-type *C. glutamicum* chromosomal DNA, constructed in the *E. coli-C. glutamicum* shuttle vector pVK9, was introduced into 2A-1R by electroporation, and transformants that grew faster on minimal medium plates were selected. From these we isolated a plasmid, designated pL5k, that abolished L-glutamate production and restored rapid growth on minimal medium. Sequencing of a 3-kb Sau3AI fragment from the plasmid showed that it contained only one intact gene, NCgl1221. The 533-amino-acid product of this gene has previously been reported to catalyze betaine efflux (21). Its deduced amino acid sequence has significant homology to mechanosensitive ion channels, such as the *E. coli yggB* gene product. Mechanosensitive channels are gated by alterations of membrane tension, thus preventing cell disruption by hypo-osmotic shock (13, 30). The NCgl1221 protein has an N-terminal region similar to that of YggB and an additional C-terminal region unlike any known sequence. This type of protein is also present in other corynebacteria, such as *C. glutamicum* ATCC 13032, *Corynebacterium efficiens* YS314, *Corynebacterium diphtheriae* NCTC 13129, and *Corynebacterium jeikeium* K411 (GenBank accession no. NC_004369, NC_004369, NC_002935, and CR931997, respectively).

The coding region of the endogenous NCgl1221 gene of 2A-1R was found to contain an insertion sequence with 99.9% identity to IS*1207* at nucleotide 1258, resulting in the production of a C-terminally truncated NCgl1221 protein of 423 amino acids. To determine whether this mutation was responsible for L-glutamate production, we cloned the mutant NCgl1221 gene [named NCgl1221(V419::IS*1207*)] and introduced it into wild-type *C. glutamicum* ATCC 13869. The cloning process requires integration into the chromosome by ho-

TABLE 1. L-Glutamate production by NCgl1221 gene mutants in the absence of induction[a]

Strain	OD_{620}	Amt of L-glutamate (mM)	Yield (wt/wt [%])
ATCC 13869 (wild type)	63.2 ± 1.4	2.4 ± 0.28	0.0 ± 0.12
BL1 [NCgl1221(V419::IS*1207*)]	41.0 ± 0.7	92.3 ± 1.78	39.6 ± 0.80
BL2 [NCgl1221(A111V)]	46.1 ± 0.7	74.9 ± 1.70	31.8 ± 0.76
BL3 [NCgl1221(W15CSLW)]	55.7 ± 1.2	36.6 ± 0.85	14.7 ± 0.38

[a] Data represent mean values ± SDs for three independent cultures.

mologous recombination, since the plasmid lacks a replication origin that functions in *C. glutamicum*. We isolated a derivative, designated BL1, in which the chromosomal NCgl1221 gene had been replaced by the NCgl1221(V419::IS*1207*) gene. This strain displayed remarkably high L-glutamate productivity without induction treatment (Table 1), showing that this mutation alone can lead to L-glutamate production.

In the course of constructing BL1, we obtained an integrant carrying both wild-type NCgl1221 and NCgl1221 (V419::IS*1207*). The L-glutamate productivity of this integrant was one-fourth that of BL1 under biotin-sufficient conditions (data not shown). It is therefore possible that the NCgl1221 gene product forms oligomers, like *E. coli* YggB, which is predicted to form hexamers (30), and that therefore NCgl1221(V419::IS*1207*) is semidominant. This might explain the ability of the wild-type NCgl1221 gene cloned in the multicopy plasmid to restore the growth of 2A-1R on minimal medium and reduce its L-glutamate productivity.

We also sequenced the NCgl1221 genes of other *odhA* disruptants of *C. glutamicum* that produce large amounts of L-glutamate. As expected, most had mutations in the NCgl1221 gene. Substitutions identified were Ala100→Thr [named NCgl1221 (A100T)], Ala111→Val [NCgl1221(A111V)], Ala111→Thr [NCgl1221(A111T)], Pro424→Leu [NCgl1221(P424L)], and Trp15→Cys-Ser-Leu-Trp [insertion of three amino acids; NCgl1221(W15CSLW)]. *odhA* disruptants that produced very little or no L-glutamate carried the wild-type NCgl1221 allele. The PHDhtm program (http://www.predictprotein.org/newwebsite/submit.html) predicts that NCgl1221 has three transmembrane segments in its N-terminal domain and one transmembrane segment in its C-terminal domain. The NCgl1221(W15CSLW), NCgl1221(A100T), NCgl1221(A111T), and NCgl1221(A111V) substitutions are located in the N-terminal domain, while the NCgl1221(V419::IS*1207*) and NCgl1221(P424L) substitutions are in the C-terminal domain (Fig. 2a).

E. coli yggB mutations have been described that confer constitutive K^+ leakage phenotypes in the absence of hypo-osmotic shock, so-called gain-of-function (GOF) mutations (15, 23). Two GOF mutations of *E. coli* YggB have substitutions, A102P and L109S, respectively, in the third transmembrane helix (Fig. 2b). The positions of NCgl1221(A100T) and probably of NCgl1221(A111V) and NCgl1221(A111T) in the third transmembrane helix resemble these substitutions, suggesting that alterations in this region cause a structural transformation, producing the GOF phenotype: constitutive excretion of L-glutamic acid. The NCgl1221(V419::IS*1207*) and NCgl1221 (P424L) substitutions in the C-terminal region also cause a GOF-like phenotype. In particular, NCgl1221(V419::IS*1207*) results in truncation of the C-terminal extracellular domain, suggesting that changes in this region can also produce alterations of the structure of the NCgl1221 protein, leading to L-glutamic acid excretion.

We constructed NCgl1221(A111V) and NCgl1221(W15CSLW) derivatives of wild-type *C. glutamicum* by replacing the NCgl1221 gene on the chromosome. These NCgl1221 gene mutants, designated BL2 and BL3, respectively, also produced remarkably high levels of L-glutamate without induction (Table 1), confirming that NCgl1221 gene mutations on their own cause L-glutamate overproduction.

NCgl1221 gene mutations render cells resistant to the L-glutamate analog, 4-fluoroglutamate. Overexpression of the *E. coli* arginine exporter YggA renders cells resistant to canavanine, an arginine analog that inhibits cell growth (19). We therefore examined the effect of NCgl1221 gene mutations on sensitivity to an L-glutamate analog. As shown in Fig. 3a, the NCgl1221(A111V) and NCgl1221(W15CSLW) strains grew faster than the wild type on minimal medium containing 4-fluoroglutamic acid. The colony-forming efficiency of the wild-type strain was only 3.2% in the presence of 1.25 mM 4-fluoroglutamic acid, while those of the NCgl1221(A111V) and NCgl1221(W15CSLW) strains were 46% and 60%, respectively (Fig. 3b). These data imply that the mutant NCgl1221 proteins reduce 4-fluoroglutamic acid entry. However, amplification of the wild-type NCgl1221 gene did not affect 4-fluoroglutamic acid sensitivity (data not shown). We speculate that the glutamate export system of wild-type *C. glutamicum* is inactive under normal growth conditions and that it is activated by conformational changes of NCgl1221 caused by certain

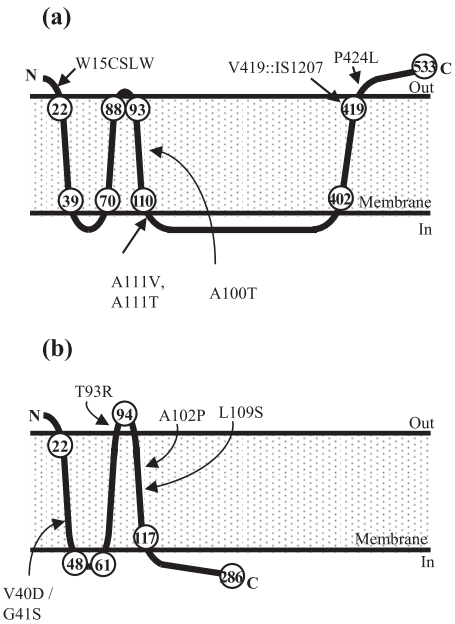

FIG. 2. Secondary structure of *C. glutamicum* NCgl1221 and *E. coli* YggB and their mutation sites. (a) Membrane topology of *C. glutamicum* NCgl1221 as predicted by the PHDhtm program. Arrows indicate positions of NCgl1221 gene mutations. Circled numbers indicate residues located on the borders of transmembrane segments. (b) Proposed membrane topology of *E. coli* YggB based on the PhoA fusion assay (24). Arrows indicate positions of GOF mutations. Circled numbers indicate residues used for the PhoA fusion assay.

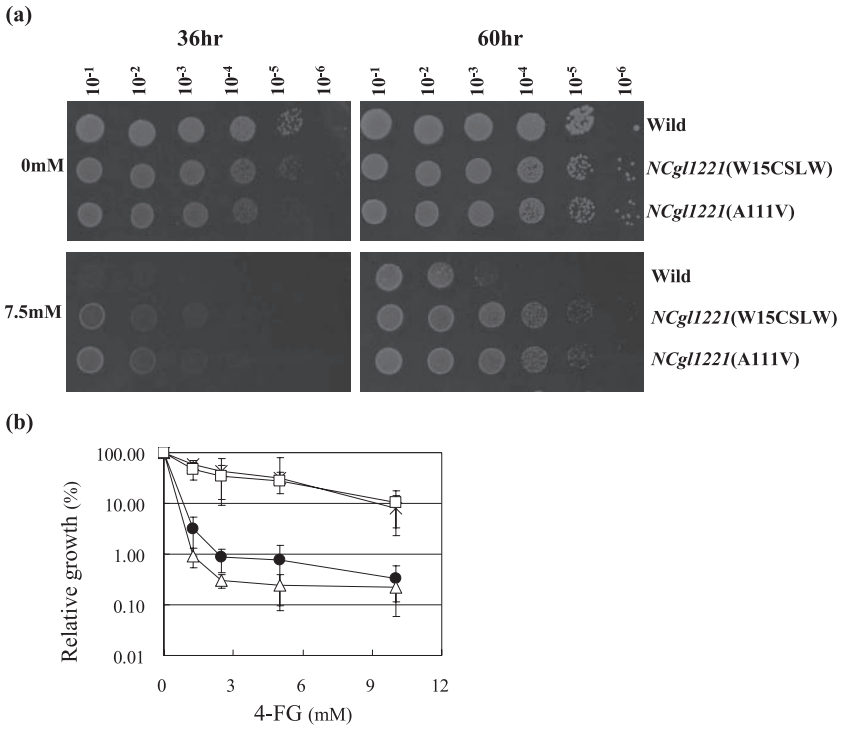

FIG. 3. Fluoroglutamic acid resistance of NCgl1221 gene mutants. (a) Serial dilutions (1/10 each) of cultures of wild-type *C. glutamicum* ATCC 13869 or NCgl1221 gene mutants were spotted onto minimal medium with or without 7.5 mM 4-fluoroglutamic acid, and the plates were incubated at 31.5°C. The upper numbers give cell titers estimated from the OD_{620} of cultures. (b) Cultures of wild-type *C. glutamicum* ATCC 13869 and its NCgl1221 gene mutants were inoculated into fresh minimal medium with or without 4-fluoroglutamic acid, and the cultures were shaken at 31.5°C. When the OD_{620} of each strain without 4-fluoroglutamic acid reached 1.0, cell numbers were examined by plating appropriate dilutions on CM2B plates. Colonies were counted after 2 days of incubation at 31.5°C. Symbols: filled circles, wild-type; open triangles, NCgl1221 gene deletion mutant; open squares, NCgl1221(A111V); X cross, NCgl1221(W15CSLW). Error bars indicate standard deviations ($n = 3$).

NCgl1221 mutations or by treatments that induce L-glutamate production.

Effect of NCgl1221 gene amplification on L-glutamate production. The amplification of an amino acid exporter gene is expected to increase excretion of the corresponding amino acid. For example, amplification of the *lysE* gene encoding the lysine exporter leads to excretion of L-lysine, although a large amount of L-lysine is not excreted by the wild-type strain without treatments to increase the intracellular L-lysine pool (35). It is reported that most amino acid exporters are regulated by intracellular metabolite concentrations. Thus, *lysE* expression is positively regulated by the transcriptional regulator LysG, which senses the intracellular L-lysine pool (2). *brnFE*, encoding the L-methionine/L-isoleucine exporter, is also positively regulated by intracellular L-methionine and L-isoleucine levels (33).

We examined the effects of amplifying the NCgl1221 gene on L-glutamate production. The resultant strain produced no L-glutamic acid in the presence of excess biotin; however, when induced by biotin limitation, it produced larger amounts of L-glutamic acid than the wild type (Table 2). Similar results were obtained using other induction conditions, such as Tween 40 or penicillin treatment. Under these conditions, the times required for L-glutamate production to reach plateaus were almost identical (data not shown), although the biomass yield of the NCgl1221 gene-amplified strain was reduced. This means that the L-glutamate secretion rate per cell was increased by NCgl1221 gene amplification. It is also suggested that L-glutamate export is activated by the NCgl1221 protein only under conditions that induce L-glutamate production.

Effects of NCgl1221 gene disruption on L-glutamate production. If the NCgl1221 protein is the major L-glutamate exporter of *C. glutamicum*, L-glutamate production should be diminished by disrupting the NCgl1221 gene. We deleted the chromosomal NCgl1221 locus and tested the resulting NCgl1221 gene disruptant for L-glutamate productivity in limiting biotin. L-Glutamate production and the glucose consumption rate

TABLE 2. Effect of NCgl1221 gene amplification on L-glutamate production[a]

Culture condition and strain description[b]	OD_{620}	Amt of L-glutamate (mM)	Yield (wt/wt [%])
Tween 40			
Vector	51.5 ± 0.5	267 ± 3.63	50.7 ± 0.7
NCgl1221^{++}	39.4 ± 0.9	303 ± 11.7	58.3 ± 1.1
Penicillin G			
Vector	66.3 ± 0.5	165 ± 1.28	29.0 ± 0.2
NCgl1221^{++}	59.7 ± 0.3	204 ± 4.00	36.0 ± 0.7
Biotin limitation			
Vector	51.8 ± 0.6	295 ± 2.31	52.9 ± 0.4
NCgl1221^{++}	45.3 ± 1.4	310 ± 2.31	55.6 ± 0.4

[a] Data represent mean values ± SDs for three independent cultures.
[b] Vector, ATCC 13869/pVK9; NCgl1221^{++}, ATCC 13869/pL5k.

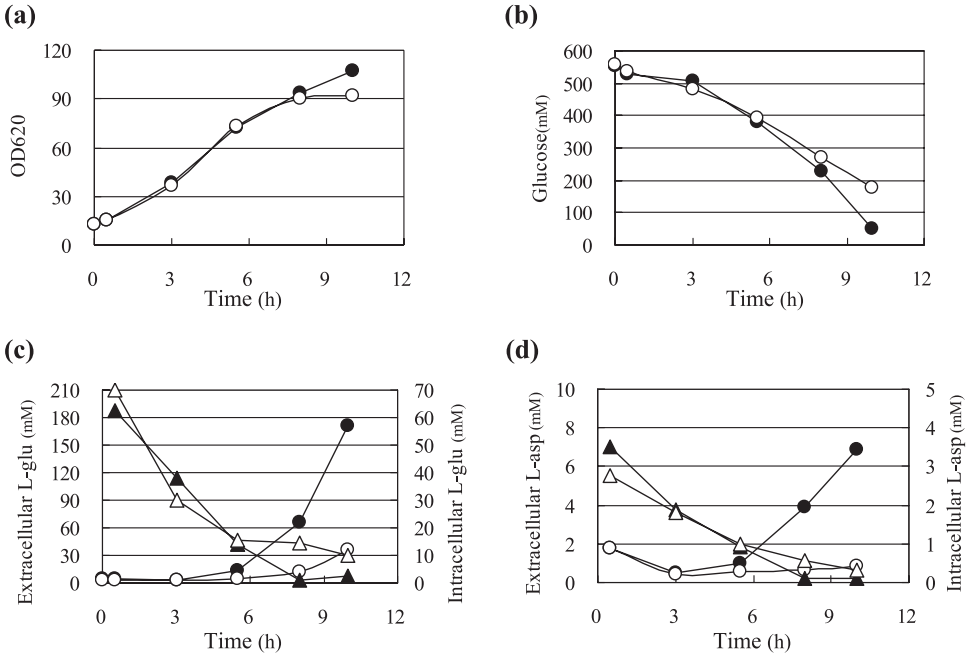

FIG. 4. Effect of NCgl1221 gene deletion on L-glutamate production and the intracellular L-glutamate pool. Wild-type *C. glutamicum* ATCC 13869 and its NCgl1221 gene deletion derivative were grown on biotin-depleted medium. (a) Growth; (b) residual glucose (symbols: filled circles, wild type; open circles, NCgl1221 gene deletion mutant); (c) extracellular and intracellular L-glutamate; (d) extracellular and intracellular L-aspartate (symbols: filled circles, extracellular pool of the wild type; open circles, extracellular pool of the NCgl1221 gene deletion mutant; filled triangle, intracellular pool of the wild type; open triangle, intracellular pool of NCgl1221 gene deletion mutant).

were both greatly diminished (Fig. 4). After 8 h of cultivation, L-glutamate accumulation in the wild type and the NCgl1221 gene disruptant reached 66.6 mM and 12.2 mM, respectively. The intracellular L-glutamate levels of the wild type and the NCgl1221 gene disruptant decreased gradually from the nonproducing period to the producing period. Although the wild type and the NCgl1221 gene disruptant had similar levels of intracellular L-glutamate in the nonproducing period, intracellular L-glutamate in the disruptant was about 4- to 10-fold higher than that of the wild type during the producing period. We also examined other metabolites. L-Aspartate behaved similarly to L-glutamate. Extracellular L-aspartate was lower in the disruptant than in the wild type, while the reverse was true of intracellular L-aspartate. By contrast, the ratio of intracellular 2-oxoglutarate to extracellular 2-oxoglutarate was almost the same in the wild type and disruptant (data not shown). Similar results were obtained when the L-glutamate production was induced with Tween 40 or penicillin treatment: the amount of L-glutamate after 24 h of cultivation was 6.8-fold or 7.1-fold lower in the disruptant than that in the wild type, respectively. These results suggest that the NCgl1221 protein is the major exporter of the acidic amino acids L-glutamic acid and L-aspartic acid in *C. glutamicum.* An alternative possibility is that it is a positive regulator that activates an acidic amino acid export system in response to those treatments that induce L-glutamate excretion.

DISCUSSION

Amplification of biosynthesis genes, attenuation of biosynthetic branch points, and interference with feedback inhibition and repression have been used to improve amino acid production for more than 50 years. However, although their existence was assumed, genes controlling amino acid export, such as the exporter gene for L-lysine, have only recently been identified. It is reported that expression of exporter genes is induced by an increase in the intracellular pool of the corresponding amino acid (2, 33). If the L-glutamate exporter of *C. glutamicum* were regulated by the intracellular L-glutamate pool, ODHC disruptants should be provoked by increases in the 2-oxoglutarate pool to produce significant amounts of L-glutamate and so to induce the expression of the L-glutamate exporter. However, as described above, ODHC disruptants do not produce large amounts of L-glutamate. This suggests that L-glutamate export activity is not induced by increases in intracellular L-glutamate.

We showed that the L-glutamate-overproducing *odhA* disruptants that we isolated also carry a NCgl1221 gene mutation. The N-terminal region of the NCgl1221 protein is similar to the mechanosensitive channels of other microorganisms. It is thought that these channels are activated by alteration of membrane tension (13, 30). By contrast, the C-terminal region of NCgl1221 does not resemble that of any known protein. From the results reported in this study, we conclude that NCgl1221 is the major exporter of L-glutamic acid in *C. glutamicum* and that it is activated by alterations of membrane tension. Treatments inducing L-glutamate production, such as biotin limitation and addition of Tween 40 or penicillin, alter membrane tension by inhibiting lipid or peptidoglycan biosynthesis and lead to excretion of L-glutamic acid by activating NCgl1221. The NCgl1221 gene mutations found in some of the ODHC mutants probably also produce structural changes in NCgl1221 that lead to the constitutive export of L-glutamate without any

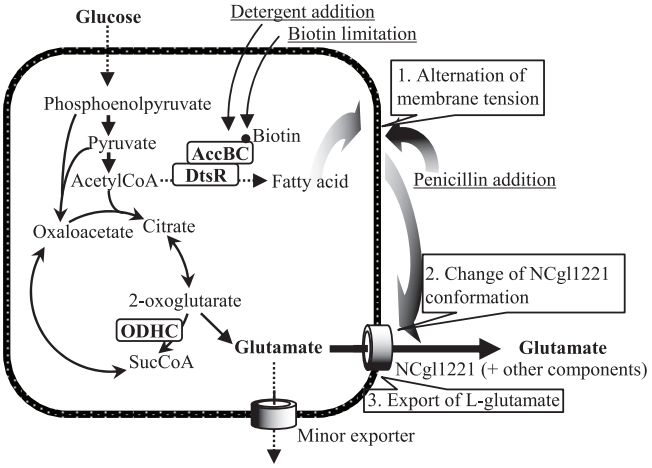

FIG. 5. Model of induction of L-glutamate production in *C. glutamicum.* Treatments inducing L-glutamate production, such as biotin limitation and detergent, alter membrane tension by inhibiting fatty acid biosynthesis. Addition of penicillin also leads to a change of membrane tension by inhibiting cell wall biosynthesis. Alteration of membrane tension triggers a structural transformation of NCgl1221 that enables it to catalyze L-glutamic acid excretion.

change in membrane tension. Similar constitutively active mutations, so-called "gain-of-function" mutations, have been reported in *E. coli yggB* (15, 23).

We observed that the intracellular L-glutamate pool under L-glutamate-producing conditions was lower than under nonproducing conditions, as previously described (6). Were attenuation of ODHC activity to trigger L-glutamate production, intracellular L-glutamate would be elevated. In order to explain a decrease in the L-glutamate pool under L-glutamate-producing conditions, it would be necessary to suppose that L-glutamate biosynthesis is downregulated or that L-glutamate export is upregulated. However, ODHC activity is reduced and GDH activity unchanged under L-glutamate-producing conditions (8). Therefore, we surmise that activation of the L-glutamate exporter is needed to trigger L-glutamate production (3, 4).

We propose the following model for L-glutamate production. (i) Treatments leading to L-glutamate production cause changes in membrane tension. (ii) The structure of NCgl1221 is altered by these changes. (iii) Activated NCgl1221 catalyzes L-glutamate excretion (Fig. 5).

Since the NCgl1221 gene disruptant still produced small but significant amounts of L-glutamate, a second minor L-glutamate export carrier may exist. It is also possible that the NCgl1221 protein is an activator of an L-glutamate exporter(s). If so, L-glutamate secretion by the unknown carrier must decrease fivefold in the absence of NCgl1221. It is reported that ATP or some related high-energy metabolite is required for L-glutamate secretion (4), but there is no ATP-binding motif in the NCgl1221 sequence. Hence, NCgl1221 may activate a putative L-glutamate exporter driven by ATP or a related high-energy metabolite under L-glutamate secretion conditions. Measurements of intracellular metabolites in the NCgl1221 gene disruptant suggest that the NCgl1221 export system exports both L-glutamate and L-aspartate but not 2-oxocarboxylic acids, such as 2-oxoglutarate.

Treatments that induce L-glutamate production also cause a decrease in ODHC activity, resulting in an increase in the intracellular L-glutamate concentration, potentially mediated by the OdhI-PknG system (20). The ODHC activity of 2A-1R carrying a mutant NCgl1221 gene decreased twofold compared with that of the wild-type strain, suggesting that the NCgl1221 gene mutations affect ODHC activity. This attenuation of ODHC activity further increases production of L-glutamate only when the NCgl1221 exporter is activated. Since a decrease in ODHC activity was also observed in response to treatments that induce L-glutamate secretion, the activated forms of NCgl1221 may affect ODHC activity. This might be mediated by the OdhI-PknG system.

Why do *C. glutamicum* cells excrete large amounts of L-glutamate when exposed to induction treatments? Since these treatments all affect cell surface structures and may therefore alter membrane tension, L-glutamate may function as a compatible solute to prevent cells from bursting. Another potential function of L-glutamate excretion is carbohydrate storage during periods when sugar is in excess and cell growth is inhibited by the absence of biotin. The excreted L-glutamate might then be used as a carbon source when biotin becomes available once more. Thus, L-glutamate production by *C. glutamicum* may be a mechanism of adaptation to environmental changes affecting cell surface structures.

ACKNOWLEDGMENTS

We thank H. Kojima and H. Yasueda for their support.

REFERENCES

1. **Asakura, Y., E. Kimura, Y. Usuda, Y. Kawahara, K. Matsui, T. Osumi, and T. Nakamatsu.** 2007. Altered metabolic flux due to deletion of *odhA* causes L-glutamate overproduction in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. **73:**1308–1319.
2. **Bellmann, A., M. Vrljic, M. Patek, H. Sahm, R. Krämer, and L. Eggeling.** 2001. Expression control and specificity of the basic amino acid exporter LysE of *Corynebacterium glutamicum*. Microbiology **147:**1765–1774.
3. **Eggeling, L., K. Krumbach, and H. Sahm.** 2001. L-glutamate efflux with *Corynebacterium glutamicum*: why is penicillin treatment or Tween addition doing the same? J. Mol. Microbiol. Biotechnol. **3:**67–68.
4. **Gutmann, M., C. Hoischen, and R. Krämer.** 1992. Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. Biochim. Biophys. Acta **1112:**115–123.
5. **Hirasawa, T., M. Wachi, and K. Nagai.** 2000. A mutation in the *Corynebacterium glutamicum ltsA* gene causes susceptibility to lysozyme, temperature-sensitive growth, and L-glutamate production. J. Bacteriol. **182:**2696–2701.
6. **Hoischen, C., and R. Krämer.** 1989. Evidence for an efflux carrier system involved in the secretion of glutamate by *Corynebacterium glutamicum*. Arch. Microbiol. **151:**342–347.
7. **Hoischen, C., and R. Krämer.** 1990. Membrane alteration is necessary but not sufficient for effective glutamate secretion in *Corynebacterium glutamicum*. J. Bacteriol. **172:**3409–3416.
8. **Kawahara, Y., K. Takahashi-Fuke, E. Shimizu, T. Nakamatsu, and S. Nakamori.** 1997. Relationship between the glutamate production and the activity of 2-oxoglutarate dehydrogenase in *Brevibacterium lactofermentum*. Biosci. Biotechnol. Biochem. **61:**1109–1112.
9. **Kennerknecht, N., H. Sahm, M. R. Yen, M. Patek, M. H. Saier, Jr., and L. Eggeling.** 2002. Export of L-isoleucine from *Corynebacterium glutamicum*: a two-gene-encoded member of a new translocator family. J. Bacteriol. **184:**3947–3956.
10. **Kimura, E., C. Abe, Y. Kawahara, and T. Nakamatsu.** 1996. Molecular cloning of a novel gene, dtsR, which rescues the detergent sensitivity of a mutant derived from *Brevibacterium lactofermentum*. Biosci. Biotechnol. Biochem. **60:**1565–1570.
11. **Kimura, E.** 2002. Triggering mechanism of L-glutamate overproduction by DtsR1 in coryneform bacteria. J. Biosci. Bioeng. **94:**545–551.
12. **Kinoshita, S., S. Udaka, and M. Shimono.** 1957. Studies on the amino acid fermentation. Part 1. Production of L-glutamic acid by various microorganisms. J. Gen. Appl. Microbiol. **3:**193–205.
13. **Levina, N., S. Totemeyer, N. R. Stokes, P. Louis, M. A. Jones, and I. R. Booth.** 1999. Protection of *Escherichia coli* cells against extreme turgor by activation of MscS and MscL mechanosensitive channels: identification of genes required for MscS activity. EMBO J. **18:**1730–1737.

14. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. **Miller, S., W. Bartlett, S. Chandrasekaran, S. Simpson, M. Edwards, and I. R. Booth.** 2003. Domain organization of the MscS mechanosensitive channel of *Escherichia coli*. EMBO J. **22:**36–46.
16. **Miwa, K., K. Matsui, M. Terabe, K. Ito, S. Nakamori, K. Sano, and H. Momose.** 1984. Cryptic plasmid in glutamic acid producing bacteria. Agric. Biol. Chem. **48:**2901–2903.
17. **Nakamura, J., S. Hirano, and H. Ito.** June 2006. L-glutamic acid producing microorganism and a method for producing L-glutamic acid. U.S. patent US20060141588A1.
18. **Nampoothiri, K. M., C. Hoischen, B. Bathe, B. Mockel, W. Pfefferle, K. Krumbach, H. Sahm, and L. Eggeling.** 2002. Expression of genes of lipid synthesis and altered lipid composition modulates L-glutamate efflux of *Corynebacterium glutamicum*. Appl. Microbiol. Biotechnol. **58:**89–96.
19. **Nandineni, M. R., and J. Gowrishankar.** 2004. Evidence for an arginine exporter encoded by *yggA* (*argO*) that is regulated by the LysR-type transcriptional regulator ArgP in *Escherichia coli*. J. Bacteriol. **186:**3539–3546.
20. **Niebisch, A., A. Kabus, C. Schultz, B. Weil, and M. Bott.** 2006. Corynebacterial protein kinase G controls 2-oxoglutarate dehydrogenase activity via the phosphorylation status of the OdhI protein. J. Biol. Chem. **281:**12300–12307.
21. **Nottebrock, D., U. Meyer, R. Krämer, and S. Morbach.** 2003. Molecular and biochemical characterization of mechanosensitive channels in *Corynebacterium glutamicum*. FEMS Microbiol. Lett. **218:**305–309.
22. **Nunheimer, T. D., J. Birnbaum, E. D. Ihnen, and A. L. Demain.** 1970. Product inhibition of the fermentative formation of glutamic acid. Appl. Microbiol. **20:**215–217.
23. **Okada, K., P. C. Moe, and P. Blount.** 2002. Functional design of bacterial mechanosensitive channels. Comparisons and contrasts illuminated by random mutagenesis. J. Biol. Chem. **277:**27682–27688.
24. **O'Mahony, M., and R. Ishii.** 1986. A comparison of English and Japanese taste languages: taste descriptive methodology, codability and the umami taste. Br. J. Psychol. **77:**161–174.
25. **Radmacher, E., K. C. Stansen, G. S. Besra, L. J. Alderwick, W. N. Maughan, G. Hollweg, H. Sahm, V. F. Wendisch, and L. Eggeling.** 2005. Ethambutol, a cell wall inhibitor of *Mycobacterium tuberculosis*, elicits L-glutamate efflux of *Corynebacterium glutamicum*. Microbiology **151:**1359–1368.
26. **Shiio, I., S. Otsuka, and M. Takahashi.** 1962. Effect of biotin on the bacterial formation of glutamic acid. I. Glutamate formation and cellular permeability of amino acids. J. Biochem. **51:**56–62.
27. **Shiratsuchi, M., H. Kuronuma, Y. Kawahara, Y. Yoshihara, H. Miwa, and S. Nakamori.** 1995. Simultaneous and high fermentative production of L-Lysine and L-glutamic acid using a strain of *Brevibacterium lactofermentum*. Biosci. Biotechnol Biochem. **59:**83–86.
28. **Simic, P., H. Sahm, and L. Eggeling.** 2001. L-Threonine export: use of peptides to identify a new translocator from *Corynebacterium glutamicum*. J. Bacteriol. **183:**5317–5324.
29. **Soga, T., Y. Ohashi, Y. Ueno, H. Naraoka, M. Tomita, and T. Nishioka.** 2003. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J. Proteome Res. **2:**488–494.
30. **Sukharev, S.** 2002. Purification of the small mechanosensitive channel of *Escherichia coli* (MscS): the subunit structure, conduction, and gating characteristics in liposomes. Biophys. J. **83:**290–298.
31. **Takinami, K., H. Yoshii, H. Tsuri, and H. Okada.** 1965. Biochemical effects of fatty acid and its derivatives on L-glutamic acid fermentation. Part III. Biotin-Tween 60 relationship in the accumulation of L-glutamic acid and the growth of *Brevibacterium lactofermentum*. Agric. Biol. Chem. **29:**351–359.
32. **Takinami, K., H. Yoshii, Y. Yamada, H. Okada, and K. Kinoshita.** 1968. Control of L-glutamic acid fermentation by biotin and fatty acid. Amino Acid Nucleic Acid **18:**120–160.
33. **Trotschel, C., D. Deutenberg, B. Bathe, A. Burkovski, and R. Krämer.** 2005. Characterization of methionine export in *Corynebacterium glutamicum*. J. Bacteriol. **187:**3786–3794.
34. **Udaka, S.** 1960. Screening method for microorganisms accumulating metabolites and its use in the isolation of *Micrococcus glutamicus*. J. Bacteriol. **79:**754–755.
35. **Vrljic, M., H. Sahm, and L. Eggeling.** 1996. A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. Mol. Microbiol. **22:**815–826.
36. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.